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The adhesive PitA pilus protein from the early dental plaque colonizer *Streptococcus oralis*: expression, purification, crystallization and X-ray diffraction analysis**Proof instructions**

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Synopsis: The recombinant production and crystallization of the PitA adhesin from the *Streptococcus oralis* pilus island 2-encoded sortase-dependent pilus is described. Limited proteolysis of PitA and its complex with terbium crystallophore gave greatly improved X-ray diffraction to 2.16 Å resolution and a sufficiently strong anomalous signal for terbium SAD phasing, respectively.

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Keywords: adhesins; PitA; PI-2 sortase-dependent pilus; *Streptococcus oralis*; proteolysis; terbium crystallophore

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The adhesive PitA pilus protein from the early dental plaque colonizer *Streptococcus oralis*: expression, purification, crystallization and X-ray diffraction analysis

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PitA is the putative tip adhesin of the pilus islet 2 (PI-2)-encoded sortase-dependent pilus in the Gram-positive *Streptococcus oralis*, an opportunistic pathogen that often flourishes within the diseased human oral cavity. Early colonization by *S. oralis* and its interaction with *Actinomyces oris* seeds the development of oral biofilm or dental plaque. Here, the PI-2 pilus plays a vital role in mediating adherence to host surfaces and other bacteria. A recombinant form of the PitA adhesin has now been produced and crystallized. Owing to the large size (~100 kDa), flexibility and complicated folding of PitA, obtaining diffraction-quality crystals has been a challenge. However, by the use of limited proteolysis with α -chymotrypsin, the diffraction quality of the PitA crystals was considerably enhanced to 2.16 Å resolution. These crystals belonged to space group *P1*, with unit-cell parameters $a = 61.48$, $b = 70.87$, $c = 82.46$ Å, $\alpha = 80.08$, $\beta = 87.02$, $\gamma = 87.70^\circ$. The anomalous signal from the terbium derivative of α -chymotrypsin-treated PitA crystals prepared with terbium crystallophore (Tb-Xo4) was sufficient to obtain an interpretable electron-density map via terbium SAD phasing.

1. Introduction

Early colonization by Gram-positive *Streptococcus* and *Actinomyces* species and their adherence to salivary pellicle-coated tooth surfaces, along with mutual co-aggregation, generate likely tipping points in the further development of oral biofilm or dental plaque (Kolenbrander *et al.*, 2006, 2010; Li *et al.*, 2004). The co-aggregation between such early colonizers helps to seed biofilm growth by establishing suitable environmental conditions for secondary or subsequent colonizers, which also include Gram-negative bacteria (Kolenbrander *et al.*, 2010). Pili (or fimbriae), the hair-like surface appendages on some early-colonizer bacteria, have been shown to play a crucial role in mediating attachment to the host and other bacterial cells during biofilm formation (Hamada *et al.*, 1998; Mishra *et al.*, 2010, 2011; Okahashi *et al.*, 2010, 2011; Reardon-Robinson *et al.*, 2014; Vitkov *et al.*, 2001; Zähler *et al.*, 2011). *S. oralis* is part of the normal microflora of the mouth in humans and other primates and is regarded as one of the earliest colonizing bacteria in dental plaque biofilm (Denapate *et al.*, 2016; Kolenbrander & London, 1993). *S. oralis*, which belongs to the mitis group of streptococci, is also known to be an opportunistic pathogen commonly associated with infective endocarditis and other infections (Douglas *et al.*, 1993; Hosokawa *et al.*, 2018; Roy *et al.*, 2016; Shelburne *et al.*, 2014; Thiagarajan *et al.*, 2016).

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Table 1

Macromolecule-production information.

Source organism	<i>S. oralis</i> (ATCC 35037)
DNA source	<i>S. oralis</i> (ATCC 35037)
Forward primer†	5'-TTCGAATTTCGATTCTACTACAGAACCTCAGACAAC-3'
Reverse primer‡	5'-TTCGCGGCCGCTCCGGTTTCAGGTACACTGTTCTATTATTTCG-3'
Cloning vector	pET-32a(+) (Novagen)
Expression vector	pET-32a(+) (Novagen)
Expression host	<i>E. coli</i> BL21(DE3) pLysS
Complete amino-acid sequence of the construct produced§	MSDKIIHLTDDSFDTDLVKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLINIDQNPGTAPKYGIR GIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSGHMHSSGLVPRGSGMKEATAAKFERQHM DSPDLGTDDEDDKAMADIGSEFDSTTEPQTTLHKITIPISGDDKYELSLDITSKLGTETQTDPLDVLVADL SGSMQNDVQSFQDRTISRIDALKNTLRGTNGRGLDITLSNSNNRLSMVGGKIDNKKVDQYWDGNKWR LFRPYWPYERMTKKYDGVSPWDDANTILGWSNNARAATAVYNMSIAGGNSIGTESGIGTGTNIGAGLTLAN QLMGARSNAKKVILLSDGFANMVYDANGYTIYNNEDPNIETAPQWFWDRLNNNLNLSYSLAPTLDFG YSIKFRYSNNVDSITSLQYYMRQHNASIPNEIFSANDEDQLRDSFKDITDKILPLGIHHVSIKDVLSKYVQL LPNGSSEFRVVEKDGSEILTENQVTFDTKTSTSEGLVEVTAKFSFNYSLELDGARYVLKFTVTSSQALDAI AGDKLEAGDAEGSDVNKLYSNKGASVTYSYGIGNSQTKTKEYSDNPTFKPSDPLTVPVEVEWQGVGTGARTV ITADQPSNVELKLQVKNKNGGSDNQDYRKTNNVSKNVSNETRNFKEKVGQYQDLIAPDVPFATKEIKNVG TESNPSFKVIYKQLPSLTIKKLEAENNLNKEFRIKVKLTSPDSKPLNGTFGEITVVNGEAEIRVEKRRWR GILSYLPRGTHYKVEEAASTNGYHVTYENQEGDLNKDETSTVTHHKLPSLSVTTKVGVFANLLKSFKITI NIRDAQNSPLNGTYTATVNNKRTPLQFTNGRASIDLNKDQTIKIDGLPLDSHYTVEETNSSRGYQVSYENQ EGKLDGDKSATVTNNKNSVPETCCR

† The EcoRI site is underlined. ‡ The NotI site is underlined. § Cloning artefacts are underlined or coloured. (Trx tag, red; hexahistidine tag, blue; thrombin recognition site, pink; S-tag, green; enterokinase recognition site, brown.)

Recently, the genomic presence of pilus-islet 2 (PI-2)-encoded pili was identified among mitis-group oral streptococci (Zähner *et al.*, 2011). In *S. oralis*, the PI-2 gene cluster encodes five proteins for the assembly of a sortase-dependent pilus: the tip PitA and backbone PitB pilin subunits, the SrtG1 and SrtG2 sortase enzymes and the SipA signal peptidase. Immuno-electron microscopy and bioinformatics analysis have indicated that PitA is positioned as the ancillary tip pilin of the PI-2 pilus and also contains the von Willebrand factor type A (vWFA) domain with a metal-ion-dependent adhesion site (MIDAS). The role of the vWFA domain in mediating protein–protein interactions is well known in both eukaryotes and prokaryotes (Whittaker & Hynes, 2002). Moreover, the vWFA domain has been identified amongst tip pilins of other types of sortase-dependent pili as well as in helping to facilitate adherence activities between host cells or other bacteria (Izoré *et al.*, 2010; Kankainen *et al.*, 2009; Kant *et al.*, 2016; Konto-Ghiorghi *et al.*, 2009; Krishnan *et al.*, 2013; Nielsen *et al.*, 2012). Presumably, *S. oralis* PitA might have a similar adhesive role, although the molecular identification of its receptor sites still remains to be uncovered. Among the other PI-2-encoded proteins, PitB represents the major pilin forming the pilus backbone, whereas SrtG1 and SrtG2 are pilin-specific C-type sortases catalyzing the pilus-polymerization process. Intriguingly, the PI-2 pilus conserved among mitis-group streptococci exhibits certain unusual features that differ from typical sortase-dependent pili (Bagnoli *et al.*, 2008; Telford *et al.*, 2006; Ton-That & Schneewind, 2004; Zähner *et al.*, 2011). These include the surface expression of only a single pilus structure, an absent basal pilin in the pilus structure, a missing YPKN pilin motif in the backbone pilin, a noncanonical C-terminal LPXTG-like motif (VTPTG/VPETG) in each pilin type and the requirement for a signal peptidase (SipA) during pilus assembly.

Thus far, the crystal structures of PitB and SipA from *S. pneumoniae* have been reported (Shaik *et al.*, 2015).

However, the X-ray structure determination of the PI-2 pilus tip adhesin from a mitis-group Gram-positive host has yet to be accomplished. This mainly arises from the large size, multiple domains, flexibility and complicated folding patterns that are inherent to the tip pilins in sortase-dependent pili. In order to reveal the structural basis for the interaction between the adhesive tip pilin and host cells or other bacteria during dental plaque biofilm formation, we have expressed, purified and crystallized *S. oralis* PitA. We further used a combination of limited proteolysis and terbium crystallophore (Tb-Xo4) to improve the diffraction quality of PitA crystals to facilitate structure solution.

2. Materials and methods

2.1. Macromolecule production

The region of the *S. oralis* (ATCC 35037) *pitA* gene corresponding to the mature PitA protein (residues 36–828) was cloned into pET-32a(+) (Stratagene) and expressed in the *Escherichia coli* BL21(DE3) pLysS expression strain (Zähner *et al.*, 2011). Recombinant PitA protein (PitA_{Trx}) lacks an N-terminal signal peptide (residues 1–35) and C-terminal transmembrane region (residues 830–863), but instead contains a thioredoxin–hexahistidine tag with an enterokinase cleavage site at its N-terminus (Table 1). For purification, 1 l Luria–Bertani (LB) medium containing 100 µg ml⁻¹ ampicillin was inoculated with 1% overnight starter culture and incubated at 310 K until an OD₆₀₀ of 0.6 was reached. PitA_{Trx} protein expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside and the cells were then allowed to grow overnight at 304 K. The cell pellets were recovered by centrifugation, resuspended in lysis buffer (20 mM sodium phosphate pH 8.0, 400 mM NaCl) containing EDTA-free protease-inhibitor (Roche) and then disrupted by sonication. The cell suspension was then centrifuged at 48 400g for 60 min

at 277 K to remove any insoluble debris. The cell-free lysate was applied onto a 5 ml HiTrap chelating column containing nickel-affinity resin (GE Healthcare) that had been pre-equilibrated with lysis buffer. Any protein contaminants were rinsed from the column with lysis buffer containing 20 mM imidazole. Bound PitA_{Trx} protein was eluted from the resin using a linear gradient of lysis buffer containing 400 mM imidazole. SDS-PAGE was used to identify PitA_{Trx}-containing eluted fractions, which were then pooled and dialysed overnight at 277 K against dialysis buffer (20 mM HEPES pH 8.0, 150 mM NaCl). To remove the N-terminal thioredoxin-hexahistidine tags, the dialyzed PitA_{Trx} protein was treated with enterokinase (which had been purified in-house according to an established protocol; Skala *et al.*, 2013) for 24 h at 310 K using a 1:10 000 protease:protein ratio. Untagged PitA_{FL} protein (full-length mature PitA) was separated from any remaining tagged protein by nickel-affinity chromatography. The PitA_{FL} protein was concentrated using an Amicon ultrafiltration centrifugal device fitted with a 10 kDa cutoff membrane and then gel-filtered on a Sephacryl 200 (26/60) column (GE Healthcare) containing dialysis buffer (Fig. 1).

Selenomethionine (SeMet)-substituted PitA (PitA_{SeMet}) was purified using the same protocol as described above. For this, the PitA-containing plasmid (see above) was transformed into auxotrophic *E. coli* B834 cells and the cells were then grown in Selenomethionine Medium Base plus Nutrient Mix (Molecular Dimensions) supplemented with 50 mg l⁻¹ L-SeMet. Purified PitA protein in dialysis buffer was treated to limited proteolysis with various proteases (trypsin, α -chymotrypsin and subtilisin) at 310 K using various concentrations and time intervals. The extent of digestion was monitored by SDS-PAGE, which showed that α -chymotrypsin was optimal for producing a major stable fragment of ~60 kDa after 24 h incubation at a 1:10 000 protease:protein ratio. A large-scale sample of α -chymotrypsin-treated protein (PitA_{Chy}) was subsequently purified by gel filtration in the presence of 1 mM phenylmethylsulfonyl fluoride.

2.2. Crystallization

Initial crystallization screening trials were performed in 96-well sitting-drop crystallization plates using a Mosquito

Figure 1 Macromolecular production of PitA. (a) Gel-filtration chromatographic profiles of PitA_{Trx} (blue trace), PitA_{FL} (green trace) and PitA_{Chy} (red trace) on a Sephacryl S200 column. The elution volume of the main peak is indicated. (b) SDS-PAGE analysis of PitA after gel filtration. Lane M, molecular-weight markers (labelled in kDa); lane 1, recombinant PitA_{Trx}; lane 2, PitA_{FL}; lane 3, PitA_{Chy}.

Figure 2 Crystallization of recombinant PitA. (a) Crystals of PitA_{FL}. (b) Crystals of PitA_{Chy}.

Table 2
Crystallization.

	PitA _{FL}	PitA _{Chy}
Method	Hanging-drop vapour diffusion	Hanging-drop vapour diffusion
Plate type	VDX plate (Hampton Research)	VDX plate (Hampton Research)
Temperature (K)	295	295
Protein concentration (mg ml ⁻¹)	30	40
Buffer composition of protein solution	20 mM HEPES pH 8.0, 150 mM NaCl	20 mM HEPES pH 8.0, 150 mM NaCl
Composition of reservoir solution	1 M potassium iodide, 25% PEG 3350, 100 mM HEPES pH 7.8	0.2 M ammonium acetate pH 7.2, 20% PEG 3350
Volume and ratio of drop	2 µl, 1:1	2 µl, 1:1
Volume of reservoir (ml)	1	1

Crystal automated liquid-dispensing system (TTP Labtech) at 295 K with a protein:precipitant ratio of 1:1. Roughly 1440 different conditions from commercial screens were used in the first round of crystallization trials. Very small plate-like crystals were observed for PitA_{FL} in a screening condition after about three months (Fig. 2, Table 2) when the protein concentration was 30 mg ml⁻¹. Further optimization was performed by altering the precipitant concentration and pH, and by using different additives with the hanging-drop vapour-diffusion method. A similar protocol was used in crystallization trials for PitA_{SeMet} and PitA_{Chy}. Although crystals of PitA_{SeMet} were obtained, their diffraction was weak and further optimization gave no improvements. However, the PitA_{Chy} crystals that appeared after a 15-day incubation showed better X-ray diffraction when the protein concentration was increased to 40 mg ml⁻¹. Further optimization of the initial conditions produced thick plate-like crystals (Fig. 2, Table 2). PitA_{Chy} crystals were soaked for 20 min in mother liquor containing 100 mM Tb-Xo4 (Polyvalan Crystallophore No. 1, Molecular Dimensions; Engilberge *et al.*, 2017, 2019) to prepare a terbium-derivatized crystal form (PitA_{ChyTb}).

To confirm the identity of the crystal, peptide mass fingerprinting (PMF) was carried out with a trypsinized sample using an AB SCIEX Triple TOF (time-of-flight) 5600 mass-spectrometer system equipped with an ESI (electron spray ionization) source. For this, crystals were washed in mother

liquor and resolved by SDS-PAGE. Stained protein bands in the gel were excised and exposed to repeated dehydration and rehydration cycles, first with a 2:1 mixture of acetonitrile (ACN) and 50 mM ammonium bicarbonate (ABC) for 5 min and thereafter with 25 mM ABC for 2 min. The gel pieces were then trypsinized (20 µg ml⁻¹ trypsin in 25 mM ABC) overnight at 310 K. For protein identification, the peptide mass-spectra data were searched against the NCBI non-redundant protein database using the *Paragon* search engine (Fig. 3).

2.3. Data collection and processing

Initial X-ray diffraction experiments were performed at the home source (Xenocs GeniX^{3D} Cu HF microbeam X-ray generator with a MAR 345 image-plate detector). High-resolution diffraction data for PitA_{FL} crystals were collected at a synchrotron source (the BM14 beamline at the ESRF, Grenoble, France) using 30%(v/v) lithium acetate dihydrate as a cryoprotectant. X-ray diffraction data for PitA_{Chy} were collected on the ID30 beamline at the ESRF. Anomalous X-ray diffraction data sets were collected from a PitA_{ChyTb} crystal containing terbium ions on the ID29 beamline at the ESRF near the *L*₁ absorption edge of terbium ($\lambda = 1.42379$ Å). 30%(v/v) ethylene glycol was used as a cryoprotectant for the PitA_{Chy} and PitA_{ChyTb} crystals at the synchrotron sources.

MNIRFDFKKV	QIIARRLVTL	LAILFLCAPI	SLLNADSTTE	PQTTLHKITIT	PISGQDDKYE	60
LSLDITSKLG	TETQTDPLDV	VLVADL SGSM	QNQDVQSFDFG	RTISRIDALK	NTLRGTNGRK	120
GLIDTILSNS	NNRLSMVGFG	GKIDNKKVDQ	YWDGNKWRLF	RPYWPYERMT	KYYDGVSPWD	180
DANTILGWSN	NARAAKTAVY	NMSIAGGNSI	GTESGIGTG T	NIGAGLTLAN	QLMGARSNA	240
KKVVILLS DG	FANMVYDANG	YTIYNNYNNED	PNIETAPQWF	WDRLLNNLNS	LSYSLAPTLD	300
GFYSIKFRYS	NNVDSITSLQ	YYMRQHNASI	PNEIFSANDE	DQLRDSFKDI	TDKILPLGIH	360
HVSIKDVLSK	YVQLLPNGSS	EFRVVKEKDG	SSEILLENQV	TFDTKTTSEG	LVEVTAKFSP	420
NYSLEDGARY	VLKFTVTSSQ	EALDAIAGDK	KLEAGDAEGS	DVNKLYSNKG	ASVTYSYGIG	480
NSQTKTKEYS	DNPTFKPSDP	LTVPVEVEWQ	GVTGART VIT	ADQPSNVELK	LVQKNKNGGS	540
DNQDYRKTNV	NVSKNVSNET	RNFEKVAKGY	QYDLIAPDVP	AFTKEIKNVG	TESKPSFKVI	600
YKQLPSLTIK	KVLEAENNLN	KEFRIKVKLT	SPDSKPLNGT	FGEITVVNGE	AEIRVEKRKR	660
WEGILSYLPR	GTHYKVEEEA	ASTNGYHVTY	ENQEGDLNKD	ETSIVTNHKL	PSLSVTKKVT	720
GVFANLLKSF	KITINIRDAQ	NSPLNGTYTA	TVNNKRTPLQ	FTNGRASIDL	NKDQTIKIYG	780
LPLDSHYTVE	EESNSSRGYQ	VSYENQEGKL	DGDKSATVTN	NKNSVPETGV	DFLSSTLMLG	840
IILPLGGIFF	TILGLYLVVH	RRK				863

Figure 3

PMF analysis of PitA_{FL} and PitA_{Chy}. Matched peptides in the PitA_{FL} (bold) and PitA_{Chy} (underlined) crystals are indicated. Residues corresponding to the MIDAS motif (DXSXS...T...D) of the vWFA domain are underlined and in italics.

Table 3
Data collection and processing.
Values in parentheses are for the outer shell.

	PitA _{FL}	PitA _{Chy}	PitA _{ChyTb}
Diffraction source	BM14, ESRF	ID30, ESRF	ID29, ESRF
Wavelength (Å)	1.7712	0.97951	1.42379
Temperature (K)	100	100	100
Detector	MAR CCD 225	PILATUS 6M	PILATUS 6M
Crystal-to-detector distance (mm)	163	336	393
Rotation range per image (°)	0.5	0.1	0.1
Total rotation range (°)	180	300	360
Exposure time per image (s)	8	0.05	0.037
Space group	<i>P</i> 2 ₁ 2 ₁ 2	<i>P</i> 1	<i>P</i> 1
<i>a</i> , <i>b</i> , <i>c</i> (Å)	52.27, 422.92, 48.39	61.48, 70.87, 82.46	61.81, 70.28, 79.88
α , β , γ (°)	90, 90, 90	80.08, 87.02, 87.70	79.88, 86.89, 87.18
Resolution range (Å)	51.87–3.26 (3.26–3.20)	69.78–2.16 (2.20–2.16)	61.65–3.00 (3.05–3.00)
Total No. of reflections	129571	208886	154689
No. of unique reflections	18638	65632	26816
Completeness (%)	99.8 (100.0)	89.9 (90.5)	98.0 (95.7)
Multiplicity	7.0 (7.1)	3.2 (3.2)	5.8 (5.3)
<i>CC</i> _{1/2}	0.99 (0.88)	0.99 (0.85)	0.99 (0.99)
<i>I</i> / σ (<i>I</i>)	11.6 (2.5)	14.0 (2.0)	21.3 (5.9)
<i>R</i> _{meas}	0.178 (0.781)	0.053 (0.559)	0.067 (0.178)
<i>R</i> _{p.i.m.}	0.068 (0.290)	0.029 (0.308)	0.027 (0.074)
Overall <i>B</i> factor from Wilson plot (Å ²)	78	46	63
DANO/sd(DANO)	1.06	—	1.198

Diffraction data were indexed and integrated using *XDS* (Kabsch, 2010) and were scaled with *AIMLESS* (Evans & Murshudov, 2013). SAD phasing calculations were performed using the *CRANK2* module in *CCP4* (Skubák & Pannu, 2013; Winn *et al.*, 2011), which identified two terbium sites with occupancies of >0.3.

3. Results and discussion

Initially, mature *S. oralis* PitA protein (residues 36–829) was cloned in pET-28a expression vector (Novagen) for soluble production in the *E. coli* BL21(DE3) strain. However, despite being soluble, homogeneous and pure, this N-terminally histidine-tagged protein did not yield any positive hits in crystallization trials. As a remedy for this, another construct (Zähner *et al.*, 2011), in which PitA had been cloned in pET-32a(+) vector as an N-terminally thioredoxin–hexahistidine-tagged protein (PitA_{Trx}) for expression in the *E. coli* BL21(DE3) pLysS strain (see Section 2.1, Fig. 1 and Table 1), was instead used in crystallization trials. Following the removal of the N-terminal tag, PitA_{FL} now gave thin plate-like crystals (Fig. 2, Table 2). PMF analysis confirmed the presence of PitA in these crystals (Fig. 3). Moreover, the PitA_{FL} crystals were suitable for X-ray analysis and diffracted to 3.2 Å resolution at a synchrotron source, belonging to space group *P*2₁2₁2 and exhibiting unit-cell parameters *a* = 52.27, *b* = 422.92, *c* = 48.39 Å (Table 3, Supplementary Fig. S1). Matthews coefficient (*V*_M = 3.205 Å³ Da^{−1}) and solvent-content (*V*_S = 59.7%) calculations indicated the presence of a single molecule in the asymmetric unit of this crystal form. PitA appears to have only limited sequence identity to other known pilin structures, as no significant similarity was found when *BLAST* searches against the Protein Data Bank were performed using the PitA_{FL} sequence as a query. On the other hand, a *DELTA-*

BLAST (Domain Enhanced Lookup Time Accelerated *BLAST*) search results identified RrgA from *S. pneumoniae* (Izoré *et al.*, 2010) and GBS104 from *S. agalactiae* (Krishnan *et al.*, 2013) as the top two best hits, with each having 24% sequence identity (Supplementary Fig. S2). Owing to the presence of seven methionines, SeMet-substituted PitA protein (PitA_{SeMet}) was produced to solve the structure using selenium SAD phasing. Unfortunately, PitA_{SeMet} crystals diffracted to a low resolution of ~4.5 Å and further improvements were unsuccessful. Since the screening solution that yielded PitA_{FL} crystals contains potassium iodide, iodide SAD phasing was also carried out using the collected data set, but was deemed to be unsuccessful owing to the weak anomalous signal.

As an alternative, limited proteolysis of PitA with α-chymotrypsin was performed and yielded an ~60 kDa stable fragment (PitA_{Chy}; Fig. 1). The crystals formed by the PitA_{Chy} protein diffracted to 2.16 Å resolution and belonged to space group *P*1, with unit-cell parameters *a* = 61.48, *b* = 70.87, *c* = 82.46 Å, α = 80.08, β = 87.02, γ = 87.70° (Table 3, Supplementary Fig. S1). As Tb-Xo4 has recently been shown to assist in nucleation and terbium SAD phasing (Engilberge *et al.*, 2017), PitA_{Chy} protein co-crystallization and PitA_{Chy} crystal-soaking experiments were both attempted. On comparison of these two methods, the Tb-Xo4-soaked crystals diffracted slightly better and achieved a greater data completeness (Table 1). Moreover, the calculated Matthews coefficient (*V*_M = 2.01 Å³ Da^{−1}) suggested the presence of two molecules in the asymmetric unit with a solvent content of 38.9%. As a result of the terbium ions (occupancies of >0.3), the anomalous signal was sufficiently strong for Tb-SAD phasing and thus enabled an interpretable electron-density map of PitA to be obtained. Model building and refinement are currently under way and the outcome will be reported shortly.

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