University of Southampton

DAD – A Novel Bacterial Dioxygenase Enzyme

A possible new enzyme for bioremediation procedures

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It is hoped that now the DAD protein has been purified and crystallised, it will be possible to use protein X-ray crystallography to determine its structure by building a 3D atomic model.

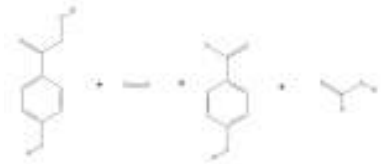
Why Study DAD?

Environmental issues dominate today's news, particularly pollution and the resultant change in climate. We are continually producing more waste and emissions than can safely and effectively be dealt with. Aromatic hydrocarbons, formed by the incomplete burning of fossil fuels, as well as those found in other sources such as plastics and pesticides, are especially damaging to the environment. They are not naturally degraded and therefore persist within the biosphere for many, many years.

Bioremediation is a relatively new way of thinking about waste disposal. Some microorganisms have evolved to catabolise aromatic compounds as their carbon source. By learning more about the enzymes involved in their catabolic pathways, like dioxygenases, it may be possible to genetically modify them to degrade pollutant hydrocarbons such as those from petroleum, significantly reducing their negative impact on the environment.

How Does DAD Work?

2,4- dihydroxyacetophenone dioxygenase (DAD) is one such enzyme from *Alcaligenes* sp. involved in the decomposition of aromatic compounds from plants. This dioxygenase is of particular interest because of the unique manner by which it catalyses the cleavage of the aromatic compound 2,4'-dihydroxyacetophenone to yield 4-hydroxybenzoate and formate, unlike any other known dioxygenase. It may offer an additional method for bioremediation as dioxygenases generally catalyse aromatic ring fission, but DAD catalyses the cleavage of an aliphatic side-chain.



2,4'-dihydroxyacetophenone + O_2 = 4-hydroxybenzoate + formate

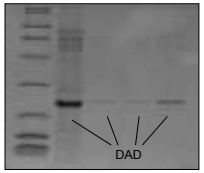
DAD shows very little sequence similarity identity other proteins. However, genome sequencing has indicated the presence of several reading frames highly homologous to those in various *Pseudomonas* bacteria. The functions of the corresponding proteins are currently unknown.

Methods

Expression of DAD

DAD DNA was transformed into BL21 bacteria for ease of expression and purification on the large scale that is needed for crystallography. They were grown on ampicillin plates, to select for colonies that successfully took up the plasmid, then used to inoculate Novagen Overnight Express Autoinduction growth media (more soluble protein).

Purification of DAD from Contaminants

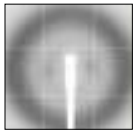


The bacterial cells were broken open by sonication, then the soluble BipD protein purified from the cell debris. This is achieved primarily using ion exchange (*Q-sepharose anion exchange*) and gel filtration (*sephadex 200*) columns, which separate the molecules by charge and size respectively.

Growth of Crystals

The purified DAD was concentrated to 4mg/ml^{-1}. Crystal screens were set up using the hanging drop method. The crystals took 10 months to grow.

X-ray Crystallography



The crystals were harvested, frozen in liquid nitrogen and taken to the ESRF where they diffracted to a resolution of about 3Å.

Analysis of Data and Model Building

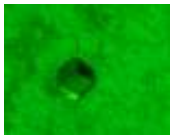
A suite of computer programmes will be used to measure the position and intensity of each spot in the diffraction pattern, and convert this to numerical data. The 3D structure of the DAD protein can then be built over a series of refinement steps, by matching the observed data as closely as possible with the proposed model.

Results

Within months 10-12 after crystal screens were set up, several small crystals of DAD were found to have grown under different conditions, based on either PEG 400 or ammonium sulphate (screens JB1 A3, B3, B6 and JB6 A1 and B1). All the crystals were of a similar cuboid shape.

1 crystal from each screen was taken to Grenoble to confirm that it was protein and see what resolution it diffracted to. No data was obtained from one crystal, but the other diffracted to approximately 3Å.

Crystal of DAD from JB screen 6 B1, 1.5M ammonium sulphate, 15% w/v glycerol, 100mM Tris-HCl, pH 8



Further Work…

The main objective of my research is to determine the three-dimensional structure of DAD by X-ray crystallography. Now that a protocol for expressing, purifying and crystallising the DAD protein has been developed, a seleno-methionine derivative will be expressed. This incorporation of the element selenium into methionine residues is necessary in order to determine the phase of the X-ray waves as they hit the electron cloud around the atoms in the DAD molecule. Without knowing the phase of the waves, it is not possible to solve the structure.

However, the data that has already been collected from the native protein can be processed enough to determine the spacegroup of the crystal (the different types of symmetry present within the unit cell). It should also be possible to use this data along with data from the selenomethionine derivative to refine the model.

It is hoped that it will also be possible to analyse complexes of the DAD enzyme and site- directed mutants for an improved understanding of its seemingly unique catalytic mechanism. This will provide additional structural information about the way in which the DAD enzyme functions to catalyse this novel reaction.

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