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Correlation of thermostability and conformational changes of catechol 2, 3-dioxygenases from two disparate micro-organisms

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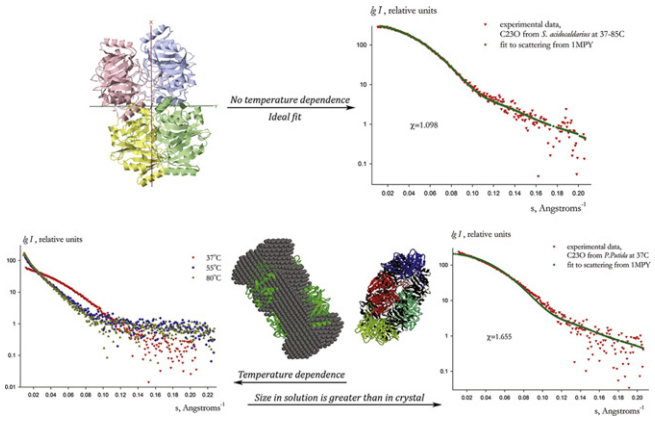
HIGHLIGHTS

- Solution structure of two C23O enzymes of different origin has been studied.
- Both C23Os present an impressive similarity in overall shape and functionality.
- Thermophilic C23O solution scattering is reproduced well from its crystal structure.
- Mesophilic C23O has a slightly extended shape in solution compared to crystal.
- Both C23Os have dramatically different thermostability and low sequence identity.

GRAPHICAL ABSTRACT



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ABSTRACT

We have investigated the structure of recombinant catechol 2, 3-dioxygenase (C23O) purified from two species in which the enzyme has evolved to function at different temperature. The two species are mesophilic bacterium *Pseudomonas putida* strain mt-2 and thermophilic archaea *Sulfolobus acidocaldarius* DSM639. Using the primary sequence analysis, we show that both C23Os have only 30% identity and 48% similarity but contain conserved amino acid residues forming an active site area around the iron ion. The corresponding differences in homology, but structural similarity in active area residues, appear to provide completely different responses to heating the two enzymes. We confirm this by small angle X-ray scattering and demonstrate that the overall structure of C23O from *P. putida* is slightly different from its crystalline form whereas the solution scattering of C23O from *S. acidocaldarius* at temperatures between 4 and 85 °C ideally fits the calculated scattering from the single crystal structure. The thermostability of C23O from *S. acidocaldarius* correlates well with conformation in solution during thermal treatment. The similarity of the two enzymes in primary and tertiary structure may be taken as a confirmation that two enzymes have evolved from a common ancestor.

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1. Introduction

Extradiol type-dioxygenases (e.g. catechol 2,3 dioxygenase or C23O) are found in a variety of bacteria and are involved in the aromatic ring fission at the *meta* position of dihydroxylated aromatics [1]. These systems are of interest from an evolutionary, geochemical and ecological perspective due to their potential application in environmental protection [2–5]. Many microorganisms are able to utilise xenobiotic aromatic compounds as the sole carbon and energy source for growth. Various effects have been made to improve their capabilities for environmental pollutant degradation and bioremediation [6].

We present here a study of two C23O enzymes of different origin: from (i) mesophilic bacteria *Pseudomonas putida* and (ii) thermophilic archaea *Sulfolobus acidocaldarius*. The two enzymes, from separate origin, present an impressive similarity in overall shape and functionality despite having dramatically different thermostability and distinguishable primary sequences. As comprehensively shown in [7], proteins adapt to higher temperatures by adopting mutations yielding increased structural stability.

P. putida is a gram-negative rod-shaped saprotrophic soil bacterium. *S. acidocaldarius* strain DSM639 belongs to the aerobic crenarchaeon and was the first hyper-thermoacidophile to be characterised from terrestrial solfataras by Brock et al. [8]. It grows optimally at 75 °C to 80 °C, at pH 2 to 3, utilising complex organic substrates. Various species can be found throughout the world in areas of volcanic or geothermal activity, such as geological formations called mud pots (or solfatare).

There are studies dedicated to determination of amino acids responsible for the thermostability of the C23O enzymes, e.g. [9,10] but the conclusions proposed there do not extend to all members of the C23O family, cf. [11]. The phylogenetic analysis of 35 extradiol dioxygenase sequences presented in [11] has revealed the evolutionary history of the enzymes. In addition, it provided a classification system and enabled conserved residues to be defined. C23O from *P. putida* is included into the set described in [11], but the sequence of C23O from *S. acidocaldarius* has not been considered. The protein sequence identity between the mesophilic and thermophilic enzymes is 31%, which is very low for making structural predictions; therefore, we determined a sequence alignment based upon the three-dimensional structural alignment of the crystal structures of the distinct C23Os reported, which represent eight different bacterial species (Table 1).

The structure of C23O from *P. putida* (Fig. 1), is one of the two structures under our investigation available at 2.8 Å resolution (PDB:1MPY, structure of catechol 2,3-dioxygenase (metapyrocatechase) from *P. putida*, [3]).

To date, little information about the high resolution structure of C23Os from thermophilic hosts is available [10,12]. Jiang et al. [13] state that the pheB gene encoding for thermostable C23O from *Bacillus stearothermophilus* has been cloned and the corresponding protein has been crystallized. The authors refer to the structural refinement and the detailed analysis of the structure and function as being in progress but, to our knowledge, the details are yet to have been published.

2. Materials and methods

2.1. Sample preparation

P. putida mt-2 was kindly provided by Dr. David T. Gibson (University of Iowa, USA). *S. acidocaldarius* DSM639 was purchased from the Bioresource Collection and Research Center (Hsin-Chu, Taiwan). The growth media and cultural conditions of these strains were according to Evans et al. [14] and Brock et al. [8], respectively.

The amplified gene fragments were subcloned into the pET28a vector (Novagen, Co.), to include N-terminal hexahistadine tags, and expressed in *Escherichia coli* BL21 (DE3) (Novagen, Co.). The recombinant strains were grown at 37 °C in Luria-Bertani medium containing kanamycin (30 μg/ml). Isopropyl β-D-1-thiogalactopyranoside (1 mM) was added to induce the expression of C23O genes.

2.1.1. Enzyme purification

The recombinant *E. coli* strains were harvested at late-log phase and the cells disrupted by sonication on ice. Clarified lysates were obtained by centrifugation at 100,000 ×*g* for 60 min at 4 °C. For protein purification, all procedures were conducted at 4 °C. C23O enzymes were purified by HisTrap affinity column (5 ml; GE Healthcare) and then on a Sephacryl S-200 HR column (145 ml, GE Healthcare) according to the manufacturer's protocols. The enzyme assay is described in [15] and the protein concentrations were determined by the Bradford method [16]. Proteins were dissolved in 50 mM Tris pH 8.0 buffer with 10% acetone used as a protein stabilizer [17,18].

2.2. Sequence alignment

The primary sequence of C23O from *S. acidocaldarius*, Uniprot: Q4J6K0, was used to BLAST [19] the PDB [20], which produced a clear set of eight cases of alignment to most of the sequence, with identity scores of 22% to 32% (Table 1). Using PROMALS3D [21], the sequence Q4J6K was then subjected to a structure based sequence alignment against eight representative structures (one for each species), using the A-chain for each structure. After rejecting one structure (PDB:1fiu) as a relative outlier, the sequence alignment shown in Fig. 3 was obtained.

2.3. SAXS data collection and processing

Small angle X-ray scattering (SAXS) data were collected on a Bruker Nanostar II SAXS camera. Protein and buffer solutions were measured using a reusable 2 mm quartz capillary. The sample volume was approximately 40 μl. The range of momentum transfer s ($s = 4\pi\sin\theta/\lambda$, where 2θ is the scattering angle and λ is wavelength = 1.54 Å using Cu K_α) was 0.01 to 0.21 $Å^{-1}$. Each data set was collected for 1 h. Sample temperature was controlled with a nominal precision of 0.1 °C.

SAXS from both C23O proteins were measured at three concentrations (9, 3 and 1.5 mg/ml) at temperatures from 4 °C to 85 °C in steps of 5 °C. At every change of the temperature, the sample was incubated for approximately half an hour with the X-ray beam off. Sample dilutions

Table 1
The sequences and structures form the PDB used in the structure based alignment by PROMALS3D. Subsequent BLASTP hits were a match to less than 50% of the length of the protein, and delineated a clear natural boundary. The last entry, Q44048, was rejected due to producing an alignment with significant insertions and deletions unlike any of the others.

Uniprot sequence	Representative PDB	BLASTP identity	Enzyme name	Species
Q4J6K0			Catechol 2,3-dioxygenase	*Sulfolobus acidocaldarius*
P06622	1mpy	31%	Metapyrocatechase	*Pseudomonas putida*
Q45135	3eck	32%	Homoprotocatechuate 2,3-dioxygenase	*Brevibacterium fuscum*
Q7WYF5	3hpy	30%	Catechol 2,3-dioxygenase	*Pseudomonas alkylphenolia*
Q0S9X1	3lm4	27%	Catechol 2,3-dioxygenase	*Rhodococcus* sp. (strainRHA1)
Q2GAG3	3b59	22%	Glyoxalase/bleomycin resistance	*Novosphingobium aromaticivorans*
Q6REQ5	2wl9	22%	Catechol 2,3-dioxygenase	*Rhodococcus* sp. DK17
P47228	1lgt	22%	Biphenyl-2,3-diol 1,2-dioxygenase	*Burkholderia xenovorans*
Q44048	1fiu	29%	3,4-Dihydroxyphenylacetate 2,3-dioxygenase	*Arthrobacter globiformis*