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Structure Report

Use of a "silver bullet" to resolve crystal lattice dislocation disorder: A cobalamin complex of Δ^1-pyrroline-5-carboxylate dehydrogenase from *Mycobacterium tuberculosis*

Thomas Lagautriere, Ghader Bashiri, Edward N. Baker *

Structural Biology Laboratory, School of Biological Sciences and Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, Auckland 1010, New Zealand

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ABSTRACT

The use of small molecules as "silver bullets" that can bind to generate crosslinks between protein molecules has been advanced as a powerful means of enhancing success in protein crystallization (McPherson and Cudney, 2006). We have explored this approach in attempts to overcome an order–disorder phenomenon that complicated the structural analysis of the enzyme Δ^1-pyrroline-5-carboxylate dehydrogenase from *Mycobacterium tuberculosis* (P5CDH, *Mtb*-PruA). Using the Silver Bullets Bio screen, we obtained new crystal packing using cobalamin as a co-crystallization agent. This crystal form did not display the order–disorder phenomenon previously encountered. Solution of the crystal structure showed that cobalamin molecules are present in the crystal contacts. Although the cobalamin binding probably does not have physiological relevance, it reflects similarities in the nucleotide-binding region of *Mtb*-PruA, with the nucleotide loop of cobalamin sharing the binding site for the adenine moiety of NAD^+.

1. Introduction

The use of small molecules as "silver bullets" that can generate crosslinks between protein molecules and so enhance success in protein crystallization has been recently advanced as an important tool for structural biology (McPherson and Cudney, 2006). Whereas reagents such as salts and polyethylene glycols are widely used to manipulate solubility and phase separation in protein crystallization, small molecules with multiple functional groups of an appropriate kind can generate the kinds of intermolecular crosslinks that can be crucial to the formation of a well-ordered crystal, suitable for structural analysis.

We report here the use of this approach for overcoming an order–disorder phenomenon that complicated the structural analysis of an essential enzyme from *Mycobacterium tuberculosis* (*Mtb*), the causative agent of TB. This NAD^+-dependent enzyme, Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH, *Mtb*-PruA), is involved in the utilisation of proline, a process implicated as an important factor in the adaptation of mycobacteria to slow growth rate and hypoxia (Berney et al., 2012). The enzyme crystallized readily using polyethylene glycol as precipitant, but initial crystals displayed an unusual arrangement of ordered and disordered molecules, resulting in gaps of ~102 Å between ordered layers of ~98 Å (Lagautriere et al., 2014). To try to overcome this, we used the Silver Bullets Bio additives screen (McPherson and Cudney, 2006) to seek a new crystal form, while still using the original crystallization condition, which were the only conditions that yielded *Mtb*-PruA crystals.

A new crystal form was indeed obtained, without disorder. The intriguing pink colour of the crystals was found to be due to the presence of cobalamin (vitamin B_{12}), from the Silver Bullets Bio screen, and analysis of the crystal structure shows that the cobalamin additive exploits the nucleotide binding site in *Mtb*-PruA and generates crystal contacts that determine the new crystal form and eliminate the disorder.

2. Protein production and crystallization

Mtb-PruA was expressed and purified as described previously (Lagautriere et al., 2014). The apo-PruA protein (15 mg/mL) was screened against the Silver Bullets Bio additive screen (Hampton

Abbreviations: P5C, Δ^1-pyrroline-5-carboxylic acid; GSA, glutamate-γ-semialdehyde; P5CDH, Δ^1-pyrroline-5-carboxylate dehydrogenase; MAD, multiwavelength anomalous dispersion; MPD, 2-methyl-2,4-pentandiol; FAD, flavin adenine dinucleotide; NAD^+, nicotinamide adenine dinucleotide; DMB, dimethylbenzimidazole.

* Corresponding author. Fax: +64 9 373 7414.
E-mail address: en.baker@auckland.ac.nz (E.N. Baker).

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Research) using sitting drop vapour diffusion at 18 °C. The original precipitation solution, from the Morpheus screen (Gorrec, 2009), was retained. This comprised 0.1 M Bicine/Tris pH 8.1, 12% polyethylene glycol (PEG) 1000, 12% PEG 3350, 12% 2-methyl-2,4-pentandiol (MPD) and 0.03 M each of $NaNO_3$, Na_2HPO_4 and $(NH_4)_2SO_4$. Crystallization drops contained reservoir solution, protein solution and Silver Bullets Bio screen at a 1:2:1 ratio, in a total drop size of 2.0 μL. New pink-coloured crystals grew within a week, in condition 65 of the Silver Bullets Bio additive screen [0.2% w/v biotin, 0.2% w/v phospho(enol)pyruvic acid monosodium salt hydrate, 0.2% w/v D-(+)-Melezitose hydrate, 0.2% w/v cobalamin and 0.02 M HEPES sodium pH 6.8]. These crystals were also soaked in 250 μM NAD^+ for 2–3 min before cryoprotection, to explore nucleotide binding.

3. Data collection and structure determination

The crystals were flash-cooled in liquid nitrogen, and X-ray diffraction data collected at 110 K on beamline MX1 of the Australian Synchrotron, using 0.5° oscillations per frame over a total range of 360° degrees for each data set. The data were indexed and processed with *XDS* (Kabsch, 2010), reindexed with *POINTLESS* (Evans, 2006) and scaled with *SCALA*. The crystals were trigonal and belong to the space group $P3_121$. The crystal structure was solved at 2.27 Å resolution, by molecular replacement with *MolRep* (Vagin and Teplyakov, 2010) using apo-PruA as a search model (PDB code 4IDM). The structure was refined by cycles of manual building using *COOT* (Emsley and Cowtan, 2004) and refinement using *REFMAC5* (Murshudov et al., 2011). Data processing and refinement statistics are outlined in Table 1.

4. Cobalamin binding creates new crystal form

The pink-coloured crystals of *Mtb*-PruA protein had a different morphology from the previously-grown crystals (Fig. 1) and proved to belong to the trigonal space group $P3_121$, with unit cell dimensions $a = b = 164.3$ Å, $c = 259.1$ Å, $\gamma = 120°$. This is a distinctly different crystal form from the original hexagonal crystals, in space group *P*622, and displays none of the disorder seen previously. The crystal structure contains three PruA dimers, arranged in a loosely packed hexamer, together with four cobalamin molecules. These cobalamin molecules occupy intermolecular sites in the crystal, bound to equivalent sites on the external faces of four PruA molecules (two dimers) but also making additional contacts with neighbouring molecules in the crystal (Fig. 2). Two PruA molecules (one dimer) have no associated cobalamin, apparently because this cobalamin-free dimer is further away from neighbouring molecules than are the other two dimers in the asymmetric unit; the closest distance between this dimer and the adjacent symmetry-related molecule is ~10 Å (Fig. 2). When compared with the original *P*622 crystals, the new crystal form contains the same PruA hexamers. There are some similarities in the packing of adjacent hexamers in the two cases, but their orientations with respect to the 6-fold and 3_1-axes is quite different, and it is clear that cobalamin binding has triggered a very substantial repacking, and a small decrease in solvent content (from 60% to 56%).

5. Structure of the PruA–cobalamin complex

The crystal structure of PruA–cobalamin complex was solved by molecular replacement using the known *Mtb*-PruA structure [(Lagautriere et al., 2014); PDB code 4IDM] as a search model and was refined at 2.27 Å resolution to a crystallographic R_{factor} of 14.8% and R_{free} of 20.4% (Table 1). Only one Ramachandran outlier was present in each molecule; Phe498, which has excellent density and is also an outlier in the original *Mtb*-PruA structure, where it is involved in helping to define the active site (Lagautriere et al., 2014). Three dimers of *Mtb*-PruA assemble about a 3-fold non-crystallographic symmetry axis to form a hexamer in the asymmetric unit (Fig. 2). Analysis with PISA (Krissinel and Henrick, 2007) shows that 6.3–6.8% of the solvent accessible surface of each dimer is buried by hexamer formation. In the centre of the hexamer, the N-terminus of each monomer packs against the dimerization domain of a monomer from a neighbouring dimer. At the dimer-dimer interfaces, the NAD^+-binding domain from one dimer (N-terminus and α3 helix) interacts with the catalytic domain (α15 helix) and the dimerization domain (β6 strand) of the other dimer. Despite these interactions, the hexamer appears to be crystallization-induced, since PruA behaves as a dimer in solution (Lagautriere et al., 2014).

Excellent electron density is present for the four cobalamin molecules, covering the corrin ring and its central cobalt atom, and the nucleotide loop that is attached to the corrin ring and provides a dimethylbenzimidazole (DMB) group as a fifth ligand to the cobalt atom. Surprisingly, although the cobalamin used in the Silver Bullets Bio screen is in its adenosylcobalamin form, there is no electron density for the adenosyl moiety in any of the four cobalamin molecules in the present structure (Fig. 3), and the cobalt atom has no sixth ligand. Similar observations have been

Table 1
Structure processing and refinement statistics.

	PruA–cobalamin	PruA–cobalamin–NAD^+
Processing		
PDB code	4LEM	4NS3
Wavelength (Å)	0.953691	0.953691
Space group	$P3_121$	$P3_121$
Cell dimensions		
a (Å)	164.3	164.9
b (Å)	164.3	164.9
c (Å)	259.1	260.2
α, β, γ (°)	90,90,120	90,90,120
Resolution (Å)[a]	20.01–2.27 (2.39–2.27)	20.05–2.38 (2.51–2.38)
R_{merge}[a]	0.175 (1.127)	0.185 (0.870)
R_{pim}[a]	0.038 (0.291)	0.054 (0.297)
Unique reflections[a]	186,040	163,553
Observed reflections	4,012,807	2,019,802
Mean $I/\sigma I$[a]	15.7 (2.5)	12.1 (2.6)
Multiplicity	21.6 (15.1)	12.3 (9.1)
Completeness (%)	99.5 (97.5)	99.6 (98.2)
CC_I mean[b]	0.733	0.742
Refinement		
Resolution range (Å)	20.02–2.27	142.77–2.38
No. reflections	176,621	155,251
R_{work}/R_{free} (%)	14.8/20.4	14.9/20.1
Number of atoms		
Protein	24,947	24,843
Ligand	364	418
Water	2515	2631
R.m.s.d. from ideality		
Bonds (Å)	0.016	0.015
Angles (°)	1.838	1.816
Average B factors (Å²)		
Protein	31.55	27.9
Waters	37.01	31.46
Ramachandran statistics		
Favored (%)	97.03	97.32
Allowed (%)	2.94	2.65
Outliers (%)	0.03	0.03
Poor rotamers (%)	1.31	2.03
Molprobity score; percentile	1.34;100th	1.52;99th

[a] Values in parentheses are for the outermost resolution shell.
[b] Pearson correlation coefficient.

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