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Structure of a novel *N*-acetyl-L-citrulline deacetylase from *Xanthomonas campestris*

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Abstract

The structure of a novel acetylcitrulline deacetylase from the plant pathogen *Xanthomonas campestris* has been solved by multiple-wavelength anomalous dispersion (MAD) using crystals grown from selenomethionine-substituted protein and refined at 1.75 Å resolution. The asymmetric unit of the crystal contains one monomer consisting of two domains, a catalytic domain and a dimerization domain. The catalytic domain is able to bind a single Co(II) ion at the active site with no change in conformation. The dimerization domain forms an interface between two monomers related by a crystallographic two-fold symmetry axis. The interface is maintained by hydrophobic interactions between helices and hydrogen bonding between two β strands that form a continuous β sheet across the dimer interface. Because the dimers are also related by two-fold crystallographic axes, they pack together across the crystal via the dimerization domain, suggesting that higher order oligomers may form in solution. The polypeptide fold of the monomer is similar to the fold of *Pseudomonas* sp. carboxypeptidase G2 and *Neisseria meningitidis* succinyl diaminopimelate desuccinylase. Structural comparison among these enzymes allowed modeling of substrate binding and suggests a possible catalytic mechanism, in which Glu130 functions as a bifunctional general acid–base catalyst and the metal ion polarizes the carbonyl of the acetyl group.

Keywords: ArgE gene; Deacetylase; *N*-acetyl-L-citrulline; *N*-acetyl-L-ornithine; Arginine biosynthesis; Crystal structure

1. Introduction

The first five steps of the canonical arginine biosynthetic pathway in yeast, plants, and bacteria utilize acetylated substrates [1]. The first non-acetylated substrate is L-ornithine, produced from *N*-acetyl-L-ornithine in a reaction catalyzed by *N*-acetyl-L-ornithine deacetylase (AODase) [2–4]. Recently, a novel arginine biosynthetic pathway was identified in *Xanthomonas campestris*, a major plant pathogen, in which *N*-acetyl-L-ornithine is the substrate for the transcarbamoylation reaction and the product is *N*-acetyl-L-citrulline [5,6]. Thus, a second novel enzyme, *N*-acetylcitrulline deacetylase (ACDase) is required to catalyze the deacetylation of *N*-acetyl-L-citrulline to produce L-citrulline [6,7].

We have cloned, expressed, and determined crystal structures of *N*-acetyl-L-citrulline deacetylase from *X. campestris* in the metal-free form and with a single Co(II) ion bound at the active site. Binding of Co(II) was investigated because many deacetylases have been shown to require at least one metal ion for catalysis [8], although their role in catalysis is still not fully understood. The structures suggest a catalytic mechanism and provide new insights into how related enzymes discriminate among different substrates. They also provide a starting point for controlling the growth of *X. campestris* by designing specific inhibitors that selectively inactivate ACDase.

2. Experimental

2.1. Purification, crystallization, and data collection

Gene cloning, protein expression and purification, and data collection were described previously [7]. Briefly, the argE' gene

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was PCR amplified from *X. campestris* genomic DNA (ATCC 3391D), cloned into a pET28a expression vector (Novagen). and expressed in *Escherichia coli* BL21(DE3) cells (Invitrogen). The protein was purified using Ni-affinity and DEAE columns (GE Healthcare). After thrombin digestion, the protein was separated from the his-tag by Ni-affinity chromatography, and concentrated to 12 mg/ml for crystallization trials. The metal-free protein was prepared by dialysis against a buffer containing 100 mM NaCl, 20 mM Tris–HCl pH 8.0, 1 mM EDTA and 5 mM β-mercaptoethanol, while Co(II)-bound protein was prepared by dialysis into a buffer containing 100 mM NaCl, 20 mM HEPES pH 8.0, and 200 mM $CoCl_2$. Crystals of metal-free native protein and selenomethionine-substituted protein grown from a solution containing 0.2 M magnesium formate, 12–15% (w/v) PEG 3350 pH 5.9 by hanging-drop vapor diffusion as reported previously [7]. The crystals of Co(II)-bound protein were grown from a solution containing 100 mM sodium acetate, 200 mM sodium formate pH 4.6.

High-resolution (1.75 Å) data for metal-free and the Co(II)-bound crystals were collected from a single frozen crystal cryoprotected by replacing the mother liquor with the reservoir solution supplemented by 20% (v/v) ethylene glycerol on beam line X26C at the Brookhaven National Laboratory. The crystals belong to space group *C*2 with unit cell parameters: $a=94.13$, $b=95.23$, $c=43.61$ Å, $\beta=93.76°$. Reasonable values for the packing density calculation suggested that there is one monomer in an asymmetrical unit [9]. High-resolution data (1.75 Å) for metal-free crystals soaked with 100 mM *N*-acetyl-L-citrulline or 100 mM *N*-acetyl-L-ornithine were also collected; however, the electron density maps provided no evidence of ligand binding.

High-resolution (1.75 Å) MAD data were also collected from a single frozen crystal of the selenomethionine-substituted protein on beam line X12B at Brookhaven National Laboratory. An X-ray excitation scan had an intense absorption edge at 12655 eV, characteristic of Se. The data were collected at three wavelengths on the Se absorption edge (0.9780 Å), inflection point (0.9761 Å) and in a low energy region remote from the absorption edge (0.9550 Å) [7]. The unit cell parameters for the selenomethionine-substituted crystals were similar to those of the metal-free native crystals. All diffraction data were processed using the HKL2000 package [10] and reduced using program TRUNCATE in the CCP4 suite [11].

2.2. Structure determination and refinement

The structure was solved using the MAD data [7]. Six selenium sites were found and refined using SOLVE [12], and 82% of the polypeptide chain was automatically traced using RESOLVE [13]. The model was manually completed using O [14].

CNS [15] was used to refine the 1.75 Å data sets collected on the metal-free and the Co(II)-bound crystals. Refinement of the metal-free structure converged with residuals $R=0.226$ and $R_{free}=0.258$. The residuals R and R_{free} for the Co(II)-bound structure were 0.198 and 0.224, respectively. 92.1% of the residues were in the most favored region of the Ramachandran plot calculated by program PROCHECK [16], with no residues in the disallowed region. Data collection and refinement statistics are summarized in Table 1. Coordinates for the metal-free and Co-bound structures have been deposited at the PDB with accession codes 2F8H and 2F7V, respectively.

2.3. Structural modeling of the substrate binding

Even though loop and helix positions are significantly different among ACDase structure and its closely related structures of succinyldiaminopimelate desuccinylase from *Neisseria meningitidis mc58*. (PDB ID: 1VGY) [17] and carboxypeptidase G2 from a *Pseudomonas* sp. (PDB ID: 1CG2) [18], their active sites are extremely similar. When the metal-binding residues of these structures are superimposed onto ACDase using the equivalent residues (His72, Asp103, Glu130–131, Glu155 and His340), the r.m.s. differences are only 0.21 and 0.46 Å, respectively. Similarly, the active site structure of ACDase is also similar to other bi-metal enzymes such as aminopeptidase from *Aeromonas proteolytica* [19].

Table 1
Data collection and refinement statistics for ACDase

	Data 1	Data 2
Protein buffer	20 mM Tris–HCl	20 mM HEPES
	pH 8.0	pH 8.0
	100 mM NaCl	100 mM NaCl
	1 mM EDTA	200 mM $CoCl_2$
Crystallization conditions	200 mM $MgCl_2$	100 mM Na acetate
	12–15% PEG 3350	200 mM Na formate
	pH 5.9	pH 4.6
Space group	*C*2	*C*2
Resolution (Å)	1.75	1.75
Unit-cell parameters	$a=94.13$ Å	$a=94.07$ Å
	$b=95.23$ Å	$b=95.43$ Å
	$c=43.61$ Å	$c=43.67$ Å
	$\beta=93.76°$	$\beta=93.76°$
Measurements	286,692	142,933
Unique reflections	37,941	38,233
Redundancy	7.6 (6.9)[a]	3.7 (3.5)
Completeness (%)	98.1 (96.0)	98.7 (97.0)
$\langle I/\sigma(I)\rangle$	22.3 (3.5)	18.4 (2.8)
R_{merg} (%)[b]	6.5 (39.7)	5.2 (35.5)
Wilson B (Å^2)	19.6	19.7
Resolution range (Å)	50–1.75	50–1.75
Number of protein atoms	2617	2698
Number of water atoms	353	370
Number of hetero-atoms	0	1
R_{msd} of bond lengths (Å)	0.005	0.005
R_{msd} of bond angle (°)	1.3	1.3
R_{work} (%)[c]	22.6	19.7
R_{free} (%)[d]	25.8	22.5
Average *B* factor (Å^2)	22.1	25.0

[a] Figures in brackets apply to the highest resolution shell.

[b] $R_{merg}=\sum_h\sum_i|I(h,i)-\langle I(h)\rangle|/\sum_h\sum_i I(h,i)$, where $I(h,i)$ is the intensity of the ith observation of reflection h, and $\langle I(h)\rangle$ is the average intensity of redundant measurements of reflection h.

[c] $R_{work}=\sum_h|F_{obs}-F_{calc}|/\sum_h F_{obs}$.

[d] $R_{free}=\sum_h|F_{obs}-F_{calc}|/\sum_h F_{obs}$ for 10% of the reserved reflections.