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Unusual NADPH conformation in the crystal structure of a cinnamyl alcohol dehydrogenase from *Helicobacter pylori* in complex with NADP(H) and substrate docking analysis

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ABSTRACT

Cinnamyl alcohol dehydrogenase is a zinc- and NADPH-dependent dehydrogenase catalyzing the reversible conversion of *p*-hydroxycinnamaldehydes to their corresponding hydroxycinnamyl alcohols. A CAD homolog from *Helicobacter pylori* (HpCAD) possesses broad substrate specificities like the plant CADs and additionally a dismutation activity converting benzaldehyde to benzyl alcohol and benzoic acid. We have determined the crystal structure of HpCAD complexed with NADP(H) at 2.18 Å resolution to get a better understanding of this class of CAD outside of plants. The structure of HpCAD is highly homologous to the sinapyl alcohol dehydrogenase and the plant CAD with well-conserved residues involved in catalysis and zinc binding. However, the NADP(H) binding mode of the HpCAD has been found to be significantly different from those of plant CADs.

Structured summary of protein interactions:
HpCAD and **HpCAD** bind by x-ray crystallography (View interaction)

1. Introduction

Cinnamyl alcohol dehydrogenases (CAD; EC 1.1.1.195) are NADPH- and Zn^{2+}-dependent enzymes and belong to the superfamily of medium-chain dehydrogenases [1]. In plants, CAD is a key enzyme in lignin biosynthesis involved in the final step of the monolignol synthesis, catalyzing the reversible conversion of *p*-hydroxycinnamaldehydes into the corresponding alcohols using NADPH as coenzyme [2–5]. However, some members of the plant CAD family appear to have different enzymatic activities not related to lignin biosynthesis but involved in plant disease resistance and possibly in the response to pathogen attacks [6,7]. Outside of plants, CADs seem to be not required for biosynthesis of lignin although they have high sequence homologies with the plant CADs. The roles of CADs in bacteria and yeasts are less well characterized. The CADs present in *Mycobacterium bovis* BCG and in *Saccharomyces cerevisiae* appear to be involved in lipid metabolism within the cell envelope and the Erlich pathway, respectively [8,9].

Helicobacter pylori, a human pathogen infecting the gastric mucosa and causing an inflammatory process leading to gastritis, ulceration and cancer [10,11], carries a single CAD gene encoding a CAD homolog protein (HpCAD) with 30∼42% amino acid sequence identity to the plant CADs. Interestingly, alcohol dehydrogenases (ADHs) contribute to the pathogenicity of *H. pylori* by metabolizing dietary alcohols to form toxic aldehydes, which interact with the gastric mucosa to cause inflammation [10,12,13]. The HpCAD carries a specific activity of dismutating benzaldehyde to form benzyl alcohol and benzoic acid in addition to the well-known enzymatic activities of the cinnamyl alcohol dehydrogenase [14]. To date, several three dimensional structures of CAD/SAD have been determined [15–17], but a bacterial CAD structure has not yet been reported.

Here, we present the first bacterial CAD structure from *H. pylori* determined in complex with NADP(H) at 2.18 Å resolution. The overall structure of HpCAD is similar to *Arabidopsis* CAD5 and *Aspen* SAD structures. But significant structural differences were observed around the NADP(H)-binding pocket, causing an

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extraordinary NADP(H) conformation of the phosphate group on the ribose ring, which is not found in other CAD structures. Furthermore, the binding modes of aromatic substrates in the substrate binding site of HpCAD were investigated by docking calculations.

2. Materials and methods

2.1. Expression and purification of HpCAD protein

The gene encoding the HpCAD (1044 bp) was amplified from *H. pylori* genomic DNA using forward primer 5′-GCGCATATGATGAGAGTTCAATCTAAAGG and the reverse primer 5′-GCGGAATTCTTAATCAACGATTTTTCATA (*NdeI* and *BamHI* sites are underlined). PCR conditions were: 1 × 94 °C for 5 min; 35 × 94 °C for 30 sec, 55 °C for 40 s, 72 °C for 30 s, 72 °C for 10 min. After restriction digestion, the gene was ligated into a pET-15b vector (Novagen, Madison, WI, USA) and transformed into DH5α competent cells. Correct clones were identified and verified by PCR, restriction digestion with *NdeI* and *BamHI* and DNA sequencing with T7 forward and T7 terminal reverse primers. The expression plasmid was constructed for the recombinant HpCAD to carry a His-tag and a thrombin site at the N-terminus. The plasmid was transformed into an *Escherichia coli* strain Rosseta™(DE3)pLysS (Novagen) for protein expression. A 10 ml aliquot of an overnight culture was seeded into 1000 ml of fresh LB (Luria–Bretani) medium containing 50 mg/ml ampicillin and cells were grown to $OD_{600\ nm}$ of 0.6 at 37 °C. The cells were cooled down in ice for 30 min and protein expression was induced for 5 h with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30 °C. The cells were harvested by centrifugation at 6000 rpm for 6 min at 4 °C. The harvested cells were washed twice in phosphate-buffered saline. The cell pellet was used directly for purification or stored at −80 °C until use.

The cell pellet was suspended in binding buffer (50 mM NaH_2PO_4 pH 8.0, 500 mM NaCl, 5 mM imidazole and 5 mM β-mercaptoethanol) and cells were disrupted by sonication. After centrifugation at 15 000 rpm for 1 h, the clear supernatant was collected, filtered (Qualitative filter paper, Advantec, Japan) and applied onto a column of Nickel Sepharose 6 Fast Flow (GE Healthcare, Sweden) beads pre-equilibrated with the binding buffer. The column was washed first with 20 column volumes of binding buffer and then with 2 column volumes of washing buffer (50 mM Tris–HCl pH 8.0, 500 mM NaCl, 30 mM imidazole). The recombinant HpCAD proteins were eluted with the elution buffer (50 mM Tris–HCl pH 8.0, 100 mM NaCl and 300 mM imidazole). The HpCAD eluted from the nickel column was further purified by size-exclusion chromatography using a Superdex 200 column (GE Healthcare, Piscataway, New Jersey, USA) equilibrated with 20 mM Tris–HCl pH 8.0 and 150 mM NaCl. The fractions containing HpCAD were pooled, exchanged into 20 mM Tris–HCl pH 8.0 and concentrated to a final concentration of 8.5 mg/ml by ultrafiltration (Microcon YM-30, Millipore Corporation, Bedford, Massachusetts, USA). The protein concentration was determined by Bradford assay [18] using bovine serum albumin (BSA) as a standard. The purity was monitored by SDS-PAGE comparing with the theoretical molecular weight of the tagged monomer ~45 kDa.

2.2. Site-directed mutagenesis

The Arg208 of the HpCAD was mutated to Ala by site-directed mutagenesis using a Stratagene site-directed mutagenesis kit (Agilten technologies, Inc., Santa Clare, CA, USA) following the manufacturer's protocol. The primers for mutagenesis were designed for use with the expression vector plasmid: Forward: 5′-TTT GTG TTC GTT TGC TGC AAA AAC GCT-3′; reverse: 5′-AGC GTT TTT GCA GCA AAC GAA CAC AAA-3′. Plasmids harboring the mutation R208A was transformed into an *E. coli* strain Rosseta™(DE3)pLysS (Novagen) for protein expression. The mutant HpCAD ($HpCAD_{R208A}$) was expressed and purified in the same way as the wild type HpCAD.

2.3. Crystallization and data collection and processing

Crystallization of HpCAD was attempted using Crystal Screens I, II and Index Screens (Hampton Research, Riverside California, USA) and Wizard Screens I, II and Cryo I, II (Emerald BioStructures, Bainbridge Island, Washington, USA) and our own laboratory screen solutions. Crystallization trials were setup at 18 °C using the microbatch method under Al's oil, an 1:1 mixture of paraffin oil (Hampton Research) and silicone oil (Fluka, Milwaukee, WI, USA), in 72-well plates. For crystallization trials, equal volumes of protein solution (8.5 mg/ml) and reservoir solution were mixed and equilibrated under the oil. Small crystals were produced with Index screening solution No. 83 (0.1 M Bis–Tris pH 6.5, 0.2 M $MgCl_2$ 25% Polyethylene glycol 3350) using the proteins in 20 mM Tris–HCl pH 8.0. Further screenings to find optimal crystallization conditions were performed by hanging drop vapor-diffusion method, varying the salt, precipitant concentration and the volume of the drop. The best crystals were grown in 0.1 M Bis–Tris pH 6.5, 0.2 M $MgCl_2$ and 19% polyethylene glycol 3350 at 18 °C using 8.5 mg/ml HpCAD in 20 mM Tris–HCl pH 8.0.

For X-ray analysis, crystals were transferred into a cryosolution containing the well solution supplemented with 21% glycerol and soaked for 30 min at room temperature. Then, the crystals were flash-frozen in liquid nitrogen. X-ray diffraction data were collected from a single crystal using an ADSC Quantum 210 CCD detector at the beamline 6C1 of Pohang Accelerator Laboratory, Pohang, Korea. Hundred and eighty rotation images were collected with an oscillation angle of 1° and an exposure time 30 s for each image at 200.0 mm detector distance. All diffraction data were indexed, integrated and scaled using the HKL2000 program suite [19]. The Matthews coefficient and solvent content were calculated using CCP4 [20].

2.4. Structure determination and refinement

The structure of HpCAD was determined by molecular replacement using the program AMoRe in CCP4 program suite [20] with the sinapyl alcohol dehydrogenase from *Populus tremuloide* (*Aspen*) (PDB ID: 1YQD) as a search model. The structure was refined by several rounds of refinement using the program CNS [21] and REFMAC5.0 [20] and manual building with the graphic program O [22] and COOT [23]. In the initial stage, refinement was carried out by positional and B group refinement with strong NCS restraints and in the final stage of the refinement by positional and individual B faction refinement with no NCS restraints. Ions, NADP(H) and water molecules were added into the electron density maps (2Fo-Fc and Fo-Fc) at the final round. Waters were placed using the automatic water picking function of by CNS [21], and waters were accepted or rejected by visual inspection of difference electron density maps by COOT [23]. The quality of the final model was validated by PROCHECK [20].

2.5. Docking calculations for substrates

Docking calculations were performed using the program AUTODOCK 4.2 [24]. The monomer model of the crystal structure of HpCAD was used for docking experiments. The model of substrates was generated by Dundee PRODRG2 Sever [25]. Hydrogen's were