Structure of homoprotocatechuate 2,3-dioxygenase from *Brevibacterium fuscum* at 1.6 Å resolution

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Introduction

The catabolism of aromatic compounds by prokaryotic organisms is critical to the global carbon cycle. A natural source for the complete breakdown of aromatics is usually attributed to soil-associated bacteria. Other significant sources are anthropogenic in origin, such as those generated from industrial processes, the incomplete burning of fossil fuels, the spraying of pesticides and herbicides, and those produced in the manufacture of household goods. The same bacteria that degrade natural aromatics have also evolved pathways to degrade a few of these relatively novel aromatics. However, many of these useful xenobiotic compounds are recalcitrant to degradation and also have environmental detractions such as toxicity and the ability to biomagnify and accumulate in the tissues of plants and animals. This has led to an immense interest in the enzymatic pathways used by bacteria in the degradation of all aromatics [1, 2].

Research has centered on the enzymes that result in the cleavage of the aromatic ring as this is generally considered to be the most crucial step. These enzymes are termed aromatic ring cleaving dioxygenases since the ring cleavage occurs concomitantly with the incorporation of both atoms of the second substrate, dioxygen. It is hoped that kinetic and structural studies will clarify the mechanism by which these enzymes catalyze the energetically difficult ring cleavage reaction. This can provide insight into the reason for the recalcitrance of certain aromatics, making it possible to design biodegradability into synthetic compounds prior to their use in the environment [3].

Homoprotocatechuate 2,3-dioxygenase from *Brevibacterium fuscum* (BF 2,3-HCPD) is a member of a subfamily of dioxygenases that catalyze the extradiol cleavage of catecholate substrates into the corresponding muconic semialdehydes. Mechanistically the reaction is proposed to occur through the activation of dioxygen by ferrous iron, leading to nucleophilic attack by the emerging superoxide on the catecholic ring [4]. The present report describes the collection of a high-resolution data set that will yield insight into the mechanism and substrate specificity of aromatic ring cleaving dioxygenases.

Methods and Materials

Bf 2,3-HPCD was overexpressed and purified from an *Escherichia coli* clone to a specific activity of 15 U/mg [5]. A limited proteolysis with trypsin (Δ41 residues) was used to cleave residues that appeared to have high mobility and could be interfering with crystallization. The proteolytic fragment (residues 1–323) crystallized as thick tetragonal rods in microbatch crystallization experiments that contained 10 mg/ml Bf 2,3-HPCD, 10–15% PEG5000, 0.2 M Mg acetate, and 50 mM Mops (pH = 7.5). This crystal form belonged to the space group $I4_1$ (a = b = 156.7 Å, c = 124.8 Å) and diffracted on a rotating anode source to 2.5 Å resolution.

A high-resolution cryogenic data set of the $I4_1$ crystal form was collected at Argonne National Labs at beamline 19-ID. Cryofreezing was accomplished by exchanging the crystal mother liquor solution with a cryoprotectant solution consisting of 15% PEG5000MME, 0.2 M Mg acetate, 100 mM Mops (pH = 7.5), and 25% glycerol. The $I4_1$ crystal form tended to crack during direct transfers into high levels of glycerol or during excessive handling. To overcome this, the glycerol concentration was increased in 5% steps, and the crystal was not handled until it was to be inserted in the beam. After the final glycerol step, the crystal was mounted in a cryo loop and flash frozen directly in a liquid nitrogen stream at the beamline. Data were produced using monochromatic radiation (λ = 1.03321Å) and collected using a 3 x 3 CCD array imaging detector. Diffraction intensities were indexed with DENZO and scaled with SCALEPACK [6].

Results and Discussion

The crystals diffracted beyond 1.5 Å, but data collection geometry at the time prevented collection of a complete data set beyond 1.6 Å. This is almost 1.0 Å higher in resolution than those collected on our home x-ray source. A combination of single isomorphous replacement, solvent flattening, and four-fold noncrystallographic symmetry averaging was used to obtain phases adequate for modeling the data. The high-resolution synchrotron data set was used for the final refinement of the crystallographic model. This is the highest resolution structure reported for an extradiol dioxygenase.

Bf 2,3-HPCD is a homotetramer of identical subunits. Each subunit is composed of two domains (N-terminal and C-terminal). Each of these domains is made of two structurally homologous βαβββ motifs that combine to form a β-barrel. The C-terminal domain β-barrel supplies all the ligands to the ferrous iron and makes up the residues of the active site.

Despite having less than 25% sequence homology, Bf 2,3-HPCD has the same fold and active site ligands seen in the recently determined structures of 2,3-dihydroxybiphenyl 1,2-dioxygenase [7] and catechol 2,3-dioxygenase [8]. The active site iron is coordinated by two axial ligands ($His214^{NE2}$ and a water molecule) and four equatorial ligands ($His155^{NE2}$, $Glu267^{O1}$, and two water molecules) in an octahedral

geometry. Based on the space available to substrate, we would predict that one hydroxyl from substrate would bind equatorially, while the other would bind in an axial mode, leaving an open site equatorially to bind dioxygen. Several residues around the active site are structurally conserved (His200, His248, and Y257) and are most likely important for the mechanism, while others are involved in the substrate specificity of Bf 2,3-HPCD (Arg243, Arg292, Arg293, Trp192, and Trp304). Asn157 may be involved in the unique stability of Bf 2,3-HPCD to oxidation, and its reported catalase activity [5].

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