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Structural insights into the mechanism of *Escherichia coli* YmdB: A 2′-*O*-acetyl-ADP-ribose deacetylase

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ABSTRACT

The *Escherichia coli* protein YmdB belongs to the macrodomain protein family, which can bind ADP-ribose (ADPr) and its derivatives. Recently, YmdB was reported to be capable of deacetylating *O*-acetyl-ADP-ribose (*O*AADPr) to yield ADPr and free acetate. To study the substrate specificity and catalytic mechanism, the crystal structures of *E. coli* YmdB in complex with ADPr, double mutant N25AD35A complexed with 2′-*O*AADPr, and Y126A/ADPr complex were solved at 1.8 Å, 2.8 Å and 3.0 Å resolution, respectively. Structural and biochemical studies reveal that YmdB has substrate specificity against 2′-*O*AADPr. The conserved residues Asn25 and Asp35 are crucial for catalytic activity, and an active water molecule is proposed as the nucleophile to attack the acetyl group of 2′-*O*AADPr. Our findings indicate that the conserved phenyl group of Tyr126 plays a crucial role in catalytic activity by stabilizing the right orientation of distal ribose and that Gly32 may be important for activity by interacting with the acetyl group of 2′-*O*AADPr. Based on these observations, a model of YmdB in complex with 2′-*O*AADPr was made to illustrate the proposed catalytic mechanism of YmdB.

1. Introduction

Sirtuins are a class of enzymes with nicotinamide adenine dinucleotide (NAD)-dependent protein lysine deacetylase activity that regulate numerous biological pathways by deacetylating various substrate proteins (Borra et al., 2002; Tanner et al., 2000; Tanny and Moazed, 2001). Sirtuins require NAD^+ to deacetylate proteins and subsequently generate 2′-*O*-acetyl-ADP-ribose (2′-*O*AADPr) as a reaction product (Jackson and Denu, 2002). Sirtuin-produced 2′-*O*AADPr is then released into solvent, where 3′-*O*AADPr is formed by intramolecular transesterification and exists in equilibrium with 2′-*O*AADPr (approximately 1:1) (Jackson and Denu, 2002).

*O*AADPr is a unique metabolite that has been implicated as a signaling molecule, of which the cellular level *in vivo* might be tightly controlled. However, the details of the metabolism of *O*AADPr remain unclear (Tong and Denu, 2010). Recently, some macrodomains have been reported either to be present in the same operon or to exist as fusion proteins with sirtuins, providing an important clue that macrodomains may have functional connections with sirtuins and might be involved in the metabolism of *O*AADPr (Chen et al., 2011).

Macrodomains are evolutionarily conserved domains that are found throughout all kingdoms of life, from viruses to mammals (Han et al., 2011). This family of proteins contains at least one copy of an approximately 130–190 residue "macrodomain" that functions as the binding site of metabolites of NAD^+, including ADP-ribose (ADPr), poly(ADP-ribose) and *O*AADPr (Allen et al., 2003; Ladurner, 2003; Pehrson and Fried, 1992; Pehrson and Fuji, 1998). In addition, macrodomain proteins also contain a variety of additional domains to interact with specific target proteins or nucleic acid regions (Ahel et al., 2009; Chen et al., 2011; Jankevicius et al., 2013; Neuvonen and Ahola, 2009). A subfamily

Abbreviations: ADPr, ADP-ribose; *O*AADPr, *O*-acetyl-ADP-ribose; NAD, nicotinamide adenine dinucleotide; ITC, isothermal titration calorimetry; PCR, polymerase chain reaction; LB, Luria–Bertani; IPTG, isopropyl β-D-1-thiogalactopyranoside; Ni-NTA, Ni-nitrilo acetic acid; SSRF, Shanghai Synchrotron Radiation Facility; TFA, trifluoroacetic acid; UPLC, ultra performance liquid chromatography.

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of sirtuin-linked macrodomains named MacroD-like proteins, including human MacroD1 and MacroD2, *Escherichia coli* YmdB and *Staphylococcus aureus* MacroD-like protein (SAV0325), have recently been identified as *O*AADPr deacetylases that hydrolyze sirtuin-produced *O*AADPr to yield ADPr and free acetate (Chen et al., 2011). Several conserved residues, especially Asp184 and Asn174 (MacroD1 numbering), were identified to be crucial for catalytic activity (Chen et al., 2011). A potential water molecule was proposed to be involved in nucleophilic attack based on transition-state analysis (Hirsch et al., 2014). However, the enzymatic mechanism and substrate specificity of *O*AADPr deacetylation catalyzed by MacroD-like proteins are poorly understood.

To learn more about the catalytic mechanism, we confirmed the deacetylase activity of *E. coli* YmdB and solved the crystal structure of YmdB in complex with its product ADPr at 1.8 Å resolution. Consistently with previous studies (Chen et al., 2011), biochemical experiments showed that the N25A or D35A mutant is partially active, whereas the activity of the double-mutant N25AD35A is severely impaired, suggesting a nucleophilic water attack assisted by both Asn25 and Asp35. Structural analysis showed that both of the two residues form hydrogen bonds with 2′-OH of the distal ribose, indicating that 2′-*O*AADPr rather than 3′-*O*AADPr or 1′-*O*AADPr is more likely the direct substrate of YmdB. To test this hypothesis, the double-mutant N25AD35A was cocrystallized with the substrate *O*AADPr (a mixture of 2′- and 3′-*O*AADPr). The crystal structure of YmdBN25AD35A was solved at 2.8 Å resolution with 18 molecules in an asymmetric unit. Two molecules bind 2′-*O*AADPr in the active site, while others bind ADPr due to the degradation of *O*AADPr (Hirsch et al., 2014), indicating that YmdB specifically deacetylates 2′-*O*AADPr. The 2′-*O*AADPr-bound structure shows a hydrogen bond interaction between Gly32 and the acetyl group of 2′-*O*AADPr, suggesting an important role of Gly32 in the reaction. Activity assays and isothermal titration calorimetry (ITC) results suggested that Tyr126 might be the crucial amino acid for substrate specificity. The crystal structure of the YmdBY126A/ADPr complex further revealed a different conformation of the distal ribose in the active site, demonstrating the critical role of Tyr126 in stabilizing the orientation of the distal ribose. All of these findings reveal the structural basis for the catalytic mechanism of 2′-*O*AADPr deacetylation by YmdB.

2. Materials and methods

2.1. Cloning, expression, and purification

Wild-type YmdB was amplified from *E. coli* BL21 (DE3) cells (Novagen) by polymerase chain reaction (PCR) with the desired primers. Site-directed mutagenesis was performed using wild-type plasmid as the template and primers with the desired mutation. The amplified products were purified and digested with *BamH I* and *Xho I* and inserted into similarly digested pET22b (Novagen) vector with an N-terminal $(His)_6$-tag to yield wild-type YmdB and mutant plasmids.

The YmdB plasmids were transformed into *E. coli* Rosetta2 (DE3) cells (Novagen) and grown with shaking at 37 °C in Luria–Bertani (LB) media containing 50 μg/mL ampicillin until reaching an OD_{600} of 0.6–0.8. The culture was then induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM and incubated at 16 °C for another 20 h.

The cells were harvested and lysed in lysis buffer (20 mM Tris–HCl, 200 mM NaCl, pH 7.5) using sonication. The lysate was centrifuged, and the supernatants were subjected to a column containing 2 mL Ni-nitrilo acetic acid (Ni-NTA) resin (QIAGEN) preequilibrated with the lysis buffer. The column was washed with 100 mL wash buffer containing 20 mM Tris–HCl, 200 mM NaCl, 40 mM imidazole, pH 7.5 to remove nonspecific binding proteins. The bound YmdB proteins were recovered with 10 mL elution buffer containing 20 mM Tris–HCl, 200 mM NaCl, 500 mM imidazole, pH 7.5. The eluted samples were further purified using a Superdex 200(16/60) or Superdex 200(10/300) size-exclusion column (GE Healthcare) with 20 mM Tris–HCl, 200 mM NaCl, pH 7.5. The fractions corresponding to YmdB proteins were collected, concentrated to 20 mg/mL and stored at −80 °C for further use.

2.2. Crystallization, data collection and processing

Crystals were grown at 10 °C using the sitting drop vapor diffusion method by cocrystallization of wild-type YmdB with the product ADPr (Sigma) or of the double-mutant YmdBN25AD35A with the substrate *O*AADPr (TRC), with a protein: ligand molar ratio of 1:4. The initial crystallization conditions were determined using the Index Screen (Hampton). The optimized conditions were 0.2 M potassium sodium tartrate tetrahydrate, 20% w/v polyethylene glycol 3350 for YmdB/ADPr and 2 M ammonium sulfate, 0.1 M BIS–TRIS pH 5.5 for YmdBN25AD35A/*O*AADPr. Crystals were soaked in a cryoprotectant solution composed of the well solution supplemented with 20% (v/v) glycerol for several seconds and flash-cooled in liquid nitrogen for data collection. Crystals of YmdBY126A were grown at 10 °C using the sitting drop vapor diffusion method. The initial crystallization conditions were determined using the Index Screen (Hampton). The optimized condition was 2 M ammonium sulfate, 0.1 M BIS–TRIS pH 5.5. Crystals were soaked in a solution composed of the well solution supplemented with 20% (v/v) glycerol and 8 mM *O*AADPr for 30–60 s and flash-cooled in liquid nitrogen for data collection.

X-ray diffraction data on YmdB/ADPr and YmdBN25AD35A/*O*AADPr were collected at the synchrotron radiation beamline BL17U1 of Shanghai Synchrotron Radiation Facility (SSRF) using an ADSC QUANTUM 315R CCD detector with a crystal to detector distance of 200 mm for YmdB/ADPr and 250 mm for YmdBN25AD35A/*O*AADPr. X-ray diffraction data on YmdBY126A/ADPr were collected at the synchrotron radiation beamline BL19U1 of SSRF using an pilatusCBF CCD detector with a crystal to detector distance of 500 mm. Individual frames were collected at 100 K using 1 s for each 1.0° oscillation over a range of 220° for YmdB/ADPr, 250° for YmdBN25AD35A/*O*AADPr, and 360° for YmdBY126A/ADPr. X-ray diffraction data were indexed, integrated, scaled, and merged using the program HKL2000 (Otwinowski and Minor, 1997). Data collection and processing statistics are shown in Table 1.

2.3. Structure determination and refinement

The structures of *E. coli* YmdB were determined by molecular replacement using the program MOLREP in the CCP4i suite (Collaborative Computational Project, 1994). The crystal structure of *E. coli* YmdB (PDB ID: 1SPV) was used as the phasing model. After several rounds of refinement using REFMAC5 (Murshudov et al., 1997) and COOT (Emsley and Cowtan, 2004), ADPr or 2′-*O*AADPr was built into the active site based on the $F_o - F_c$ electron density, and water molecules were then added to the model. The structure of YmdB/ADPr was refined to a 1.8 Å resolution with a final R_{work} of 18.78% and R_{free} of 21.73%. The structure of YmdBN25AD35A/*O*AADPr was refined to a 2.8 Å resolution with a final R_{work} of 26.08% and R_{free} of 29.01%. The structure of YmdBY126A/ADPr was refined to a 3.0 Å resolution with a final R_{work} of 22.07% and R_{free} of 24.96%. Geometry was verified using Molprobity in PHENIX (Adams et al., 2002). The final refinement statistics are listed in Table 1.