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Combining site-specific mutagenesis and seeding as a strategy to crystallize 'difficult' proteins: the case of *Staphylococcus aureus* thioredoxin

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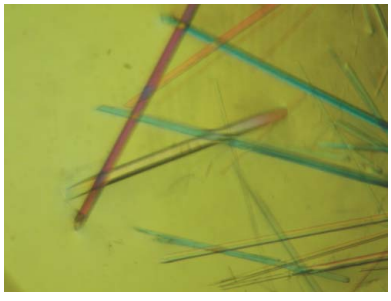

The P31T mutant of *Staphylococcus aureus* thioredoxin crystallizes spontaneously in space group $P2_12_12_1$, with unit-cell parameters a = 41.7, b = 49.5, c = 55.6 Å. The crystals diffract to 2.2 Å resolution. Isomorphous crystals of wild-type thioredoxin as well as of other point mutants only grow when seeded with the P31T mutant. These results suggest seeding as a valuable tool complementing surface engineering for proteins that are hard to crystallize.

1. Introduction

Although it is generally recognized that any protein that adopts a unique and stable folded conformation is amenable to crystallization, the crystallization behaviour of individual proteins remains difficult to predict. Properties that influence the success of crystallization experiments include solubility, surface charge and charge distribution, side-chain and main-chain flexibility, conformational heterogeneity, shelf-life and thermodynamic stability. Often, one encounters the situation where a particular protein fails to crystallize, but where crystals of variants (close homologues or single or multiple point mutants) can be successfully grown. In many studies, the structural information obtained from such a variant is equivalent to that of the original target. In other cases, the difference between the biological activities and structures of the original target and the variant may be of interest or is sufficiently pronounced to hamper interpretation of the results. In such studies, structural data on the original target remain essential.

Staphylococcus aureus and several major classes of Gram-positive bacteria lack glutathione and its cognate enzymes and must therefore employ thioredoxin and alternative systems (Fahey *et al.*, 1978). The thioredoxin-reduction system is essential for maintaining the intracellular thiol–disulfide balance in *S. aureus* and for its growth (Uziel *et al.*, 2004). The structure of *S. aureus* thioredoxin (Sa_Trx), a small 12 kDa oxidoreductase, is not known. All thioredoxins from archaea to humans have similar three-dimensional structures comprising a central core of five β-strands surrounded by four α-helices. All feature a conserved active-site loop containing two redox-active cysteine residues in the sequence Trp28-Cys29-Gly30-Pro31-Cys32 (Sa_Trx numbering; Eklund *et al.*, 1991). The oxidized form (thioredoxin S_2) contains a disulfide bridge that is reduced to a dithiol by the NADPH-dependent flavoprotein thioredoxine reductase (Holmgren *et al.*, 1975; Arner & Holmgren, 2000; Holmgren, 1968). The reduced form [thioredoxin $(SH)_2$] is a powerful protein disulfide oxidoreductase.

2. Materials and methods

2.1. Cloning and site-specific mutagenesis

The *trxA* gene from *S. aureus* was cloned into the *Nde*I and *Bam*HI sites of pET-14b (Novagen, Madison, WI, USA). The *trxA* wild-type plasmid was used as a DNA template in PCR amplification with primers designed to introduce the P31S and P31T mutations. The resulting fragments were digested with *Nde*I and *Bam*HI and cloned into the pET-14b vector (Novagen). The C32S mutant was constructed as described in Messens *et al.* (2004). The mutant

Table 1
Crystallization conditions for Sa_Trx P31T in a 15 mg ml^{-1} protein solution in 20 m*M* Tris pH 8.0, 100 m*M* KCl, 1 m*M* DTT.

	Dimensions	
Precipitant solution	Length	Width/thickness
30% PEG 4000, 0.1 *M* Tris–HCl pH 8.5, 0.2 *M* $MgCl_2$	Up to 2 mm	Up to 150 µm
2.0 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 8	Up to 300 µm	Less than 10 µm
30% PEG 4000, 0.1 *M* sodium citrate pH 5.6, 0.2 *M* ammonium acetate	Up to 2 mm	Up to 100 µm
30% PEG 4000, 0.1 *M* sodium acetate pH 4.6, 0.2 *M* ammonium acetate	Up to 2 mm	Up to 100 µm
20% PEG 8000, 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* magnesium acetate	Up to 2 mm	Up to 100 µm
30% PEG 4000, 0.1 *M* Tris–HCl pH 8.5, sodium acetate	Up to 2 mm	Up to 100 µm
30% PEG 8000, 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* sodium acetate	Up to 1 mm	Up to 50 µm
1.4 *M* sodium citrate, 0.1 *M* Na HEPES pH 7.5	Up to 300 µm	Less than 10 µm
18% PEG 8000, 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* calcium acetate	Up to 1 mm	Up to 50 µm

combination P31T with C32S was made with the QuikChange Site-Directed Mutagenesis kit (Stratagene). In this case, the P31T mutant was used as the DNA template.

2.2. Expression and purification

All mutants were transformed and expressed in *Escherichia coli* strain BL21(AI) (Invitrogen) and grown for 4 h at 310 K in Terrific broth (TB) with ampicillin (100 µg ml^{-1}). Induction at a cell density of OD_{600} = 0.9 was carried out overnight with 0.2% arabinose at 301 K. Cells were harvested, disrupted and purified as described previously (Messens *et al.*, 2002). Prior to crystallization, the N-terminal His_6 tag was removed by adding thrombin protease (5 units per milligram of recombinant protein; Calbiochem) to the protein. After incubation for 24 h at 299 K, thrombin was removed on a benzamidine Sepharose column (GE Healthcare, Uppsala, Sweden). To remove residual His-tagged proteins, the sample was applied onto an Ni^{2+}-Sepharose column (GE Healthcare). The flowthrough fractions were pooled, dialyzed against 20 m*M* Tris–HCl pH 8.0, 100 m*M* KCl, 1 m*M* DTT and concentrated to 15 mg ml^{-1}.

2.3. Crystallization and seeding

Crystallization conditions for wild-type Sa_Trx and for the P31S mutant were screened using the hanging-drop method employing a series of commercially available sparse-matrix screens: Hampton Research Crystal Screen, Crystal Screen II, Natrix Screen, PEG/Ion Screen and Malonate Grid Screen. All crystallization experiments were preformed at 293 K by the hanging-drop vapour-diffusion method in 24-well plates. In each trial, a hanging drop of 1.0 µl protein solution (15 mg ml^{-1}) mixed with 1.0 µl precipitant solution was equilibrated against a reservoir containing 500 µl precipitant solution. The initial crystallization conditions for the P31T mutant were screened by the sparse-matrix method using the Hampton Screen kit.

Crystallization trials on Sa_Trx C32S were performed using the sitting-drop vapour-diffusion method at 293 K using a nanodrop-dispensing robot (PixSys 4200 Cartesian Inc.) in 96-well Greiner crystallization plates (Sulzenbacher *et al.*, 2002). Several commercial kits, Wizard Screen 1 and 2 (Emerald Biosystems), MDL Screens 1 and 2, Stura Screen, ZetaSol Screen and Clear Strategy Screens 1 and 2 (Molecular Dimensions Ltd), were used for screening of the C32S mutant. 300, 200 and 100 nl drops of protein solution (either at 8.25 mg ml^{-1} in 5 m*M* Tris–HCl pH 8.0, 100 m*M* KCl or at 9.6 or 20 mg ml^{-1} in 10 m*M* HEPES pH 8.0, 50 m*M* KCl) were mixed with 100 nl mother liquor.

Streak-seeding was performed using a cat whisker cleaned with a 20% ethanol solution prior to use. The crystal was touched with the whisker so that seeds were dislodged by friction. To avoid the formation of too many crystals along the seeding line, the whisker was first dipped into the precipitant solution. The seeding tool was then passed through several droplets (serial seeding), thereby decreasing the number of seeds transferred to later drops. Crystals appeared after a couple of days.

2.4. X-ray diffraction

Prior to data collection, crystals were mounted in quartz capillaries or frozen in a cold stream at 100 K using a variety of potential cryoprotectants: PEG 4000, PEG 400, PEG 20 000, glycerol, MPD and paraffin oil. All X-ray data were collected using synchrotron radiation on the EMBL beamlines at the DESY synchrotron (Hamburg, Germany) and at the ESRF synchrotron (Grenoble, France). In each case, at last 90° of data were collected using 1° oscillations and exposure times of 1–10 s. Data were indexed and integrated using *DENZO*, *XDISPLAYF* and *SCALEPACK* from the *HKL* package (Otwinowski & Minor, 1997). Intensities were converted to structure-factor amplitudes using the program *TRUNCATE* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

3. Results

3.1. Crystallization of Sa_Trx P31T and Sa_Trx P31T/C32S

Screening of the crystallization conditions for Sa_Trx P31T led to crystals using several conditions (Table 1) which were also suitable for crystallizing the P31T/C32S double mutant. Although varying in size, the shape of all the crystals was the same and those grown in 25–30% PEG 4000, 0.1 *M* Tris pH 8.5 and 0.2 *M* $MgCl_2$ were selected for data collection (Fig. 1). They belong to space group $P2_12_12_1$, with unit-cell parameters a = 41.7, b = 49.5, c = 55.6 Å and contain a single thioredoxin molecule in their asymmetric unit, corresponding to a solvent content of 51% (V_M = 2.5 Å^3 Da^{-1}). Despite many attempts to collect data at cryogenic temperature, we never succeeded in successfully freezing the crystals. Usually, freezing led to a complete loss of diffraction, although in a single instance poor diffraction was observed to 3.5 Å. This crystal indexed in a unit cell with an apparently doubled c axis (a = 39.2, b = 48.6, c = 96.9 Å) and a very high

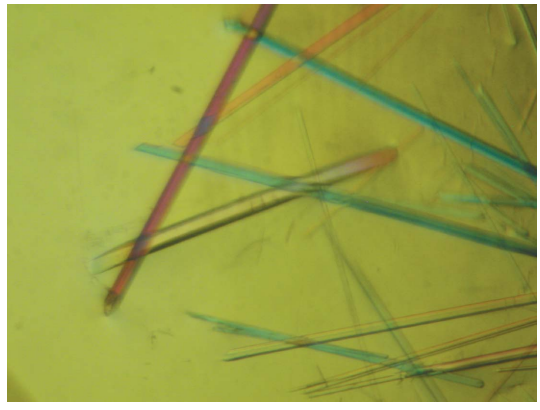

Figure 1
Typical crystals of Sa_Trx P31T.

Table 2
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Wild type	P31T	P31S	P31T/C32S
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Beamline	X11	BW7A	X13	ID14-1
Unit-cell parameters				
a (Å)	41.1	41.7	41.2	41.3
b (Å)	49.8	49.5	49.5	49.2
c (Å)	54.6	55.6	54.7	54.9
Resolution limits (Å)	15.0–2.2 (2.1–2.2)	15.0–2.2 (2.1–2.2)	15.0–2.2 (2.5–2.4)	15.0–2.55 (2.45–2.55)
$I/\sigma(I)$	14.8 (4.3)	16.9 (4.2)	13.2 (4.3)	9.9 (3.5)
No. of measured reflections	43223 (3076)	29248 (2788)	18019 (1806)	14609 (1378)
No. of unique reflections	6123 (587)	6140 (590)	4694 (464)	3928 (376)
Completeness (%)	97.7 (98.5)	99.4 (99.0)	99.7 (99.6)	99.4 (99.2)
$R_{\rm merge}$	0.093 (0.449)	0.058 (0.431)	0.071 (0.311)	0.094 (0.454)

mosaicity (>2°). Subsequent annealing by interrupting the cryostream for a few seconds abolished this diffraction. Subsequently, all data were collected at room temperature with exposure times kept sufficiently short to avoid unacceptable levels of radiation damage. Under these conditions, the largest crystals diffracted to a resolution of 2.2 Å (Table 2 and Fig. 2).

3.2. Crystallization of wild-type Sa_Trx and other site-specific mutants

The double mutant Sa_Trx P31T/C32S crystallizes spontaneously under the same conditions as Sa_Trx P31T. These crystals are isomorphous to those of Sa_Trx P31T (Table 2). The resolution obtained (2.7 Å) was not as good as for the P31T mutant owing to the needles being systematically thinner.

No crystals of wild-type Sa_Trx, of the C32S mutant or of the P31S mutant were obtained despite extensive screening. We hypothesized that the wild-type thioredoxin as well as the P31S mutant might have a dynamic equilibrium between two conformational states (oxidized and reduced) that prevents nucleation. Thus, we attempted to obtain crystals of wild-type Sa_Trx as well as of the mutant P31S by streak-seeding using crystals of Sa_Trx P31T as seed material. Using protein solutions consisting of 20 m*M* Tris pH 8.0, 100 m*M* KCl and 1 m*M* DTT, crystals grew overnight in each case that were morphologically very similar to those of Sa_Trx P31T. However, these crystals were not stable over time. The crystals of the P31S mutant and especially those of the wild-type protein become a brownish colour and lose their diffraction capacity 1–2 d after their first appearance. Consequently, for those two proteins data were collected immediately after the crystals appeared. Diffraction analysis showed that both are isomorphous to Sa_Trx P31T. Complete data sets were collected at room temperature (Table 2).

4. Discussion

In recent years, much attention has been given to surface engineering of proteins to improve crystallizability and crystal quality by altering crystal-packing interactions (Dale *et al.*, 2003; D'Arcy *et al.*, 1999; Dyda *et al.*, 1994; Jenkins *et al.*, 1995; Lawson *et al.*, 1991; Schwede *et al.*, 1999; Zhang *et al.*, 1997; McElroy *et al.*, 1992; Derewenda, 2004). Most popular among these techniques are the removal of amino acids with long charged side chains such as lysine and glutamate (Longenecker *et al.*, 2001; Mateja *et al.*, 2002; Czepas *et al.*, 2004), but genetic natural variants have also been used to enhance crystallization (Buts *et al.*, 2005).

Here, a site-specific mutant of *S. aureus* thioredoxin crystallized spontaneously under a number of conditions while the wild-type protein as well as other mutants remained recalcitrant to crystallization. Several reasons may lay behind this observation. Since Pro31 is a surface residue, it is possible that in Sa_Trx P31T a crystal contact is engineered that is crucial for nucleation. Such a situation is similar to what has been observed for the lectin from *Pterocarpus angolensis* seeds (Loris *et al.*, 2005). In this case, only the complex with Man(α1–3)Man crystallized spontaneously. The sugar ligand is involved in a lattice contact that apparently plays a role in nucleation. However, once the crystals are formed the ligand can be removed or replaced, indicating that this particular lattice contact is not necessary for maintenance of the crystals.

An alternative explanation would involve the oxidation state of the protein. Pro31 is not only a surface residue, but is also part of the conserved WCGPC active site. It is well documented that the central residues of the active-site motif can alter the redox properties (Mossner *et al.*, 1999). Consequently, the P31T mutation might influence the redox behaviour of the protein, reducing the conformational heterogeneity between the oxidized and reduced forms and hampering crystallization of the wild type and most mutants. At this stage, we could only presume that the oxidized form of P31T mutant is conformationally more homogeneous. Using the P31T mutant crystals as seeds could then favour crystal growth from protein molecules that adopt the same conformation, while molecules with different conformations remain in solution or form amorphous precipitates.

Although other possibilities cannot be excluded at this point, the latter explanation seems the most likely as crystals from both wild-type and P31S Sa_Trx, but not those from P31T or P31T/C32S mutants, have an unusually short shelf-life. They tend to visibly deteriorate and lose their diffraction capacity within 24–48 h of growth. Moreover, mutation of a surface proline to a threonine is unlikely to reduce surface entropy.

However, we show here that once crystals from a mutant protein are obtained, these crystals may be used in a microseeding experi-

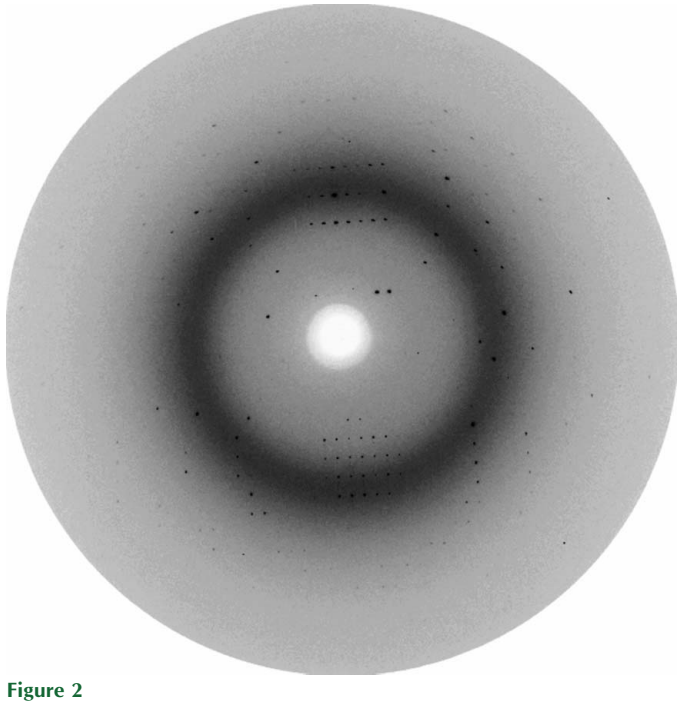

Figure 2
Diffraction pattern of wild-type Sa_Trx.

ment to obtain crystals of the wild-type protein. While in many cases the crystal structure of a variant will provide answers to most of the questions posed in the study, structural knowledge of the wild-type protein may in some cases remain essential for correct understanding.

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