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Conformational stability and crystal packing: polymorphism in *Neurospora crassa* CAT-3

Polymorphism is frequently observed from different crystallization conditions. In proteins, the effect on conformational variability is poorly documented, with only a few reported examples. Here, three polymorphic crystal structures determined for a large-subunit catalase, CAT-3 from *Neurospora crassa*, are reported. Two of them belonged to new space groups, *P1* and *P4*₃2₁2, and a third structure belonged to the same space group, *P2*₁2₁2₁, as the previously deposited 2.3 Å resolution structure (PDB entry 3ej6), but had a higher resolution (1.95 Å). Comparisons between these polymorphic structures highlight the conformational stability of tetrameric CAT-3 and reveal a distortion in the tetrameric structure that has not previously been described.

1. Introduction

Protein crystallization is a multi-parametric process that depends mainly on how intrinsic features of the protein are influenced by their environment. Different crystallization conditions can lead to altered crystal packing and in some cases to different crystal shapes owing to differential growth rates along the axes. This effect is called polymorphism (Bonnetfond *et al.*, 2011). There are a few explicitly documented examples of proteins which present polymorphism: HEWL, with six different space groups (Vaney *et al.*, 2001), and human haemoglobin, with two different space groups (Park *et al.*, 2006), are examples of this behaviour.

Catalases dismutate hydrogen peroxide into dioxygen and two water molecules. Haem-dependent monofunctional catalases are divided into two groups according to their molecular mass: small-subunit catalases (SSCs) have a molecular mass of between 55 and 65 kDa, while large-subunit catalases (LSCs) have a molecular mass of approximately 80 kDa owing to an additional C-terminal domain of 150–210 amino-acid residues. To date, there are only four LSC structures in the Protein Data Bank from three different organisms: *Neurospora crassa*, *Penicillium vitale* and *Escherichia coli*.

Catalase 3 from *N. crassa* (CAT-3) is an LSC with a mass of 77.8 kDa per subunit. CAT-1 and CAT-3 from *N. crassa* have been crystallized in the resting state (Díaz *et al.*, 2004, 2009), while for PVC from *P. vitale* a resting state and a compound I structure have been reported (Alfonso-Prieto *et al.*, 2007). For HP11 from *E. coli* several structures can be found in the PDB: two for the resting state, one of a C-terminally truncated enzyme and several singly mutated constructs (Bravo *et al.*, 1995; Melik-Adamyan *et al.*, 2001; Chelikani *et al.*, 2003, 2005). While CAT-1 and CAT-3 crystallize in space groups *C2* and *P2*₁2₁2₁, respectively, PVC crystallizes in space group *P3*₁21 and HP11 in space group *P2*₁. Despite the different space groups, one of the crystal axes always presents a dimension of approximately 130 Å: the *c* axis for PVC, the *b* axis for HP11 and the *a* axis for CAT-1 and CAT-3.

The oligomeric state of all deposited LSCs is a tetramer, although during purification steps of CAT-1 (Díaz *et al.*, 2001) and CAT-3 (Zárate-Romero, in preparation) dimeric fractions were isolated. These dimeric forms show catalase activity, but their stability and presence under physiological conditions have not yet been established. Our analysis of subunit interfaces in CAT-3 using the PISA server (Krissinel & Henrick, 2007) shows that the dimer formed

between the *P*-related subunits (the *P*-, *Q*- and *R*-related subunits are as described by Díaz *et al.*, 2009) has a contact surface of $5460.6 \text{ \AA}^2$ and a Δ^iG value of $-31.9 \text{ kcal mol}^{-1}$ (1 cal = 4.186 J), while the dimer formed between *Q*-related subunits has a contact surface of $4794.1 \text{ \AA}^2$

and a Δ^iG value of $-43.1 \text{ kcal mol}^{-1}$. Finally, the *R*-related subunits have a contact surface of $2039.1 \text{ \AA}^2$ and a Δ^iG value of $-22.5 \text{ kcal mol}^{-1}$.

In this work, we present an analysis of three structures of a recombinant CAT-3 expressed in *E. coli*, two of which belong to new space groups for CAT-3 (*P*1 and *P*₄₃₂₁²). This is to our knowledge the first example of polymorphism for LSCs. A third structure in space group *P*₂₁₂₁², the same as PDB entry 3ej6 (Díaz *et al.*, 2009), was obtained with almost identical unit-cell parameters but at a higher resolution of 1.95 Å.

2. Materials and methods

2.1. Cloning, overexpression and purification

The *cat-3* gene cloned in pSM3 (Michán *et al.*, 2003) was subcloned into pCold I (TaKaRa) using the *Eco*RI restriction enzyme. The construct codes for the whole polypeptide chain of CAT-3 plus 27 additional residues at the N-terminus including a 6×His tag.

Competent *E. coli* strain BL21 (DE3) cells were transformed with the pCold-*cat-3* construction and were grown on a Luria–Bertani (LB) agar plate supplied with $200 \mu\text{g ml}^{-1}$ ampicillin. A single colony was picked up and inoculated in a culture flask containing 500 ml LB broth and $200 \mu\text{g ml}^{-1}$ ampicillin. This culture was grown overnight at 310 K and 225 rev min⁻¹ and was used to inoculate 4.5 l LB broth supplemented with $200 \mu\text{g ml}^{-1}$ ampicillin. The culture was further incubated at 310 K and 225 rev min⁻¹ until an OD₆₀₀ of 0.670 was reached. IPTG was then added to a final concentration of 0.25 mM and the temperature was changed to 289 K. After 24 h the culture was centrifuged at 3500g for 15 min. The pellet was resuspended in 100 ml lysis buffer (50 mM Na₂HPO₄/NaH₂PO₄ pH 8, 500 mM NaCl, 10 mM imidazole) and Complete protease inhibitor (Roche Molecular Biochemicals), 1 mg ml⁻¹ lysozyme and 1 μg ml⁻¹ DNAase were added. A cell lysate was obtained by sonication, keeping the bacterial suspension in an ice bath. The lysate was centrifuged for 50 min at 13 000g and the supernatant was applied onto a HiTrap column (GE Healthcare) previously equilibrated with ten volumes of lysis buffer. After washing with ten volumes of 50 mM Na₂HPO₄/NaH₂PO₄ pH 8, 500 mM NaCl, 30 mM imidazole, the protein was eluted with an increasing gradient of imidazole and monitored by native gel electrophoresis. The identity of the protein was confirmed by mass spectrometry and zymograms (Díaz *et al.*, 2001).

After dialysis in 50 mM Na₂HPO₄/NaH₂PO₄ pH 8, the protein solution was applied onto High-Q Sepharose anion-exchange resin (Bio-Rad) and eluted with an increasing gradient of 50 mM Na₂HPO₄/NaH₂PO₄ pH 8, 500 mM NaCl. The protein started to elute when the NaCl concentration reached 200 mM; the protein solution was further concentrated and dialyzed in 50 mM Na₂HPO₄/NaH₂PO₄ pH 8, 100 mM NaCl. Finally, to obtain the CAT-3 dimer, the protein solution was applied onto a Superdex 200 column (GE Healthcare) equilibrated with 50 mM Na₂HPO₄/NaH₂PO₄ pH 8, 100 mM NaCl; the protein eluted with a retention volume of 140 ml. The sample had an approximate purity of 95% according to an SDS–PAGE gel, with a yield of 2.5 mg from 1 l culture.

2.2. Crystallization

CAT-3 was successfully crystallized in three different crystal systems: triclinic, orthorhombic and tetragonal. The triclinic form of CAT-3 was obtained by the hanging-drop vapour-diffusion method. Protein droplets consisting of 1 μl CAT-3 solution (3.5 mg ml⁻¹ in 10 mM HEPES pH 7) and 1 μl reservoir solution [Crystal Screen 2 condition No. 38; 100 mM HEPES pH 7.5, 20% (w/v) PEG 10 000]

Figure 1

Brown crystals of the resting state of CAT-3 were obtained in three different crystal forms. (a) Triclinic. The crystals grew as plates using the hanging-drop vapour-diffusion method at 277 K, the arrow indicates the crystal chosen for the diffraction data collection. (b) Orthorhombic. Crystals grew as elongated bars using the microbatch method at 277 K. (c) Tetragonal crystals grew as short bars using the hanging-drop method at 277 K. The photographs in (a) and (b) were taken with the same magnification; photograph (c) was taken before data collection at LANEM, UNAM.

Table 1

Summary of crystallographic data.

Values in parentheses are for the highest resolution shell.

Structure	Triclinic	Orthorhombic	Tetragonal
Space group	<i>P</i> 1	<i>P</i> ₂ ₁ ₂ ₁	<i>P</i> ₄ ₃ ₂ ₁ ₂
Unit-cell parameters (Å, °)	<i>a</i> = 85.44, <i>b</i> = 88.20, <i>c</i> = 104.39, α = 82.08, β = 82.47, γ = 62.33	<i>a</i> = 131.52, <i>b</i> = 154.80, <i>c</i> = 160.74	<i>a</i> = 207.51, <i>b</i> = 207.51, <i>c</i> = 137.04
PDB code	3zj4	3zj5	4bim
Resolution (Å)	29.5–3.10	42.5–1.95	30.0–2.95
Unique reflections	47857	270245	62656
Completeness (%)	98.6 (97.1)	95.0 (87.8)	99.3 (98.0)
$\langle I/\sigma(I) \rangle$	8.5 (2.8)	7.0 (2.6)	9.1 (2.8)
<i>R</i> _{merge} (%)	17.4 (51.1)	14.2 (43.2)	11.1 (46.3)
Multiplicity	4.0 (3.9)	3.8(3.8)	4.1 (4.1)
Mosaicity (°)	0.23 [<i>XDS</i>]	0.80 [<i>iMosflm</i>]	0.13 [<i>XDS</i>]
<i>B</i> from Wilson plot (Å ²)	35.66	12.10	66.94
Matthews coefficient (Å ³ Da ^{−1})	2.21	2.63	2.37
Solvent content (%)	44.30	53.18	48.07
Refinement			
Resolution limits (Å)	29.5–3.10	42.5–1.95	30.0–2.95
<i>R</i> (%)	19.70	15.64	25.53
<i>R</i> _{free} (%)	24.51	19.65	30.44
Protein atoms	21333	21606	21,339
Haem atoms	172	172	172
Tartrate atoms	—	10	—
PEG atoms	—	144	—
Solvent atoms	—	2189	321
R.m.s.d., bond lengths (Å)	0.003	0.007	0.009
R.m.s.d., bond angles (°)	0.863	1.120	1.110
Average <i>B</i> values (Å ²)			
Protein	43.8	11.9	77.11
Haem	24.7	5.0	59.7
Solvent	—	17.5	34.65
PEG	—	30.7	—
Tartrate	—	26.8	—

were equilibrated against 1 ml reservoir solution at 277 K. Brown crystals appeared after 2 weeks, with the brown colour indicating the ferric resting state of the catalase (Fig. 1a).

The orthorhombic form of CAT-3 was obtained by the microbatch crystallization method (Chayen, 1998). Protein droplets consisting of 1 µl protein solution (6 mg ml^{−1} in 10 mM HEPES pH 7) and 1 µl mother liquor [PEG/Ion condition No. 38; 200 mM ammonium dibasic tartrate, 20% (w/v) PEG 3350] were mixed, covered with 10 µl paraffin oil and kept at 277 K in a temperature-controlled room. Brown crystals appeared after 4 d and continued growing for 2 weeks (Fig. 1b).

The tetragonal form of CAT-3 appeared in experiments using the hanging-drop vapour-diffusion method: an initial hit was obtained from condition No. 43 of Crystal Screen [200 mM ammonium monobasic phosphate, 100 mM Tris pH 8.5, 50% (v/v) MPD]. Crystals suitable for X-ray diffraction experiments were only obtained after several optimization trials. Protein droplets consisting of 1 µl CAT-3 solution (6 mg ml^{−1} in 10 mM HEPES pH 7) and 1 µl reservoir solution [100 mM MES pH 5.5, 50% (v/v) MPD] were equilibrated against 1 ml reservoir solution at 277 K; the characteristic brown crystals appeared after 2 months (Fig. 1c).

2.3. Data collection and analysis

One CAT-3 triclinic crystal with dimensions of 0.12 × 0.08 × 0.01 mm was cryoprotected and cryocooled in liquid nitrogen using 100 mM HEPES pH 7.5, 40% (v/v) PEG 400 as a cryoprotectant. 360 frames were collected on beamline X6A at NSLS with an exposure time of 30 s and $\Delta\varphi = 1^\circ$ at a fixed distance of 300 mm and a wavelength of 0.9795 Å.

The orthorhombic crystals were cryoprotected with 200 mM ammonium dibasic tartrate, 40% (v/v) PEG 400 and cryocooled in

liquid nitrogen. 220 frames were collected from a crystal with dimensions 0.70 × 0.04 × 0.04 mm on beamline X6A at NSLS with an exposure time of 30 s and $\Delta\varphi = 0.5^\circ$ at a fixed distance of *d* = 220 mm and a wavelength of 0.9794 Å.

The crystals of the tetragonal form were cryoprotected with 100 mM MES pH 5.5, 50% (v/v) MPD. The crystals were cryocooled in liquid nitrogen and one data set comprising 100 frames was collected using a MicroMax-007 HF rotating-anode generator with VM-HR optics fitted with an R-Axis IV⁺⁺ detector at Laboratorio Nacional de Estructura de Macromoléculas (LANEM) at the UNAM with an exposure time of 1500 s and $\Delta\varphi = 0.5^\circ$ at a fixed distance of 155 mm and a wavelength of 1.5418 Å.

The images for the triclinic and tetragonal data sets were indexed and integrated using the *XDS* suite (Kabsch, 2010) in space groups *P*1 and *P*₄₃₂₁₂, respectively. The orthorhombic data set was indexed and integrated using *MOSFLM* (Leslie & Powell, 2007) in space group *P*₂₁₂₁₂. Scaling for each data set was performed with *SCALA* from *CCP4* (Evans, 2006). Phases were obtained by molecular replacement in *Phaser* (McCoy *et al.*, 2007) using as the initial model one monomer of CAT-3 (PDB entry 3ej6; Díaz *et al.*, 2009).

For the *P*₂₁₂₁₂ and *P*1 space groups, a tetramer was obtained in the asymmetric unit, both models were refined using rigid-body refinement in *REFMAC5* (Murshudov *et al.*, 2011) and subsequent refinement was performed using *PHENIX* (Afonine *et al.*, 2012). The models were constructed in *Coot* (Emsley *et al.*, 2010). The *P*1 and *P*₂₁₂₁₂ structures were validated using the *MolProbity* server (Chen *et al.*, 2010) and deposited with PDB entries 3zj4 and 3zj5, respectively. Analyses for intermolecular contacts for both structures were performed using the *PISA* server (Krissinel & Henrick, 2007).

For the tetragonal crystals, the solution obtained from molecular replacement with *Phaser* using a monomer from PDB entry 3ej6 (Díaz *et al.*, 2009) as the search model was a trimer, with a log-likelihood gain of 5532, an RFZ of 9.5 and a TFZ of 45.2. However, according to the *Matthews Probability Calculator* (Kantardjieff & Rupp, 2003), a tetramer was the assembly with the highest probability of fitting into the asymmetric unit and a visual analysis of the electron density and the crystal packing supported this possibility. A new molecular-replacement process using the trimer obtained in the first attempt as a fixed solution and the different domains of the monomer as search models gave a better answer, with a log-likelihood gain of 8749, an RFZ of 9.4 and a TFZ of 2.95. Through the modelling of different fragments, the remaining monomer was built after the use of several cycles of *Buccaneer* (Cowtan, 2008) together with *Phaser*. This final model comprising four monomers was refined using *BUSTER-TNT* (Blanc *et al.*, 2004). In this structure, the asymmetric unit was formed by two separate dimers, which after applying symmetry operators generated two tetramers which were not identical; the structure was validated with *MolProbity* and deposited with PDB entry 4bim. Data-collection and refinement statistics for all the structures are summarized in Table 1; in the three structures determined here, conservative but consistent criteria based on $I/\sigma(I)$ and *R*_{merge} values were used to define resolution cutoffs.

3. Results and discussion

The r.m.s.d. value between the tetramer in the asymmetric unit determined from the triclinic crystal (unit-cell parameters *a* = 85.44, *b* = 88.20, *c* = 104.39 Å, α = 82.08, β = 82.47, γ = 62.33°) and the orthorhombic structure (PDB entry 3ej6) is 0.42 Å considering 2721 C^α atoms. Owing to the resolution, the triclinic data set was refined using NCS and one atomic displacement parameter (commonly called

B value) per residue; neither solvent atoms nor double conformations or partial occupancies were modelled. An overview of crystal packing for the triclinic structure is shown in Fig. 2(a).

The orthorhombic unit-cell parameters ($a = 131.52$, $b = 154.80$, $c = 160.74$ Å) are almost identical to those reported for PDB entry 3ej6. A superposition of the orthorhombic asymmetric unit content

Figure 2 Organization of CAT-3 copies inside the unit cell in space groups (a) $P1$, (b) $P2_12_12_1$ and (c) $P4_32_12$; the left, centre and right correspond to the *ab*, *ac* and *bc* faces of the unit cell, respectively, for each structure. Monomers *A*, *B*, *C* and *D* are presented in red, blue, green and yellow, respectively, in (a) and (b); monomers *A*, *B*, *C* and *D* are presented in yellow, red, blue and green, respectively, in (c). The structure with the lowest solvent content is that belonging to space group $P1$, followed by those in space groups $P4_32_12$ and $P2_12_12_1$.

Figure 3

A comparison between the tetramers generated by applying symmetry operators to dimer *AB* (*a*; monomer *A* in yellow and monomer *B* in red) and dimer *CD* (*c*; monomer *C* in blue and monomer *D* in green) shows the alteration in the conformation of the tetramer generated based on the dimer *CD* present in the tetragonal structure of CAT-3. The scheme in (*b*) represents changes between tetramers which correspond to a rotation of almost 5°.

with PDB entry 3ej6 gave an r.m.s.d. value of 0.40 Å considering 2705 C α atoms. An overview of crystal packing for the orthorhombic structure is shown in Fig. 2(*b*). When water molecules near the active site are compared, monomer *B* is the only subunit that has two clear water molecules in the vicinity of the active site, the first at 3.0 Å from the haem iron and the second at 4.5 Å, while in the remaining three monomers of the orthorhombic structure (monomers *A*, *C* and *D* from PDB entry 3zj5) there is only one clear water molecule at ~4.5 Å from the iron. In catalases, the presence of one or two water molecules in the proximity of the iron haem has been described (Carpena *et al.*, 2003; Díaz *et al.*, 2004, 2009), but with no explanation of why these water molecules are present in some cases but not in others. In horseradish peroxidase and horse heart myoglobin the existence of such water molecules is related to the oxidation state of the system; in particular, the absence of a water molecule at ~2.4 Å from the iron haem is linked to the ferrous state (Berglund *et al.*, 2002; Hersleth & Andersson, 2011). In LCSs, compound I and the ferric state have been described; however, the existence of a ferrous state has not been demonstrated.

In PDB entry 3zj5 there are only a few residues that present double conformations and remarkably one of them is Ser388, which is next to Tyr389, the fifth ligand of the haem iron. This serine is conserved in all of the LCSs deposited in the PDB, but the conserved serine does not present a double conformation in any of them, regardless of the resolution. Furthermore, apart from CAT-3, in which all of the haem groups are modelled in the *b* conformation, and CAT-1 (PDB entry 1sy7; Díaz *et al.*, 2004), in which a partial occupancy between the *b* and *d* conformations was modelled, the remaining known LSC structures present their haem groups in the *d* conformation.

The two different dimers in the asymmetric unit of the tetragonal structure (4bim) were superposed on the tetramer of PDB entry 3ej6. When symmetry operators are applied to the dimer *AB*, a tetramer almost identical to that observed in PDB entries 3ej6, 3zj4 and 3zj5 is generated, with an r.m.s.d. value of 0.47 Å using 2574 C α atoms, but if the same operators are applied to the dimer *CD* a distorted tetramer is generated. An overview of crystal packing is shown in Fig. 2(*c*). Regardless of the contacts inside the tetramer, in the tetragonal structure the surface-contact area for each monomer is at least twice the surface-contact area calculated for the triclinic and orthorhombic structures. Nevertheless, in the tetragonal structure monomer *D* possesses a lower surface-contact area (829.2 Å²) compared with the three other monomers in the asymmetric unit (1994.9, 1571.2 and 1795.4 Å² for monomers *A*, *B* and *C*, respectively). The distortion in the symmetry-generated *CD* tetramer can be described as a rotation point with a centre at the extreme of the interface between monomer *C* and its symmetry-related copy, with a rotation of almost 5° (Fig. 3); the r.m.s.d. value of this tetramer in the superposition on 3ej6 is 1.61 Å considering 2544 C α atoms. Interestingly, the comparison of Δ^iG of the dimers related by *R*, calculated using the PISA server (Krissinel & Henrick, 2007), for the normal (monomers related by *R* of PDB entry 3ej6) and the distorted tetramers shows a $\Delta\Delta^iG$ of -7 kcal mol⁻¹, indicating that the normal tetramer seems to be more stable than the distorted tetramer. However, from the thermal stability and structural denaturalization studies reported for LCSs (Wasserman & Hultin, 1981; Switala *et al.*, 1999; Díaz *et al.*, 2001), the existence of a distorted tetramer seems controversial, even though the existence of two different tetramer populations in the tetragonal crystal is undeniable. It is important to remark that an additional sequence of 27 residues at the N-terminus, including the 6×His tag, prior to the first methionine is contained in the expressed protein used here. It is possible that these additional 27 residues, which are not visible in any of the electron-density maps for any of these

structures, are responsible for the observed distortion. Nevertheless, in the $P1$ and $P2_12_12_1$ structures described here the tetramer is identical to those previously described for CAT-3 in which CAT-3 that was crystallized was purified directly from *N. crassa* mycelia (Díaz *et al.*, 2009).

Attention is drawn to the fact that CAT-3, a member of a group of enzymes that are poorly represented in the PDB, presents an opportunity to explore the effects of crystal packing on the conformational arrangement of LSCs. In addition to the conditions described here, CAT-3 could be crystallized over a wide pH range from 4.6 to 8 with several precipitating agents such as PEG (in a molecular-mass range from 3350 to 20 000), MPD (at concentrations from 30 to 60%) and ammonium sulfate. Many of those crystallization conditions produced microcrystals which are in the process of being optimized or nicely shaped crystals with poor X-ray diffraction qualities (which usually diffracted to between 8 and 14 Å resolution). Even the crystallographic parameters of these putative CAT-3 polymorphs remain undetermined; the possibility of using them to find different conformational states seems feasible. Analysis of the biological implications of the various structures is still in progress.

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