Multiple crystal forms of *N,N'*-diacetylchitobiose deacetylase from *Pyrococcus furiosus*

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Native *N,N'*-diacetylchitobiose deacetylase from *Pyrococcus furiosus* (Pf-Dac) and its selenomethionine derivative (Se-Pf-Dac) were crystallized and analyzed in the presence and absence of cadmium ion. The four crystal structures fell into three different crystal-packing groups, with the cadmium-free Pf-Dac and Se-Pf-Dac belonging to the same space group, with homologous unit-cell parameters. The crystal structures in the presence of cadmium contained distorted octahedral cadmium complexes coordinated by three chlorides, two O atoms and an S or Se atom from the N-terminal methionine or selenomethionine, respectively. The N-terminal cadmium complex was involved in crystal contacts between symmetry-related molecules through hydrogen bonding to the N-termini. While all six N-termini of Se-Pf-Dac were involved in cadmium-complex formation, only two of the Pf-Dac N-termini participated in complex formation in the Cd-containing crystal, resulting in different crystal forms. These differences are discussed in light of the higher stability of the Cd—Se bond than the Cd—S bond. This work provides an example of the contribution of cadmium towards determining protein crystal quality and packing depending on the use of the native protein or the selenomethionine derivative.

1. Introduction

N,N'-Diacetylchitobiose deacetylase (Dac) catalyzes the hydrolysis of the *N*-acetyl group of the nonreducing residue of the disaccharide *N,N'*-diacetylchitobiose (GlcNAc₂). Dac is a key enzyme in the archaea-specific chitinolytic pathway, in which GlcNAc₂, the end product of chitinase, is deacetylated prior to degradation into monosaccharides (Tanaka *et al.*, 2004). To further structural studies on the archaeal chitinolytic pathway (Nakamura *et al.*, 2007, 2008; Tsuji *et al.*, 2010; Mine, Nakamura *et al.*, 2014), we have elucidated the crystal structures of Dacs from *Pyrococcus horikoshii* and *P. furiosus* (Pf-Dac) (Mine, Niiyama *et al.*, 2014). Dac is a homohexameric protein that consists of two trimers, with its active site at the deep end of the cleft formed by the boundary of the trimeric assembly. The active site consists of a central zinc coordinated by two histidines and one aspartic acid, and side chains from two polypeptides are involved in the catalytic cycle (Mine, Niiyama *et al.*, 2014).

Crystallized protein molecules are periodically arranged with nonphysiological crystal-packing contacts (Carugo & Argos, 1997). Crystal contacts are central to the properties of the crystal, and mutagenesis at the contact region can improve the resolution of the crystal structure (Mizutani *et al.*, 2008).

Table 1

Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Protein	Pf-Dac	Pf-Dac	Se-Pf-Dac	Se-Pf-Dac
Cd in reservoir	No	Yes	No	Yes
Data collection				
X-ray source	BL44XU, SPring-8	BL44XU, SPring-8	BL38B1, SPring-8	BL38B1, SPring-8
Wavelength (Å)	0.90000	0.90000	1.00000	0.99522
Space group	$P2_1$	$P1$	$P2_1$	$P1$
Unit-cell parameters (Å, °)	$a = 81.634$, $b = 121.825$, $c = 92.133$, $\beta = 113.882$	$a = 74.353$, $b = 77.847$, $c = 93.511$, $\alpha = 66.386$, $\beta = 84.699$, $\gamma = 61.947$	$a = 82.005$, $b = 121.926$, $c = 92.271$, $\beta = 114.205$	$a = 93.895$, $b = 93.880$, $c = 93.877$, $\alpha = 74.691$, $\beta = 74.681$, $\gamma = 74.677$
Resolution range (Å)	50–2.30 (2.34–2.30)	50–1.80 (1.86–1.80)	50–2.80 (2.85–2.80)	50–1.51 (1.54–1.51)
Z	12	6	12	6
V_M (Å ³ Da ^{−1})	2.25	2.33	2.26	4.04
Solvent content (%)	45.35	47.32	45.58	69.59
$R_{\text{merge}}^\dagger$ (%)	9.8 (40.3)	8.6 (39.9)	6.8 (36.7)	5.5 (31.8)
Completeness (%)	99.6 (99.2)	97.7 (95.8)	99.7 (99.9)	95.2 (81.7)
Total reflections	376328	546611	277954	816365
Unique reflections	72889	152603	40647	435340
Multiplicity	5.2 (4.4)	3.6 (3.2)	6.8 (6.2)	1.9 (1.9)
$\langle I/\sigma(I) \rangle$	12.5 (3.8)	10.4 (2.6)	15.0 (4.9)	15.8 (2.6)
Refinement				
Resolution range (Å)	46.57–2.30 (2.359–2.300)	41.13–1.80 (1.845–1.799)	50.00–2.80 (2.874–2.801)	31.16–1.51 (1.552–1.513)
No. of reflections	69115 (5062)	144947 (10259)	38561 (2824)	413484 (28795)
$R_{\text{cryst}}^\ddagger/R_{\text{free}}^\S$ (%)	20.9 (25.6)/27.6 (30.6)	23.5 (40.1)/28.7 (41.4)	26.2 (30.6)/34.6 (45.1)	15.2 (25.2)/16.7 (26.8)
R.m.s.d., bond lengths (Å)	0.018	0.024	0.012	0.035
R.m.s.d., bond angles (°)	1.705	1.988	1.410	2.911
Protein atoms	13057	12914	12854	13182
Zn atoms	6	0	6	0
Cd atoms	0	8	0	18
Water molecules	114	368	30	1412
Average B factor (Å ²)				
Protein atoms	49.6	40.1	65.1	19.7
Water molecules	37.1	33.0	33.6	31.9
Ramachandran plot ¶ (%)				
Favoured	86.2	88.1	78.9	91.2
Allowed	13.5	11.7	20.5	8.7
PDB code	4xm2	4xm1	4xm0	4xlz

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of observation i and $\langle I(hkl) \rangle$ is the average of symmetry-related observations of a unique reflection. $^\ddagger R_{\text{cryst}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$, where F_{obs} and F_{calc} are observed and calculated structure-factor amplitudes, respectively. $^\S R_{\text{free}}$ was calculated using a randomly selected 5% of the data set that was omitted from all stages of refinement. ¶ Ramachandran plots were prepared for all residues other than Gly and Pro.

Although in many cases protein crystal packing is unchanged by minor mutations or modification of the crystallization conditions, some proteins such as hen egg-white lysozyme crystallize in multiple forms depending on the conditions.

Cadmium can facilitate protein crystallization (Ames *et al.*, 1998; Huang *et al.*, 2002). Indeed, the selenomethionine derivative of Pf-Dac (Se-Pf-Dac) diffracted to high resolution (1.51 Å) in the presence of cadmium (Mine, Niiyama *et al.*, 2014). To further evaluate the effect of cadmium on the crystallization of (Se-)Pf-Dac, we investigated the crystal packing of these proteins in the presence and absence of cadmium. In this paper, we describe multiple crystal-packing forms of Pf-Dac and Se-Pf-Dac and discuss a crystallization contact involving a cadmium complex containing an Se or S atom.

2. Materials and methods

2.1. Protein preparation and crystallization

Pf-Dac and Se-Pf-Dac were prepared as described previously (Mine, Niiyama *et al.*, 2014). Crystals of Pf-Dac and

Se-Pf-Dac were obtained using the hanging-drop vapour-diffusion method with one of three reservoir solutions: 0.1 M Tris–HCl pH 8.0, 0.2 M MgCl₂, 3.0 M 1,6-hexanediol, 5 mM CdCl₂ (reservoir 1), 0.1 M Tris–HCl pH 8.0, 0.2 M MgCl₂, 3.4 M 1,6-hexanediol, 5 mM CdCl₂ (reservoir 2) or 0.1 M Tris–HCl pH 8.0, 0.2 M MgCl₂, 3.2 M 1,6-hexanediol, 8% acetone (reservoir 3). To crystallize Pf-Dac, 1 µl protein solution (9 mg ml^{−1} Pf-Dac in 20 mM Tris–HCl pH 8.1, 150 mM NaCl) was mixed with an equal volume of reservoir 2 or 3 and equilibrated against 0.4 ml of the corresponding reservoir solution at 293 K. For crystallization of Se-Pf-Dac, 1 µl protein solution (12 or 15 mg ml^{−1} Se-Pf-Dac in 20 mM Tris–HCl pH 8.1, 150 mM NaCl) was mixed with an equal volume of reservoir 1 or 3, respectively, and equilibrated similarly.

2.2. Data collection and processing

Crystals of Pf-Dac and Se-Pf-Dac were flash-cooled in a nitrogen flow at 100 K without cryoprotection. Diffraction data were collected on beamline BL38B1 or BL44XU at SPring-8, Hyogo, Japan. Diffraction data were integrated and scaled with *HKL-2000* (Otwinowski & Minor, 1997). The

initial phase was determined by molecular replacement with *MOLREP* (Vagin & Teplyakov, 2010) in the *CCP4* suite (Winn *et al.*, 2011). The previously determined Se-Pf-Dac structure (Mine, Niiyama *et al.*, 2014) was used as the search model. Structural refinement was performed with *REFMAC* (Murshudov *et al.*, 2011). Stereochemical analysis was performed with *PROCHECK* (Laskowski *et al.*, 1993). Theoretical calculations were performed with *Gaussian 09*.

3. Results

3.1. Multiple crystal forms of Pf-Dac and Se-Pf-Dac

Pf-Dac and Se-Pf-Dac were crystallized as described in §2. The reservoir conditions did not differ significantly except for the presence or absence of CdCl_2 . As summarized in Table 1, however, the crystal packing varied depending on the presence of cadmium and whether the native protein or its selenomethionine derivative was used.

In the absence of cadmium, the crystals of Pf-Dac and Se-Pf-Dac belonged to space group $P2_1$, with similar unit-cell parameters (Table 1). The barrel-shaped hexameric biological assemblies of Pf-Dac and Se-Pf-Dac were stacked along the noncrystallographic threefold axis (Fig. 1*a*). However, when crystallized with cadmium, the space group and unit-cell parameters of Pf-Dac and Se-Pf-Dac were different from

those in the absence of cadmium. The crystal of Pf-Dac belonged to space group $P1$. Although the Cd-containing Se-Pf-Dac crystal belonged to the hexagonal space group $H3$, as described previously (Mine, Niiyama *et al.*, 2014), the diffraction data were processed in space group $P1$ to refine the crystal structure of each protomer independently. In the Cd-containing crystals, the hexameric barrels did not directly stack but contacted the oblique hexamer (Figs. 1*b* and 1*c*), which was the only crystallization contact in the Cd-containing Se-Pf-Dac crystal (Fig. 1*b*), leading to a solvent content of as high as 69.6% (Table 1). The Cd-containing Pf-Dac crystal involved, in addition to the oblique contacts, lateral contacts between the barrels (Fig. 1*c*).

3.2. Comparison of the crystal structures of Pf-Dac and Se-Pf-Dac

Although the tertiary structures of Pf-Dac and Se-Pf-Dac with and without cadmium were almost identical, minor differences were observed. High electron density was observed at the central metal of Pf-Dac and Se-Pf-Dac in the Cd-containing conditions (data not shown), suggesting that the catalytic zinc was replaced by cadmium.

In all crystal forms, the asymmetric unit contained one biological unit comprising six molecules, thus enabling comparison of the *B* factors among chains (Fig. 2). The *B*

Figure 1

Crystal packing of (a) Cd-free Pf-Dac, (b) Cd-containing Se-Pf-Dac and (c) Cd-containing Pf-Dac. The crystal packing of Cd-free Se-Pf-Dac is the same as that in (a). Blue spheres in (b) and (c) indicate Cd atoms involved in oblique crystallization contacts between N-termini (see text).

factors of Cd-containing Se-Pf-Dac were relatively low and homogeneous across the six molecules (Fig. 2, bottom left), reflecting that the Se-Pf-Dac crystal data could be processed in space group *H*3 with two molecules in the asymmetric unit. For the three other crystal forms, however, several differences were observed among chains in the hexameric assembly. In particular, chains *C* and *F* of Cd-containing Pf-Dac had low N-terminal *B* factors (Fig. 2, arrow 1) and were the only chains with clear N-terminal electron density. Similarly, around position 225, chains *A* and *D* had relatively lower *B* factors than the other chains (Fig. 2, arrow 2). The notable properties of these chains are discussed below in relation to the cadmium complex.

3.3. Crystallization contact with the cadmium complex

The oblique crystallization contact in the Cd-containing Se-Pf-Dac crystal was formed by the N-termini (Fig. 3*a*), which showed large additional electron densities that fitted well to a model containing cadmium and chloride ions (Fig. 3*b*, top). The cadmium ion was chelated by six atoms: selenium from the N-terminal selenomethionine, two Glu225 side-chain O atoms (adjacent chain) and three chloride ions from the crystallization solution. This atomic configuration indicated that the cadmium formed a distorted octahedral complex (Fig. 3*c*, top). The negativity of the three chloride ligands facilitates intermolecular hydrogen bonding to the amide proton of Phe2, the residue after the selenomethionine. Thus, two N-termini interact in a head-to-tail manner involving two cadmium complexes (Figs. 3*a* and 3*b*), leading to stabilization

Table 2

Bond distances and angles of cadmium complexes.

	Se-Pf-Dac†	Pf-Dac‡
Distances (Å)		
Cd—Se/S	2.88 ± 0.004	2.885, 2.889
Cd—O1	2.36 ± 0.030	2.465, 2.535
Cd—O2	2.50 ± 0.033	2.605, 2.531
Cd—Cl1	2.54 ± 0.034	2.652, 2.475
Cd—Cl2	2.56 ± 0.034	2.453, 2.246
Cd—Cl3	2.56 ± 0.016	2.344, 2.515
Angles (°)		
∠Se/S—Cd—O1	78.95 ± 0.48	75.87, 79.08
∠Se/S—Cd—O2	82.70 ± 0.74	85.44, 84.46
∠Se/S—Cd—Cl1	90.34 ± 0.44	87.74, 81.45
∠Se/S—Cd—Cl2	166.56 ± 0.49	164.83, 164.69
∠Se/S—Cd—Cl3	90.63 ± 0.22	85.16, 86.01

† Values are the average ± SD for the six complexes in the asymmetric unit. ‡ Values for two complexes in the asymmetric unit are shown.

of the oblique crystallization contact of Se-Pf-Dac in the presence of cadmium. Although Figs. 3(*a*) and 3(*b*) (top) show only a representative crystallization contact involving the N-termini of chains *A* and *E*, all other N-termini (*i.e.* those of chains *B* and *D* and chains *C* and *F*) participate in the same type of contact (data not shown). These contacts lead to packing with a hexamer of Se-Pf-Dac (Fig. 1*b*, green) surrounded by the six symmetry-related hexamers in the Cd-containing crystal (Fig. 1*b*).

In contrast to Se-Pf-Dac, only two of the Pf-Dac N-termini (chains *C* and *F*) participated in complex formation in the Cd-containing crystal. The cadmium ion was chelated by the S atom of Met1, two O atoms from Glu225 and three chloride

Figure 2
Distribution of *B* factors. *B* factors of *C* α atoms are plotted *versus* the residue number. See text for arrows 1 and 2.

ions (Fig. 3*b*, bottom). The atomic configuration was almost the same as that of the cadmium complexes in the Se-Pf-Dac crystal (Fig. 3*c*, Table 2). In the other four polypeptides, however, the N-termini were not structurally determined because of poor electron density. The cadmium complexes at the N-termini of chains *C* and *F* involved Glu225 of chains *A* and *D*, respectively. The low *B* factors of two chains of Cd-containing Pf-Dac (Fig. 2, arrow 2) are likely to be a consequence of the presence of two cadmium complexes per Pf-Dac hexamer.

Figure 3
Structure of the cadmium complex. (a) Schematic of an oblique crystallization contact in the Cd-containing Se-Pf-Dac crystal. (b) Stereoviews of the structure around the crystal contact at the N-termini of Se-Pf-Dac (top) and Pf-Dac (bottom) in the presence of cadmium. Orange and blue maps are $2F_o - F_c$ and $F_o - F_c$ electron-density maps contoured at 1.5σ and 3σ , respectively. Blue and magenta balls are cadmium and chloride ions, respectively. (c) Stereoviews of the cadmium complexes of Se-Pf-Dac (top) and Pf-Dac (bottom).

4. Discussion

Addition of cadmium to the crystallization solutions altered the crystal packing of (Se-)Pf-Dac (Table 1). The crystal-packing patterns of Pf-Dac and Se-Pf-Dac in the presence of cadmium differed, whereas they were same without cadmium (Table 1, Figs. 1*b* and 1*c*). For Se-Pf-Dac, clear electron density was observed for all six molecules of the biological assembly, with all of the N-termini involved in crystal contacts through cadmium complexes. In Pf-Dac, however, four of the N-termini were structurally flexible as judged from poor electron density, and only two chains (*C* and *F*) were involved in the oblique crystal contact through the cadmium complex.

The bond angles and distances of the cadmium chelate are nearly equivalent in Se-Pf-Dac and Pf-Dac (Table 2). However, the complex is more stable in Se-Pf-Dac: the Cd—Se bond should be stronger than the Cd—S bond because selenium has a larger atomic radius than sulfur. The quantum-chemical calculations for an isodesmic reaction of the model compounds (Fig. 4) support the Se-atom effect on the stability of the complex. Thus, the model reaction was calculated to be exothermic in enthalpy by 5.0 kJ mol^{-1} at the B3LYP/3-21G level of theory. This suggests that the higher propensity for chelation enables Se-Pf-Dac to crystallize only with the crystallization contact involving the cadmium complex. In the native protein, on the other hand, a combination of the oblique chelate-involving contact and the lateral contact of the hexameric barrels (Fig. 1*c*) is suitable.

We found that cadmium improved the quality of (Se-)Pf-Dac crystals despite its lack of any physiological relevance to

Figure 4

The isodesmic reaction considered in quantum-chemical calculations.

the enzyme. In some protein crystals, cadmium complexes have been observed in intramolecular interactions (Zhang *et al.*, 2013), intermolecular interactions (Florence *et al.*, 2012) or intermolecular crystal contacts (Zanotti *et al.*, 1998). In (Se-)Pf-Dac crystals, the cadmium complex contributed to the formation of the crystal contacts, thereby changing the crystal packing and improving the quality. Since Pf-Dac and Se-Pf-Dac exhibited different crystal packing in the presence of cadmium, utilization of selenomethionine derivatives can be useful in developing soakable crystals (Ingram *et al.*, 2006) for studying ligand interactions by varying the crystal packing.

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