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Crystallization of peptidase T from *Salmonella typhimurium*

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Aminotripeptidase (peptidase T) from *Salmonella typhimurium* and a derivative carrying a C-terminal His tag have been crystallized. In both cases, the space group was found to be *C2*, with a single molecule in the asymmetric unit. Crystals of the native peptidase T diffract to 2.9 Å, but a selenomethionine derivative of this protein did not yield good crystals. Crystals of the His-tag peptidase T diffracted to 2.6 Å, however, and could be used for the production of good-quality selenomethionine crystals. All 15 methionines, a native metal ion and two mercury reactive sites could be located and crystals suitable for MAD data collection have been produced.

1. Introduction

Peptidase T from *S. typhimurium* catalyzes the release of N-terminal amino acids with hydrophobic side chains from tripeptides (Strauch & Miller, 1983). This reaction is highly specific; dipeptides, tetrapeptides or tripeptides with the N-terminus blocked are not cleaved. While peptidases in general enable the cell to use exogenously supplied peptides as carbon and nitrogen sources, the expression level of peptidase T is insufficient to play a metabolic role under aerobic conditions (Strauch & Miller, 1983). However, under anaerobic conditions the expression of the *pepT* gene is induced 30-fold, allowing mutants deficient in other peptidases to grow on tripeptides (Lombardo *et al.*, 1997; Strauch *et al.*, 1985). The *pepT* operon is regulated by FNR, a transcriptional activator that is the product of the *fnr* (fumarate and nitrate reductase regulator) gene and responds to anaerobiosis (Miller *et al.*, 1991; Spiro & Guest, 1990). Peptidase T is a 45 kDa metalloenzyme that is inhibited by EDTA (Strauch & Miller, 1983). Peptidase T is found in a number of microorganisms including *Escherichia coli*, *Haemophilus influenzae*, *Bacillus subtilis* and *Lactococcus lactis*. Peptidase T lacks significant overall similarity with other proteins, with the exception of a hypothetical protein from *B. subtilus*. However, a 45 amino-acid long region of peptidase T is similar to a short region in carboxypeptidase G2, peptidase D and Iap (Miller *et al.*, 1991). This segment contains two of the five amino acids that coordinate to the two zinc ions in the active site of carboxypeptidase G2, for which the three-dimensional structure is known (Rowsell *et al.*, 1997). A third ligand, a histidine, can be identified as part of a HXDT motif which is conserved throughout available peptidase T and carboxypeptidase G2 sequences, as well as in some other metalloenzymes with related activity (PROSITE PDOC00613). It is therefore probable that the structure of peptidase T will be related to that of carboxypeptidase G2, but the lack of extensive sequence similarity suggests that there might be regions where the two structures will differ. To investigate the structural rationale behind the unique and interesting specificity of peptidase T, we have crystallized both the native enzyme and a selenomethionine His-tag derivative, determined the space group and unit-cell parameters and located the Se-atom positions.

2. Methods

2.1. Construction of peptidase T expressing strains TN5445, TN5446 and TN5619

A 1.4 kbp *Ssp*I/*Pst*I fragment of the plasmid pJG17 (Miller *et al.*, 1991) was cloned into *Sma*I/*Pst*I-digested pSE380, creating a *pepT* overproducing plasmid pCM385. This plasmid was transformed into TN1379 (*leuBCD485*), creating strain TN5445, and into TN1470 (*metE862*::Tn*10*), creating strain TN5446.

The *pepT* gene was amplified using PCR and 5′-modified primers to generate terminal restriction sites for subsequent cloning into the His_6-tagged expression vector pET28a (Novagen). The sequences of the oligonucleotides were as follows (restriction sites are in bold): *pepT*-5′*Nco*I, 5′-CGA AAA GTG AGG GTG ACT **CCA TGG** ATA AAC TAC TTG AGC G-3′; *pepT*-3′*Xho*I, 5′-GCT TCA CCA CAA TGC GTA ACA **CTC GAG** ACT GCC CGC CCT TAG CC-3′. The blunt PCR product was cloned into blunt vector pCRII-TOPO (Invitrogen) to create plasmid pCM549. The 1.2 kbp *Nco*I/*Xho*I *pepT* fragment from pCM549 was cloned into pET28a digested with

Table 1
Data statistics for crystals of peptidase T and its derivatives.

Values in parentheses refer to the highest resolution shell.

	His-tag	Se-Met His-tag	Native	Apo enzyme	Hg derivative
Resolution (Å)	20.0–2.6	20.0–2.6	20.0–2.9	20.0–3.1	20.0–3.5
Completeness (%)	95.4 (95.1)	97.4 (95.6)	95.5 (97.6)	92.6 (93.8)	92.3 (91.9)
$R_{\rm merge}$ (%)	7.2 (32.8)	6.4 (26.4)	8.1 (33.6)	8.9 (33.1)	14.7 (35.7)
No. of observations	29737	32656	28839	16894	11099
No. of unique reflections	15591	15940	11259	8746	6189
$I/\sigma(I)$	10.1 (2.4)	11.0 (3.6)	7.9 (3.0)	7.9 (2.5)	4.7 (2.5)
$R_{\rm scale}$ against His-tag peptidase T	—	14.3 (23.3)	10.6 (21.5)	22.1 (25.9)	19.7 (25.4)

*Nco*I/*Xho*I. *E. coli* B834 cells were transformed with the resulting plasmid pCM550 to yield strain TN5619 overexpressing peptidase T with a C-terminal His tag.

2.2. Purification of peptidase T

S. typhimurium strain TN5445 was grown in Lennox L Broth containing 50 µg ml^{-1} ampicillin in an aerated fermentor under vigorous stirring. Peptidase T overexpression was induced by addition of 1 m*M* isopropyl β-D-thiogalactopyranoside (IPTG) at an OD_{600} of 0.5. Frozen cell pellets from 5 l of culture were suspended and sonicated in a minimal amount of 50 m*M* Tris–HCl pH 7.5. After removal of the cell debris by centrifugation, the supernatant was subjected to ion-exchange chromatography on a Q-Sepharose column (70 ml) in 50 m*M* Tris pH 7.5 and a 0–1 *M* NaCl gradient. This was followed by gel filtration on Superdex 200 (350 ml) and MonoQ ion exchange. The enzyme was finally concentrated to 10 mg ml^{-1} in 10 m*M* Tris pH 7.5 and stored frozen until required.

The His-tag derivative of peptidase T could be prepared in a single step. Cells of strain TN5619 were grown in LB medium at 310 K in the presence of 100 µg ml^{-1} kanamycin. 1 m*M* IPTG was added at an OD_{600} of 0.5 and the cells were harvested 3 h later. Frozen pellets from 100 ml of culture were suspended in 50 m*M* Tris pH 7.5 and sonicated. A minimal amount of DNAase was added to the supernatant in order to reduce the viscosity. This was loaded onto a 3.5 ml nickel–agarose gel column (Qiagen), washed with 50 m*M* Tris, 50 m*M* NaCl, 20 m*M* imidazole pH 7.5 and eluted with 250 m*M* imidazole and stored as before.

Selenomethionine derivatives of peptidase T and His-tag peptidase T were produced by methionine auxotrophic strains TN5446 and TN5619, respectively. Cells were grown in 1–2 l of LeMaster medium (LeMaster & Richards, 1985) containing selenomethionine and 10 µ*M* $ZnSO_4$. The cultures were induced after 8 h and grown overnight. 1 m*M* mercaptoethanol was included during nickel–agarose chromatography and 1 m*M* DTT was included during all other steps.

2.3. Crystallization and data collection

Crystals were obtained by hanging-drop vapour diffusion at 293 K. 2 µl of protein solution was mixed with 2 µl of precipitant and equilibrated against the latter. Crystals formed in a variety of salts over a broad pH range. Best results were obtained using either sodium/potassium tartrate or ammonium sulfate as precipitant in the pH range 7–9 with bis-tris or Tris buffer. Mercury derivatization was achieved by soaking the crystals in 5 m*M* thimerosal for 2 h followed by back-transfer to the mother liquor for 0.5 h. Apoenzyme crystals were obtained by adding 10 m*M* dipicolinic acid to the hanging drop after crystallization, followed by overnight incubation.

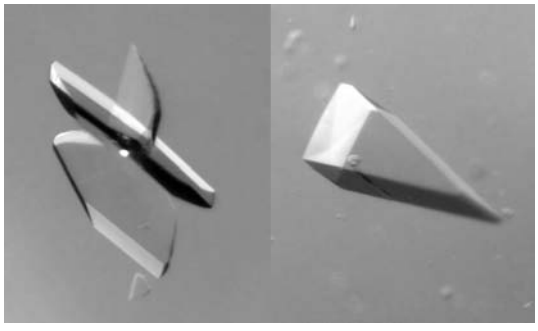

Figure 1
Crystals of native peptidase T (left) and His-tag selenomethionine peptidase T (right) grown from ammonium sulfate solutions at pH 7.5. The His-tag peptidase T crystals frequently grow as triangular plates that diffract better than the native peptidase T crystals.

X-ray data were collected using an R-AXIS II image-plate detector and a Rigaku rotating-anode X-ray generator (with λ = 1.54 Å) on crystals flash-frozen at 120 K. The crystals were transferred into mother liquor with glycerol (30%) or sucrose (50%) in a single step and were immediately mounted on rayon loops and inserted into the Cryostream. The data were processed using *DENZO* (Otwinowski, 1993) and *SCALEPACK*. Se-atom positions were determined using *CNS* (Brunger *et al.*, 1998). Most other calculations were performed with the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994).

3. Results

Crystals of peptidase T were easily formed within 24–48 h and continued to grow for up to a week. The symmetry of the diffraction pattern is consistent with space group *C*2. Initially, crystallization took place in sodium/potassium tartrate, with unit-cell parameters a = 130.9, b = 42.1, c = 111.3 Å, β = 115.6°. Freezing in 30% glycerol resulted in a very high mosaicity and a change in unit-cell parameters to a = 132.9, b = 45.1, c = 97.7 Å, β = 115.9°. Crystals grown in ammonium sulfate behaved better and had unit-cell parameters a = 135.1, b = 45.5, c = 99.6 Å, β = 117.4° and a V_m (Matthews, 1968) of 3.0 Å^3 Da^{-1}. These crystals were frozen in 50% sucrose, with a much smaller and reproducible change in unit-cell parameters: a = 132.7, b = 45.9, c = 96.6 Å, β = 116.4°. Data to 2.9 Å resolution could be collected using these crystals. Two Hg atoms were isomorphously incorporated by soaking the crystals in thimerosal followed by data collection either at room temperature (crystals grown in tartrate) or frozen in 50% sucrose (crystals grown in ammonium sulfate). The position of one of these Hg atoms was determined from a difference Patterson map and the second from a difference Fourier map, although the resolution (3.5 Å) and the Hg-atom occupancy were low.

Similarly, data from crystals treated with 10 m*M* dipicolinic acid were used to calculate a $|F_{\rm native}| - |F_{\rm apo}|$ difference Fourier map using phases from the mercury derivative. A single strong signal could be identified, which is somewhat surprising since carboxypeptidase G2 is known to have two metal ions in the active site. Crystal growth of selenomethionine peptidase T was inferior and was inhibited by the presence of DTT.

His-tag peptidase T yielded crystals diffracting to 2.6 Å, although they were often smaller than the native enzyme crys-

tals. The crystals of the His-tag derivative usually have a more distinct morphology, growing as triangular plates (Fig. 1). They belong to the same space group and data collected on the His-tag enzyme crystals scale well with native data (Table 1). Moreover, the selenomethionine derivative of this specimen also grew well in the presence of DTT. All 15 Se atoms were picked up from difference Patterson data with the *CNS* program package (Brunger *et al.*, 1998). These positions were refined with *MLPHARE*, resulting in modest but significant occupancies. In contrast, when some of the weaker sites were replaced by randomly chosen coordinates, the occupancy vanished and the phasing power decreased. Moreover, phases calculated from these data and $|F_{\rm Hg}| - |F_{\rm native}|$ structure factors were used to independently locate the Hg-atom positions. The result was consistent with the initial determination, although the enantiomorph ambiguity has not yet been resolved. Owing to the low quality of the mercury derivative and presumably also to the low number of centric reflections in the monoclinic space group, the available phases do not permit us to solve the structure of the enzyme. However, the methionine positions, a zinc position and two mercury reactive sites have been identified. The selenomethionine peptidase T crystals will be subjected to multiple-wavelength anomalous diffraction (MAD) data collection. The present work demonstrates that the use of a His-tag derivative not only facilitates protein purification but can also result in crystals of improved quality.

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