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Crystallization and preliminary X-ray analysis of dmpFG-encoded 4-hydroxy-2-ketovalerate aldolase–aldehyde dehydrogenase (acylating) from *Pseudomonas* sp. strain CF600

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The final two steps of the *meta*-cleavage pathway for catechol degradation in *Pseudomonas* sp. strain CF600 involve the conversion of 4-hydroxy-2-ketovalerate to pyruvate and acetyl coenzyme A by the enzymes 4-hydroxy-2-ketovalerate aldolase and NAD^+-dependent acylating aldehyde dehydrogenase. Biochemical studies indicate that these two enzymes comprise a bifunctional heterodimer (DmpFG, molecular mass 71 kDa) and suggest that the product of the aldolase reaction is transferred to the dehydrogenase active site *via* a channeling mechanism. Crystals of the DmpFG complex grow in multiple fan-like clusters of thin plates by the hanging-drop method and are improved by streak-seeding. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 102.0, b = 140.7, c = 191.3 Å, and diffract to 2.1 Å resolution. The asymmetric unit contains four DmpFG heterodimers. Heavy-atom derivative screening identified three isomorphous derivatives.

1. Introduction

Environmental pollution by recalcitrant organic chemicals is a widespread problem that requires new technologies to solve. One promising approach is the use of microbial degradation activities to transform or destroy these compounds. Although microbes have evolved the ability to deal with a wide range of naturally occurring compounds, they may fail for a variety of reasons to use these activities for the degradation of related man-made chemicals. Understanding these processes at the molecular level can allow microbial metabolic pathways to be tailored by genetic engineering for the degradation of compounds that may not normally be degraded (Timmis *et al.*, 1994).

Microbial degradation of one class of recalcitrant compounds, the aromatics, involves oxygenative ring cleavage followed by one of several central pathways for conversion to primary metabolites that can sustain microbial growth. One such pathway is the *meta*-cleavage pathway for catechol, which is involved in the degradation of phenols, toluates, naphthalene, biphenyls and other compounds. Enzymes of the phenol-degradation pathway of *Pseudomonas* sp. strain CF600 are encoded by the *dmp* operon of the megaplasmid pVI150 (reviewed in Powlowski & Shingler, 1994).

In the *meta*-cleavage pathway, the final steps for catechol degradation involve conversion of 4-hydroxy-2-ketovalerate to pyruvate and acetyl coenzyme A by the enzymes 4-hydroxy-2-ketovalerate aldolase (HOA) and aldehyde dehydrogenase (acylating) (ADA) (Fig. 1). After the original description of the ADA and HOA encoding genes, *dmpF* and *dmpG*, from *Pseudomonas* sp. strain CF600 (Shingler *et al.*, 1992), related sequences from a variety of catabolic operons have been reported (Eaton, 1996; Habe *et al.*, 1996; Harayama & Rekik, 1993; Hwang *et al.*, 1999; Kikuchi *et al.*, 1994; Platt *et al.*, 1995).

ADA and HOA enzymes were first purified from *Pseudomonas* sp. strain CF600 grown at the expense of phenol (Powlowski *et al.*, 1993). Interestingly, DmpG and DmpF co-purified to homogeneity: the molecular weights of DmpG and DmpF are 37.5 and 32.5 kDa, respectively, and the active oligomeric unit for the molecule appears to be a dimer (two molecules of DmpFG). Close physical association has also been demonstrated for *nah* operon encoded HOA and ADA (Platt *et al.*, 1995). Although dehydrogenase activity is catalyzed by DmpF only, aldolase activity in DmpG alone has never been successfully demonstrated (Shingler *et al.*, 1992 and our unpublished results). However, the aldolase activity is greatly enhanced by the presence of the dehydrogenase (Powlowski *et al.*, 1993). It has been suggested that the close association of these two enzymes may enhance the metabolic flow of the toxic short-chain aldehyde intermediate formed by the aldolase to the dehydrogenase while maintaining it in a sequestered environment (Powlowski *et al.*, 1993).

Thus far, no structural studies of this interesting complex or related enzymes have been

Table 1
Data statistics for crystals of DmpFG.

Values in parentheses refer to the outer resolution shell. The outer shell is 2.18–2.1 Å for native, 2.38–2.3 Å for PCMBS, 2.59–2.50 Å for $Sm(OAc)_3$ and 3.31–3.2 Å for the double derivative [PCMBS + $Sm(OAc)_3$].

	Native	PCMBS	$Sm(OAc)_3$	PCMBS + $Sm(OAc)_3$
Wavelength (Å)	1.07	1.01	1.25	1.01
Resolution (Å)	2.1	2.3	2.5	3.2
Total reflections	959825	837017	602442	292309
Unique reflections	159161	121023	94667	46122
Completeness (%)	99.5 (97.8)	99.8 (99.3)	98.7 (87.2)	99.7 (99.6)
R_{merge}† (%)	11.3 (74.7)	15.0 (65.7)	11.3 (63.0)	15.1 (55.3)
Average $I/\sigma(I)$	13.6 (1.9)	10.7 (2.5)	11.3 (1.9)	10.2 (2.7)
R_{deriv}‡	—	22.9 (30.0)	34.7 (40.7)	32.9 (41.6)

† $R_{merge} = \sum\sum|I - \langle I\rangle|/\sum I$ (summed over all intensities). ‡ $R_{deriv} = \sum|F_{deriv_h} - F_{nat_h}|/\sum F_{nat_h}$.

reported. In order to examine the organization of the active sites, the possibility of substrate channeling and the catalytic mechanisms of the two reactions, we have undertaken structural studies of this heterodimeric bifunctional enzyme. Here, we report crystals of DmpFG grown in the presence of the NAD^+ cofactor.

2. Material and methods

2.1. Protein purification and crystallization

The DmpFG complex was purified from phenol-grown *Pseudomonas* sp. strain CF600 following a modification of the previously described procedure (Powlowski *et al.*, 1993). Briefly, the final three chromatography steps were replaced by chromatography on an NAD^+-agarose affinity column (Sigma N9505, 1.6 × 2.5 cm). After loading, the column was washed with 50 m*M* sodium/potassium phosphate buffer pH 7.5 containing 1 m*M* dithiothreitol to remove contaminants, followed by elution of the enzyme with an identical buffer containing either NAD^+ (1 m*M*) or NaCl (0.1 *M*). The resulting preparations were purer than those obtained using the earlier procedure and were obtained much more rapidly. Full details will be published elsewhere.

The purified DmpFG complex was dialyzed into 50 m*M* Tris pH 7.4 using a collodian membrane (Schleicher & Schuell, New Hampshire) with a 10 kDa cutoff in the presence of 1 m*M* DTT and was concentrated to 9 mg ml^{-1} as determined by the method of Bradford using BSA as a standard (Bradford, 1977). Prior to crystallization, the protein was equilibrated with 2 m*M* NAD^+ in order to obtain a holoenzyme complex. Crystallization trials were carried out using the sparse-matrix method (Jancarik & Kim, 1991). Crystal Screen kits 1 and 2 (Hampton Research) were employed for the initial trials using the hanging-drop method. Each drop, containing 1 µl of protein solution and 1 µl of reservoir solution, was suspended over 1 ml of reservoir solution and was equilibrated at 290 K.

The results of this screening gave granular precipitate from polyethylene glycol (PEG) 8000. Further systematic screens around these conditions yielded multiple fan-like crystal clusters within 3 d from 15% PEG 8000, 100 m*M* ammonium sulfate, 100 m*M* PIPES pH 6.5 (Fig. 2*a*).

The streak-seeding method (Stura & Wilson, 1991) was used to further improve the quality of these crystals. Two or three single fan-like crystals were extracted from the multiple clusters and crushed using a Teflon ball in a microfuge tube containing the same mother liquor as used to grow the initial crystals. Dilution of this seed-stock solution was performed to obtain the ideal concentration of seed crystals. A fresh drop of 1 µl of protein solution mixed with 1 µl of mother liquor was prepared and a probe (whisker) that had been dipped into the diluted seed stock was passed through the drop. Very thin rectangular plate-like crystals appeared from these seeded drops within 3 d.

To further improve the thickness of these crystals, the mother liquor was modified to 15% PEG 8000, 100 m*M* ammonium sulfate and 100 m*M* PIPES pH 7.5 rather than pH 6.5 as had been used for the streak-seeding experiments. This increase in pH results in a slight increase in the protein solubility, thus altering the growth rate of the crystallization so that thicker crystals appear. The final rectangular plate-like crystals appeared within 5 d and had typical dimensions of 0.3 × 0.2 × 0.02 mm (Fig. 2*b*).

2.2. Heavy-atom derivatives

Heavy-atom derivative screening identified three potential isomorphous derivatives. Mercury derivatization was achieved by soaking crystals in 0.5 m*M* *p*-chloromercuribenzenesulfonic acid (PCMBS) for 10–15 min. Samarium-derivative crystals were obtained by soaking crystals in 10 m*M* samarium acetate [$Sm(OAc)_3$] for approximately 10–15 min. The double-derivative

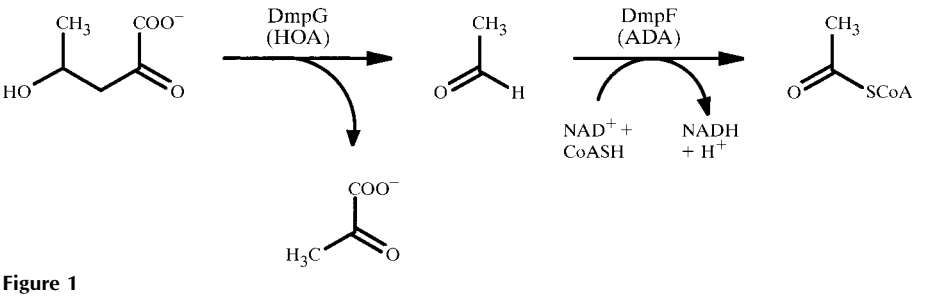
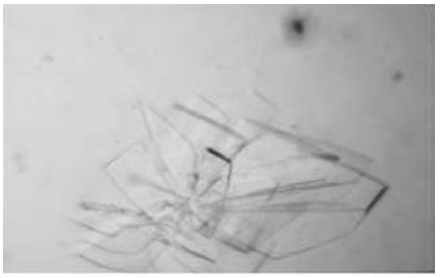


Figure 1
Reactions catalyzed by the bifunctional enzyme DmpFG. The first reaction, catalyzed by 4-hydroxy-2-ketovalerate aldolase (HOA), results in the formation of acetaldehyde. The second reaction, catalyzed by aldehyde dehydrogenase (acylating) (ADA), uses the product of the aldolase reaction to give the final product, acetyl-coenzyme A.


(*a*)

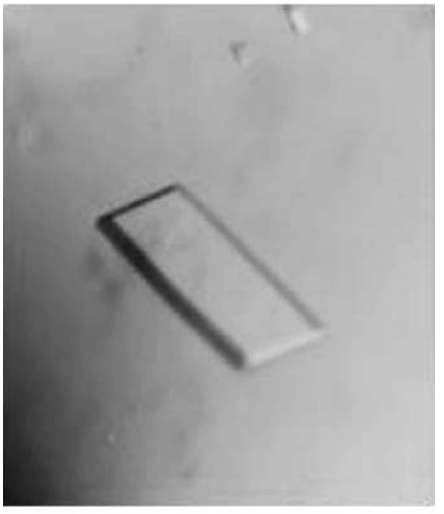

(*b*)

Figure 2
Crystals of DmpFG grown by the hanging-drop method. (*a*) Initial fan-like crystal clusters; (*b*) single crystal obtained by streak seeding.

crystals were obtained by soaking crystals in a mixture of 5 m*M* $Sm(OAc)_3$ and 0.25 m*M* PCMBS for 10–15 min.

2.3. Diffraction experiments

Diffraction data of native crystals and the three derivatives were measured at beamline X8C, Brookhaven National Laboratory, Upton, NY, USA. The crystals were placed into Paratone 8277 (Exxon Oil) as cryoprotectant for 10–15 s followed by immediate flash-cooling to 100 K in a nitrogen stream generated by a Cryostream (Oxford Cryosystems, Oxford, England). This initial flash-cooling resulted in weakly diffracting crystals (less than 6 Å resolution). This initially frozen crystal was then removed from the cold nitrogen-gas stream and quickly transferred to the cryoprotectant (Paratone 8277). The crystal was warmed in the cryoprotectant for 15–20 s before the next cycle of flash-cooling. After this annealing protocol (Harp *et al.*, 1998, 1999) was undertaken, a dramatic improvement in diffraction quality was observed and the crystals diffracted to 2.1 Å resolution at the synchrotron-radiation facility. A second cycle of flash-cooling does not further enhance diffraction quality; rather, it leads to a complete loss of diffraction. Native and derivative data sets were collected on a Quantum-4 CCD area detector in 0.5° oscillation steps for the range $0 \leq \varphi \leq 180°$.

All data were processed and scaled with *DENZO* and *SCALEPACK* from the *HKL* suite of software (Otwinowski, 1993; Otwinowski & Minor, 1997). The data-collection statistics are shown in Table 1. Subsequent calculations were performed with programs from the *CCP*4 suite of software (Collaborative Computational Project, Number 4, 1994). Initial MIR phases were derived using the automated program *SOLVE* (Terwilliger *et al.*, 1999). Density modification by solvent flattening and non-crystallographic averaging calculations were undertaken using the program *DM* (Collaborative Computational Project, Number 4, 1994).

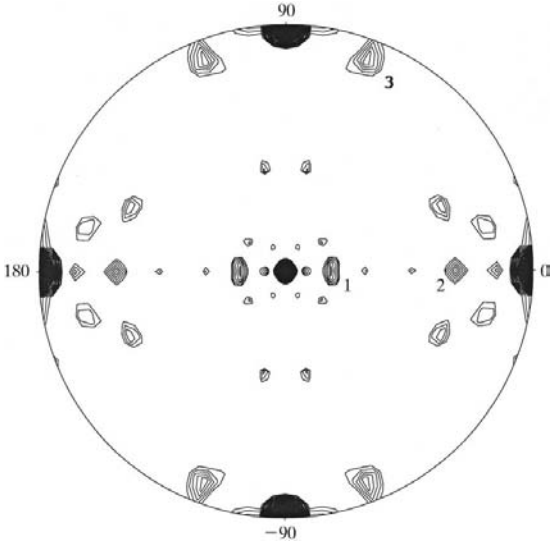


Figure 3
Self-rotation function showing the $\kappa = 180°$ section. Peaks are scaled from 0 to 100% with 100% representing the origin peak. φ angles and the position of the axes are marked on the circumference. The twofold axes are numbered from 1 to 3. Contour lines are plotted for all peaks greater than 14% of the origin peak in intervals of 2%.

3. Results and discussion

The crystals of DmpFG belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 102.0, b = 140.7, c = 191.3 Å, and diffract to 2.1 Å resolution. Assuming four DmpFG complex molecules in the asymmetric unit, the V_M value is 2.4 $Å^3\ Da^{-1}$, which is within the expected range (Matthews, 1968). This V_M value corresponds to a solvent content of approximately 48%. A self-rotation function calculated using the program *POLARRFN* (Collaborative Computational Project, Number 4, 1994) revealed three significant peaks in the $\kappa = 180°$ section (Fig. 3), suggesting the presence of three non-crystallographic twofold axes relating the four DmpFG molecules in the asymmetric unit.

Sequence-homology searches using the SWISS-PROT database (Bairoch & Apweiler, 1999) and the program *PREDICTPROTEIN* (Rost, 1996) on the amino-acid sequence of DmpG revealed approximately 32% identity in primary sequence with a chicken analog, hydroxymethylglutaryl-(HMG)-CoA lyase (P35915; Mitchell *et al.*, 1993). The fully automated fold-recognition program *3D-PSSM* (Kelley *et al.*, 2000) was applied to the sequence of DmpG in order to search for an initial homology model for the enzyme. The program predicts, with an *E* value of 0.243, that the enzyme is composed of a β/α TIM-barrel fold similar to that observed in the structure of the α-subunit of tryptophan synthase (Hyde *et al.*, 1988; PDB code 2tsy). Given these results, we attempted to use the α-subunit of tryptophan synthase as a molecular-replacement (Navaza, 1994) search object to phase the structure of DmpFG; however, these efforts proved unsuccessful.

A search for suitable heavy-atom derivatives for MIR phasing yielded three derivatives. Because of the low number of heavy-atom sites and the relatively poor occupancies of these sites, the automated program *SOLVE* (Terwilliger & Berendzen, 1999) was unable to phase the full structure. Initial phases developed by MIR were further improved using solvent flattening and non-crystallographic averaging procedures, but the resulting electron-density map was discontinuous making it difficult to trace the entire structure. Most recently, we have succeeded in expressing a selenomethionyl mutant of DmpFG (the heterodimer contains 27 methionine residues) in anticipation of pursuing a structure solution by the method of MAD phasing. We are currently pursuing crystallization of this mutant and anticipate that the structure will be readily solved by incorporating carefully measured MAD data as well as the MIR phases that we currently have.

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