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Family of phenylacetyl-CoA monooxygenases differs in subunit organization from other monooxygenases

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ABSTRACT

The phenylacetate degradation pathway is present in a wide range of microbes. A key component of this pathway is the four-subunit phenylacetyl-coenzyme A monooxygenase complex (PA-CoA MO, PaaACBE) that catalyzes the insertion of an oxygen in the aromatic ring of PA. This multicomponent enzyme represents a new family of monooxygenases. We have previously determined the structure of the PaaAC subcomplex of catalytic (A) and structural (C) subunits and shown that PaaACB form a stable complex. The PaaB subunit is unrelated to the small subunits of homologous monooxygenases and its role and organization of the PaaACB complex is unknown. From low-resolution crystal structure, electron microscopy and small angle X-ray scattering we show that the PaaACB complex forms heterohexamers, with a homodimer of PaaB bridging two PaaAC heterodimers. Modeling the interactions of reductase subunit PaaE with PaaACB suggested that a unique and conserved 'lysine bridge' constellation near the Fe-binding site in the PaaA subunit (Lys68, Glu49, Glu72 and Asp126) may form part of the electron transfer path from PaaE to the iron center. The crystal structure of the PaaA(K68Q/E49Q)-PaaC is very similar to the wild-type enzyme structure, but when combined with the PaaE subunit the mutant showed 20–50 times reduced activity, supporting the functional importance of the 'lysine bridge'.

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1. Introduction

Microbes evolved a variety of metabolic strategies, both aerobic and anaerobic, to degrade abundant aromatic compounds (Cao et al., 2009). In aerobic metabolism the aromatic ring undergoes oxygenation and becomes substituted with two hydroxyl groups, either by a dioxygenase or by the action of two successive monooxygenases (Ullrich and Hofrichter, 2007). The resulting cathechol is subsequently cleaved between these two hydroxyl groups or adjacent to one of them in an oxygen-dependent manner. In anaerobic metabolism the aromatic compound is covalently linked to the coenzyme A (CoA) and reduced, resulting in a saturated ring (Fuchs, 2008). Yet another pathway has been identified in microbes operating under fluctuating oxygen concentration. This hybrid pathway combines the features of aerobic and anaerobic pathways in that the aromatic compound is processed as a CoA derivative, which is oxygenated by an oxygenase (Fuchs, 2008). The *Escherichia coli* phenylacetate (PA) degradation pathway is representative of this hybrid pathway. Analysis of the sequenced bacterial genomes indicates that such a hybrid pathway is present in approximately one-sixth of the genomes. The pathway comprises eight reaction steps which convert phenylacetate to acetyl- and succinyl-CoA (Fig. 1) (Teufel et al., 2010). Ten proteins encoded within the *paa* operon are involved in this pathway (Ferrandez et al., 1998). Phenylacetyl-CoA (PA-CoA), undergoes oxygenation to 1,2-epoxyphenylacetyl-CoA by the phenylacetyl-coenzyme A monooxygenase (PA-CoA MO) complex and is further rearranged into a seven-member O-heterocyclic enol ether, an oxepin. The oxepin ring is opened and degraded via steps similar to fatty acid β-oxidation (Fig. 1) (Teufel et al., 2010).

The composition of the MO complex and the chemistry of the PA-CoA MO catalyzed reaction were only recently established (Grishin et al., 2011; Teufel et al., 2010). Although primary studies *in vivo* led to the conclusion that PA-CoA MO is composed of five subunits – PaaA,B,C,D,E (Fernandez et al., 2006), subsequent pull-down assays combined with structural and activity studies showed that only four subunits, PaaA,B,C,E (Grishin et al., 2011; Teufel et al., 2010) but not PaaD are necessary for activity. The function

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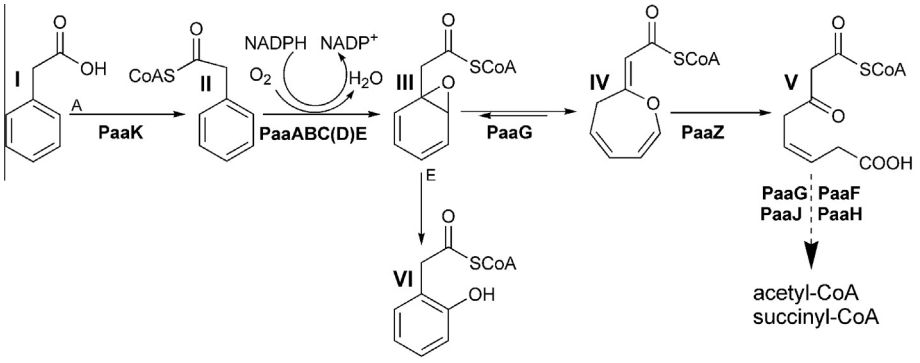

Fig. 1. The phenylacetate utilization pathway.

of PaaD, which shows sequence similarity to SufT from iron-sulfur cluster assembly *suf* operon, remains unknown. In this complex the PaaE component is the plant-type ferredoxin-NAD(P)H reductase, which shuttles electrons from NADPH through FAD and the iron-sulfur cluster to the terminal oxygenase (Grishin et al., 2011). The structure of the PaaAC subcomplex showed significant similarity to catalytic hydroxylase components of other bacterial multicomponent monooxygenases (BMMHs), including soluble methane monooxygenase hydroxylase (sMMOH), toluene/*o*-xylene monooxygenase hydroxylase (ToMOH) and others. This structural similarity allowed us to assign to PaaAC the function of a terminal oxygenase (Grishin et al., 2011). However, the small PaaB subunit, which was found essential for functioning of the complex unclear lacks homology to small subunits of other BMMs (Grishin et al., 2011) and as such, is a novel component of the monooxygenase complex. Its function is unclear and the organization of the monooxygenase components is unknown.

To address the question of the structural role of the small subunit PaaB and its location within the MO complex we have crystallized the PaaACB complex. Although the crystals diffracted only to ~4.5 Å resolution we were able to determine the quaternary arrangement of the subunits where PaaAC heterodimers are linked *via* PaaB homodimer. Further support for this arrangement came from small angle X-ray scattering and electron microscopy. This represents a new oligomeric arrangement, different from previously observed in BMMs. Mutagenesis of residues involved in salt bridges in the vicinity of the iron center and close to the PaaA surface suggests a path for electron transfer from the PaaE reductase to the di-iron center at PaaA.

2. Materials and methods

2.1. Cloning, mutagenesis, expression and purification

The purification of PA-CoA MO and kinetic measurements followed previously described protocols (Grishin et al., 2011). The part of the *Klebsiella pneumonia paa* operon containing *paaA*, *paaB* and *paaC* was cloned into the vector pFO4 (modified pET15b, Novagen).

The overlap extension PCR technique (Ho et al., 1989) was applied to obtain site-directed mutants of *E. coli* PaaA (*Ec*PaaA), using the plasmid pMJ591 containing *paaA* inserted into the pFO4 vector as a template for amplification. The proteins were co-expressed using compatible plasmids, pZL72-*paaA* and pFO4-*paaC* to obtain the *Ec*PaaAC, and pZL72-*paaA*, pFO4-*paaC* and pZL71-*paaB* (pCDFDuet modification, Novagen) in *E. coli* strain BL21(DE3) (Novagen) to obtain *Ec*PaaABC, and induced by 0.1 mM IPTG. After overnight incubation at 20 °C, cells were disrupted by sonication and soluble fraction was obtained by centrifugation at 20,000*g* at 4 °C.

All complexes were purified on Ni-nitrilotriacetic acid agarose using 50 mM HEPES, pH 7.5, 400 mM NaCl, 10 mM imidazole, 0.5 mM DTT as the lysis buffer. The column was washed sequentially with the same buffers, first supplemented with 1 M NaCl and second with 40 mM imidazole. The proteins were eluted with 50 mM HEPES, pH 7.5, 400 mM NaCl, 250 mM imidazole, 0.5 mM DTT. The final purification step was gel filtration chromatography on Superose 12 column (GE Healthcare) equilibrated with the 50 mM HEPES, pH 7.5, 50 mM NaCl, 5 mM DTT.

2.2. Reaction studies

The mixture of PaaE, PaaB and wild-type or mutant *Ec*PaaAC were added to the reaction buffer (25 mM Tris, pH 7.5, 500 μM NADPH, 10 μM $Fe(NH_4)(SO_4)_2$, 10 μM FAD, 100 μ PA-CoA) to a final concentration of each subunit of 1 μM. Reactions were incubated at 37 °C and stopped at various time points. The appearance of 2-hydroxyphenylacetate was quantitatively monitored by LC-MS/MS on a Agilent 1200 HPLC system coupled to a Agilent QQQ6410 mass spectrometer (Agilent Technologies, Inc., Palo Alto, CA).

2.3. Crystallization

The purified *Kp*PaaACB complex was screened extensively for suitable crystallization conditions by sitting drop method. In only one of the many screening drops a single, small rod-shaped crystal of *Kp*PaaACB was found at room temperature with protein concentrated to 2.5 mg/ml and well containing 100 mM MES, pH 6.5, 12% 1-propanol and 10% PEG 5000 MME. The crystal was cryoprotected by transfer into the reservoir solution supplemented with 20% 2-methyl-2,4-pentanediol and diffracted to ~4.5 Å resolution. Despite significant efforts we could not reproduce this crystal.

The crystals of PaaA K68Q, E49Q-PaaC mutant were obtained from the Classic I screen (Qiagen) in condition containing only 25% ethylene glycol.

2.4. X-ray data collection, structure solution and refinement

X-ray diffraction data were collected at the Canadian Light Source, CMCF-1 beamline, using a Mar300CCD detector. Diffraction data integration and scaling was performed with HKL2000 (Otwinowski and Minor, 1997) (Table 1). The $CC_{1/2}$ and CC^* (Karplus and Diederichs, 2012) were calculated in Phenix (Adams et al., 2010) from unmerged dataset processed for this purpose by XDS (Kabsch, 2010). The structure of *Kp*PaaACB was solved by molecular replacement using PHASER (McCoy et al., 2007) with the *Ec*PaaAC structure 3PW1 (Grishin et al., 2011) and the *Ralstonia eutropha* PaaB (*Re*PaaB) structure 3EGR (Joint Center for Structural

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