Protein-Protein Interactions in the β-Oxidation Part of the Phenylacetate Utilization Pathway

CRYSTAL STRUCTURE OF THE PaaF-PaaG HYDRATASE-ISOMERASE COMPLEX

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Received for publication, June 5, 2012, and in revised form, August 29, 2012. Published, JBC Papers in Press, September 7, 2012, DOI 10.1074/jbc.M112.388231

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* This research was supported by Canadian Institutes of Health Research Grant MOP-48370 and the Canada Research Chair Program (to M. C.). X-ray diffraction data for this study were measured at the Canadian Macromolecular Crystallography Facility at the Canadian Light Source, which is supported by the Canadian Foundation for Innovation, the Natural Sciences and Engineering Research Council of Canada, and Canadian Institutes of Health Research.

This article contains supplemental Tables S1 and S2 and Figs. S1–S8.

Background: The phenylacetate utilization pathway (paa operon) is the main pathway for degradation of aromatic compounds.

Results: We have identified protein-protein interactions among paa enzymes and determined the structure of the stable PaaF-PaaG complex.

Conclusion: Only two complexes exist among these enzymes, PaaA–PaaB–PaaC and PaaF–PaaG.

Significance: The four-step β-oxidation part of the aromatic compounds degradation pathway contains only one stable enzyme complex.

Microbial anaerobic and so-called hybrid pathways for degradation of aromatic compounds contain β-oxidation-like steps. These reactions convert the product of the opening of the aromatic ring to common metabolites. The hybrid phenylacetate degradation pathway is encoded in Escherichia coli by the paa operon containing genes for 10 enzymes. Previously, we have analyzed protein-protein interactions among the enzymes catalyzing the initial oxidation steps in the paa pathway (Grishin, A. M., Ajamian, E., Tao, L., Zhang, L., Menard, R., and Cygler, M. (2011) J. Biol. Chem. 286, 10735–10743). Here we report characterization of interactions between the remaining enzymes of this pathway and show another stable complex, PaaFG, an enoyl-CoA hydratase and enoyl-CoA isomerase, both belonging to the crotonase superfamily. These steps are biochemically similar to the well studied fatty acid β-oxidation, which can be catalyzed by individual monofunctional enzymes, multifunctional enzymes comprising several domains, or enzymatic complexes such as the bacterial fatty acid β-oxidation complex. We have determined the structure of the PaaFG complex and determined that although individually PaaF and PaaG are similar to enzymes from the fatty acid β-oxidation pathway, the structure of the complex is dissimilar from bacterial fatty acid β-oxidation complexes. The PaaFG complex has a four-layered structure composed of homotrimeric discs of PaaF and PaaG. The active sites of PaaF and PaaG are adapted to accept the intermediary components of the Paa pathway, different from those of the fatty acid β-oxidation. The association of PaaF and PaaG into a stable complex might serve to speed up the steps of the pathway following the conversion of phenylacetyl-CoA to a toxic and unstable epoxide-CoA by PaaABCE monoxygenase.

Aromatic compounds are one of the most abundant and persistent environmental pollutants. Although these compounds are quite resistant to degradation, some microbes can metabolize aromatic molecules to serve as the source of nutrition, under both aerobic and anaerobic conditions. In aerobic metabolism, a multicomponent monoxygenase or dioxygenase introduces two hydroxyl groups into the aromatic ring converting it into catechol, which is subsequently cleaved adjacent to or between these two hydroxyl groups in an oxygen-dependent reaction. In anaerobic metabolism, the aromatic compound is first covalently attached to coenzyme A and then enzymatically reduced. A third mechanism, the so-called hybrid pathway, thought to be advantageous under fluctuating microaerobic conditions, was discovered while studying phenylacetate utilization. In this pathway the aromatic compound is first attached to CoA, as in the anaerobic pathway, and next the aromatic ring is oxygenated via a multicomponent monoxygenase to yield an epoxide (Fig. 1A, compounds I–III) (6, 7). The epoxide is rearranged into an oxepin, a seven-membered O-heterocyclic enol ether (compound IV), and in subsequent reactions cleaved to simple components. This hybrid pathway, called the phenylacetate catabolon, is present in one of known bacterial genomes (6), where it serves as the central route for degradation of a wide variety of aromatic compounds such as 2-phenylacetate, 2-phenylethylamine, tropic acid, and styrene.

In Escherichia coli the phenylacetate pathway is encoded by the paa (gene related to the phenylacetic acid degradation pathway) operon and comprises 10 enzymes. The upper part of the pathway (Fig. 1A) consists of phenyl-CoA acetate ligase (PaaK)
(8) and a multicomponent monooxygenase complex consisting of four proteins (PaaABCE) (6, 9). The roles of its subunits, the chemistry of the catalyzed reaction (6), the composition of stable subcomplexes, and the crystal structure of PaaA-PaaC (9) have been described recently. PaaABCE represent a distant member of di-iron multicomponent monooxygenases and converts PA-CoA to the 1,2-epoxide derivative.

The lower part of the hybrid pathway involves further conversion of nonaromatic CoA derivatives (6). The epoxide derivative is rearranged to an oxepin (Fig. 1B, compound IV) by the isomerase PaaG and cleaved by PaaZ to yield a /H9252/3-keto C8-intermediate (Fig. 1B, compound V). The latter is cleaved by the thiolase PaaJ to yield acetyl-CoA and a C6-dehydroadipyl-CoA, which PaaG isomerizes to the /H9251/H9252/H9251/H9251-unsaturated thioester 2,3-dehydroadipyl-CoA (Fig. 1B, compound VI). Then PaaF hydrates compound VI to 3-hydroxyadipyl-CoA (Fig. 1B, compound VII), which undergoes a PaaH-catalyzed NAD-dependent oxidation to 3-oxoadipyl-CoA (Fig. 1B, compound VIII) (6). This final intermediate is cleaved by PaaJ to yield the common metabolites acetyl-CoA and succinyl-CoA (10).

These last steps of the phenaacetate utilization pathway show biochemical similarity to the fatty acid degradation and β-oxidation pathway (Fig. 1B) (5, 11). The catalyzed reactions include Δ3-Δ2-enoyl-CoA isomerization, Δ2-enoyl-CoA hydration, oxidation, and cleavage. The isomerases are Δ3-Δ2-enoyl-CoA isomerase (ECI) or Δ3,5-dienoyl-CoA isomerase (DCI) (12–15). The β-oxidation steps in bacteria are performed by the αββα heterotetrameric complex (called FOM), where the α-subunit contains domains with 2-enoyl-CoA hydratase (ECH) and 3-hydroxyacyl-CoA dehydrogenase activities, whereas β-subunit is a 3-ketoacyl-CoA thiolase (16). In animal cells fatty acid β-oxidation takes place in mitochondria and peroxisomes. In mitochondria the oxidation steps may be performed by multifunctional enzymes: ECH (17, 18), 3-hydroxyacyl-CoA dehydrogenase (19), and 3-ketoacyl-CoA thiolase (20), or by a multifunctional membrane-bound enzyme complex similar to the bacterial FOM (21). In peroxisomes the multifunctional enzymes, similar to the α-subunit of the bacterial FOM, catalyze 2-enoyl-CoA hydration and 3-hydroxyacyl-CoA dehydrogenation (22), whereas 3-ketoacyl-CoA thiolase is a separate monofunctional enzyme (23). Among these enzymes, the same type of activity is catalyzed by enzyme domains with similar architecture.

Apart from the biochemical similarity of catalyzed reactions, Paa enzymes show sequence and structure similarity to enzymes of β-oxidation pathway. PaaG is a functional analog of DCI and ECI, and PaaF and PaaG are both structural homologs of ECI, ECH, and DCI; PaaH is a homolog of 3-hydroxyacyl-CoA dehydrogenase, and PaaJ is a homolog of 3-ketoacyl-CoA thiolase. The structures of PaaG from Thermus thermophilus (Protein Data Bank code 3HRX (24)), E. coli PaaH (Protein Data Bank code 3MOG)3 and T. thermophilus PaaJ (Protein Data Bank code 1ULQ)4 have been previously determined.

The abbreviations used are: ECI, Δ3-Δ2-enoyl-CoA isomerase; CTD, C-terminal domain; ECH, 2-enoyl-CoA hydratase; FOM, bacterial fatty acid β-oxidation multienzyme complex; Ni-NTA, nickel-nitrilotriacetic acid; TCEP, tris(2-carboxyethyl)phosphine; DCI, Δ3,5-dienoyl-CoA isomerase; ECH, 2-enoyl-CoA hydratase; hm, human mitochondrial; yp, yeast peroxisomal.

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Crystal Structure of the PaaF-PaaG complex

The biochemical similarity of the reactions and the homology between the Paa enzymes and enzymes of fatty acid degradation prompted us to investigate whether enzymes of the lower part of the phenylacetate utilization pathway also associate into a multiprotein complex or complexes. To this end we have used a co-expression and co-purification strategy to identify stable complexes among enzymes PaaA, PaaB, PaaC, PaaF, PaaG, PaaH, PaaJ, and PaaZ. The only stable complex identified was between PaaF and PaaG, not counting the previously identified PaaABC complex. We solved the crystal structure of the PaaF-PaaG complex at 2.5 Å resolution. Both proteins possess a crotonase fold and form homotrimERIC discs, and the PaaG and PaaF discs interact with each other. The reaction catalyzed by PaaF, hydration of 2,3-dehydroadiPyl-CoA, is similar to that performed by ECH, and the comparison of their structures shows adaptation to different substrates. The structure of PaaG provides clues to how the enzyme accommodates two structurally diverse substrates to perform two different chemical reactions: the unusual rearrangement of the epoxide to an oxepin and the rearrangement of the double bond from the $\Delta^1$ to $\Delta^2$ position. Although the architecture of the PaaFG complex bears no similarity to structures of the fatty acid $\beta$-oxidation complex FOM (16) and multifunctional enzyme peroxisomal multifunctional enzyme 1 (22), it proves that monofunctional enzymes can also assemble into highly oligomeric structures with possible functional benefits.

EXPERIMENTAL PROCEDURES

Cloning—Genes encoding PaaF, G, H, J, and Z from E. coli K-12 strain were cloned into a modified pET15b (pFO4, ampicillin resistance), pRDSFDuet-1 multiple cloning site 2 (pZL72, kanamycin resistance), and pCDFDuet-1 multiple cloning site 2 (pZL71, streptomycin resistance) vectors (Novagen). Part of the paa operon, encoding the paaFGH and paaABC segments were cloned into pFO4 and pZL71 vectors. Proteins expressed from pFO4-based plasmids possessed noncleavABLE N-terminal His$_6$ tag, whereas those expressed from pET-Duet vectors had no tags. E. coli expression strains BL21(DE3) (Novagen) or BL21(DE3)Star (Invitrogen) were used for protein production.

Protein Expression—An overnight inoculum of a transformed E. coli expression strain was diluted 100-fold with fresh TB medium with appropriate antibiotics, and cells were allowed to grow at 37 °C until the absorbance at 600 nm reached 0.6. Protein expression was induced by the addition of isopropyl $\beta$-D-galactopyranoside to a final concentration of 0.5 mM. After overnight incubation at 20 °C, the cells were harvested by centrifugation and disrupted by sonication, and the soluble fraction was obtained by centrifugation at 14,000 × g for 45 min at 4 °C.

Protein-Protein Interaction Assay—For evaluating protein-protein interaction different combinations of subunits were co-expressed with only one of the subunits having a His tag (see Table 1): pFO4-paaF + pZL72-paaH + pZL71-paaZ; pFO4-paaG + pZL72-paaH + pZL71-paaF; pFO4-paaH + pZL72-paaZ + pZL71-paaG; pFO4-paaZ + pZL72-paaF + pZL71-paaG; pFO4-paaF + pZL72-paaZ + pZL71-paaFGH; pFO4-paaJ + pZL72-paaZ + pZL71-paaFGH; pFO4-paaABC + pZL72-paaFGH + pZL71-paaJ; pFO4-paaABC + pZL72-paaFGH + pZL71-paaZ; pFO4-paaJ + pZL72-paaFGH + pZL71-paaABC; and pFO4-paaZ + pZL72-paaFGH + pZL71-paaABC. The cells co-expressing three proteins were mixed with cells expressing the fourth protein prior to lysis to enable the search for interactions among all four proteins. As a negative control for the specificity of binding to Ni-NTA resin, we used the proteins co-expressed from plasmids pZL72-paaZ + pZL71-paaFGH, encoding untagged proteins.

The cells from overnight expression culture (20 ml of TB media) were collected and lysed as described above. Lysis buffers with different pH values were tested to evaluate the effect of pH on protein interactions. The lysis buffers were based on (a) 50 mM MES, pH 6.5; (b) 50 mM Tris, pH 7.5; or (c) 50 mM Hepes, pH 8.4, supplemented with 5% glycerol, 0.5 mM benzamidine, and 10 μM leupeptin. After binding, the column was thoroughly washed with the lysis buffer and then with the lysis buffer but supplemented with 40 mM imidazole. The retained proteins were eluted with the same buffer supplemented with 250 mM imidazole and analyzed by SDS-PAGE (see Table 1).

Purification of the PaaFG Complex—To express the PaaFG complex for purification and crystallization, two tag combinations were co-expressed: pFO4-paaF(tagged) + pZL71-paaG(untagged) (hisF-G) or pZL71-paaF(untagged) + pFO4-paaG(tagged) (F-hisG). The complex was purified using metal affinity chromatography and gel filtration. The cells were resuspended in lysis buffer (50 mM Tris, pH 8.5, 400 mM NaCl, 10 mM imidazole, 1 mM TCEP) and sonicated for 1 min with 10-s intervals. The lysate was centrifuged at 14,000 × g for 45 min at 4 °C, and the supernatant was applied to the Ni-NTA column. After binding the column was washed with 10 column volumes of the lysis buffer. To decrease nonspecific binding and increase purity of the protein after immobilized metal affinity chromatography, the washing was continued with the high salt buffer (the same as lysis buffer but with increased salt concentration to 1 M NaCl) and then with high imidazole buffer (the lysis buffer but with increased imidazole concentration to 40 mM). The protein was eluted with buffer containing 50 mM Tris, pH 8.5, 200 mM NaCl, 250 mM imidazole, and 1 mM TCEP. The eluted protein was loaded on a PD10 desalting column (Bio-Rad), and the buffer was exchanged to 20 mM Tris, pH 8.5, 200 mM NaCl, 1 mM TCEP. The final step was gel filtration on a Superdex 200 column (GE Healthcare) equilibrated with 20 mM Tris, pH 8.5, 200 mM NaCl, and 1 mM TCEP. The peak fraction was collected for analysis and crystallization. The PaaFG complex showed the single peak on gel filtration and a final purity of more than 95%, according to SDS-PAGE (Fig. 2).

Mass Spectrometry—Protein was precipitated with TFA, added to the final concentration of 0.1% (v/v), and loaded on C18 reverse phase column (Pierce) pre-equilibrated with 0.1% TFA, 50% acetonitrile (v/v). The column was washed with 0.1% TFA, and the protein was eluted with 80% acetonitrile. The elution fraction was diluted to 50% acetonitrile, and formic acid was added to 0.1% (v/v). The sample was injected directly into a Micromass Waters Q-TOF Ultima Global mass spectrometer equipped with a Z-spray ion source and NanoLockSpray (Waters, Mississauga, Canada) source, and measurements were...
performed in a positive ion electrospray mode (+ ESI). The analysis on the Q-TOF Global was carried out in V mode with instrument resolution between 9,000 and 10,000 based on full width at half-maximum. The source and desolvation temperature were set to 80 and 150 °C, respectively. The TOF was operated at an acceleration voltage of 9.1 kV, a cone voltage of 100 V, radio frequency lens of 45 V, and a capillary voltage of 3.8 kV. Collision energy was set to 10 V. Operating parameters of the ESI interface were optimized by infusing standard solutions of [Glu1]-fibrinopeptide B (residues 1–14 of the fibrinopeptide B) (Sigma) as a reference compound 100 fmol/μl in water:acetonitrile 50:50 (v/v) with 0.1% formic acid. All of the measurements were performed at room temperature. The Mass Lynx 4.0 chromatographic software was used to control the equipment and to analyze data.

Western Blotting—The protein was transferred from SDS-PAGE gel to PVDF membrane (Millipore) using wet Western blotting technique (Bio-Rad) according to the manufacturer’s procedure. The detection of His-tagged protein was accomplished with Penta-His antibody (Qiagen) using the ECL detection kit (GE Healthcare) according to the manufacturer’s instructions.

Crystalization—Both PaaF-PaaG constructs were concentrated to 6 mg/ml and screened for crystallization conditions by the sitting drop vapor diffusion method in 96-well plates using the protein complex (Qiagen) and ICSG+ (Qiagen) screens, as well as an in-house buffer screen based on the optimum solubility screen (39). The PaaF-PaaG complexes crystallized readily and in a variety of conditions. The hits were optimized by a hanging drop vapor diffusion method mixing 1 μl of the well solution with 1 μl of protein solution. The best results were obtained with protein concentration of 3 mg/ml. Several conditions led to well diffracting crystals in two crystal forms, space group P321 and P1. The P321 crystals were prone to twinning, with the F-hisG showing significantly lower twinning than the hisF-G (supplemental Table S1). The best diffracting crystals and with minimal amount of twinning as detected by Phenix XTRIAGE program (25), were obtained from drops equilibrated against 0.1M sodium citrate, pH 5.5, 0.2M sodium acetate, and 5% (w/w) PEG 4000 (supplemental Table S1). The crystals were cryoprotected by soaking in a solution containing 0.1 M sodium citrate, pH 5.5, 0.2 M sodium acetate, 5% (w/w) PEG 4000, and 20% 2-methyl-2,4-pentanediol.

Structure Determination—Diffraction data were collected with Mar300 CCD detector at the Beamline CMCF-1 at the Canadian Light Source at 100 K. Data integration and scaling was performed with HKL2000 (26). The pertinent details are presented in Table 2. The structure PaaFG was solved by molecular replacement using program MOLREP (27) with the starting model Protein Data Bank code 2DUB (enoyl-CoA hydratase (18)) for PaaF and Protein Data Bank code 3HRX (PaaG from T. thermophilus (24)) for PaaG. Subsequent model building and refinement was performed using COOT (28) and REFMAC5 (29), respectively. The twin refinement option did not reduce the R factors, indicating negligible twinning contribution, and was not included in further refinement. Noncrystallographic symmetry was used in refinement to improve density map, applying tight restraints for backbone atoms and medium restraints for the side chains. In the crystal form P321, the asymmetric unit contains two PaaF molecules and two PaaG molecules. The refinement statistics are given in Table 2. Analysis of the structures using MolProbity (30) showed good quality of the models.

Electron Microscopy—The samples of PaaF–His$_6$G were prepared for electron microscopy using the conventional negative staining technique as previously described (31). The sample was diluted to ∼0.05 mg/ml, adsorbed to a glow-discharged carbon-coated copper grid, and stained with uranyl acetate. The images were recorded at room temperature using low dose procedures with a FEI Tecnai F20 electron microscope (FEI Inc.) operated at 200 kV at a calibrated magnification of 68,000, resulting in a sampling of 2.2 Å at the specimen level. The images were collected on a Gatan Ultrascan 4k × 4k Digital (CCD) Camera System Camera (Gatan Inc.) with a defocus ranging from 1.5 to 2.5 μm. A total of 620 single particles were manually selected from 10 images using the program Boxer (32) in 128 × 128 pixel images. Image processing was done using SPARX (33). Several rounds of classification and multi-reference alignment were performed, and a three-dimensional initial volume was calculated using the common lines procedure. Several iterations using the projection matching were executed, and finally D3 symmetry was imposed. The final volume was low pass filtered at 40 Å.

Modeling—Modeling of the substrate within PaaF and PaaG was performed by manual docking. Comparison of human mitochondrial ECI (hmECI) (13), yeast peroxisomal ECI (ypECI) (12, 14), rat liver ECH (17), and rat mitochondrial ECH (18) showed that the position of coenzyme A moiety is highly conserved, allowing us to confidently model it in PaaF and PaaG. The structures of PaaF, PaaG, and ypECI were superimposed, and the CoA moiety was placed in the active center of PaaF and PaaG. The moieties 1,2-epoxidephenylacetyl, hex-3-enedioic acyl, and hex-2-enedioic acyl and libraries of bond lengths and angles were generated using the Dundee PRODRG server (34). CoA and the acyl moieties were connected in proper orientation. The torsion angles of the models in the active sites of PaaF and PaaG were manipulated using Coot software (28).

RESULTS AND DISCUSSION

Protein-Protein Interactions—We have previously investigated the interactions between PaaA, B, C, D, and E (9). Now, we have expanded this search for protein-protein interactions to the entire phenylacetate degradation pathway including the PaaABC complex (PaaA, 35.5 kDa; PaaB, 10.9 kDa; PaaC, 27.9 kDa) and enzymes of the lower part of the pathway: PaaZ (73 kDa), PaaG (28.4 kDa), PaaF (27.2 kDa), PaaH (51.7 kDa), and PaaJ (42.3 kDa).

We were able to overexpress individually the PaaF, G, H, J, and Z proteins in soluble forms. To investigate their interactions, they were co-expressed in E. coli in various combinations using Novagen pET-Duet system of compatible vectors, which allows co-expressing of up to eight proteins, and/or cells expressing individual proteins were mixed together before lysis (Table 1). In each case only one protein was tagged with His$_6$, leading to an additional molecular mass of 1.35 kDa, whereas

Crystal Structure of the PaaF-PaaG complex
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TABLE 1
Search for protein-protein complexes between Paa proteins
The co-expression combination is shown in the left column. The purification columns depict whether the protein complex was found.

| Co-expression combination | Added cells | Purification |
|--------------------------|-------------|--------------|
| Paa-His$_6$F + PaaH + PaaZ | PaaG         | Nonspecific  |
| Paa-His$_6$G + PaaH + PaaF | PaaZ         | Nonspecific  |
| Paa-His$_6$H + PaaG + PaaZ | PaaF         | Paa-His$_6$G |
| Paa-His$_6$Z + PaaF + PaaG | PaaH         | Paa-His$_6$G |
| Paa-His$_6$F + PaaG + PaaH + PaaJ + PaaZ | Paa-His$_6$FG | Paa-His$_6$FG |
| Paa-His$_6$J + PaaF + PaaG + PaaH + PaaZ | Paa-His$_6$FG | Paa-His$_6$FG |
| Paa-His$_6$A + PaaB + PaaC + PaaF + PaaG + PaaH + PaaJ | Paa-His$_6$ABC | Paa-His$_6$ABC |
| Paa-His$_6$B + PaaF + PaaG + PaaH + PaaA + PaaB + PaaC | Paa-His$_6$ABC | Paa-His$_6$ABC |
| Paa-His$_6$Z + PaaF + PaaG + PaaH + PaaA + PaaB + PaaC | Paa-His$_6$ABC | Paa-His$_6$ABC |

PaaF + PaaG + PaaZ (control)  

The other proteins were expressed without tags. After sonication the mixture was loaded onto a Ni-NTA column, and the protein(s) retained on the column were analyzed by SDS-PAGE after the imidazole elution step (supplemental Fig. S1). We have performed this affinity purification with each of the five proteins having a His$_6$ tag to avoid potential artifacts because of the presence of a tag. We have also tested the effect of pH in the range of 6.5–8.5 on complex formation. To prevent disruption of weak interaction by high ionic strength conditions, we have used low concentration of salt in lysis, wash, and elution buffers (9).

When the purification was conducted at pH 7.5 and 8.5, we detected only one complex, comprised of PaaF and PaaG, among these proteins (supplemental Fig. S1 and Table 1). Because the molecular masses of PaaF and PaaG are quite similar, we confirmed the identity of the proteins by a combination of Western blots and mass spectrometry. The former confirmed the presence of the His-tagged bait (either His$_6$PaaF or His$_6$PaaG), whereas the mass spectrometry showed the presence of the untagged prey (either PaaG or PaaF, respectively). The molecular masses measured by mass spectrometry were 28,778 Da for the untagged PaaG (expected 28,779 Da) and 27,611 Da for the untagged PaaF (expected 27,612 Da). To exclude the possibility of nonspecific binding to the resin, we tested by SDS-PAGE the extracts from the cell co-expressing untagged PaaF and PaaG. Neither one was retained on the affinity column. We have further purified the complexes with His-tagged PaaF or PaaG at pH 7.5 and 400 mM NaCl in the lysis, wash, and elution buffers and with an additional wash step with 1 M NaCl. The PaaFG complex was retained on the Ni-NTA column, indicating that the complex is stable even at a very high ionic strength.

When the mixtures were purified at pH 6.5 multiple bands were observed on the SDS-PAGE of the elution fraction. As a control, we co-expressed untagged PaaF, PaaG, PaaH, and PaaZ and applied the cell lysate to Ni-NTA beads at low pH. The elution mixture from the Ni-NTA beads contained bands for PaaF, PaaG, and PaaZ, similar to the results with tagged proteins. Therefore, we concluded that under low pH conditions these proteins bind nonspecifically to the Ni-NTA beads, precluding conclusions about complex formation.

We have also investigated the potential interactions between the stable PaaABC complex and any other enzymes from the lower part of the pathway. No such interactions were detected. These experiments showed that the only stable complex in the phenylacetate degradation pathway, in addition to previously identified PaaABC, is that of PaaF-PaaG, which was detected under all tested conditions, independent of the location of the His tag (hisF-G and F-hisG complexes, see Table 1). No complex between PaaF, PaaH, and PaaJ, equivalent to the fatty acid β-oxidation complex, was detected.

Characterization of the PaaFG complex—The PaaF-His$_6$PaaG and His$_6$PaaF-PaaG complexes eluted as a single peak from the size exclusion column (Superdex 200), with elution volumes of 11.8 and 11.5 ml (Fig. 2), respectively, corresponding to an apparent molecular mass of ~250 kDa. Because the molecular masses of PaaF and PaaG are ~30 kDa, this indicated a large hetero-oligomer in solution with several copies of each molecule. Dynamic light scattering measurements of the PaaF-PaaG complex indicated a similar molecular mass of ~210 kDa, with a low percentage of polydispersity, confirming the presence of large oligomers in solution.

The Structure of the PaaFG Complex—The crystal structure of the F-His$_6$G complex was determined to 2.55 Å resolution (Table 2). The $R_{free}$ factor is higher than expected for this resolution, which is likely due to a high solvent content of the crystal of 64%. Blobs of electron density present along the 3-fold axis and distant from the polypeptide chains are most likely partially ordered solvent molecules and were not modeled. A typical section of electron density map is shown in supplemental Fig. S2. The good quality of the model was confirmed by MolProbity (30).

PaaG and PaaF share 35% sequence identity and were expected to have similar overall three-dimensional structures. However, differences in their side chains allowed for unequivocal assignment of the electron densities to specific molecules. The superposition of PaaF and PaaG resulted in a root mean square deviation of 1.35 Å for 220 common C$_{\alpha}$ atoms. PaaF and PaaG associate into homotrimeric rings; two PaaF homotrimers pack face-to-face into a homohexamer, and the PaaG trimers bind to the outside surfaces of the PaaF hexamer forming a four-layered sandwich structure (Fig. 3). The same arrangement was found in a second crystal form (P1 space group, form 3 in supplemental Table S1) that formed under different crystallization conditions and diffracted to 3.5 Å resolution. The Matthews coefficient suggested the presence of 10–15 monomers/asymmetric unit. Molecular replacement using the full oligomeric structure of PaaFG complex showed a clear solu-
tion. The subsequent rigid body refinement led to plausible statistics ($R_{\text{free}} = 0.30$) (data not shown). Therefore, the observed association of the four discs was independent of the crystallization conditions.

PaaF possesses a canonical crotonase fold (Fig. 4A). It comprises a core domain built of ββα units forming two nearly perpendicular β-sheets surrounded by six α-helices. An additional C-terminal α-helical domain (CTD) contains three α-helices (α8, α9, and α10). The three PaaF molecules form a disc-shaped homotrimer with a diameter of 70 Å and thickness of about 35 Å (Fig. 4B). Each PaaF molecule within the disc interacts with the other two subunits. The interactions are mediated by the CTD of the first PaaF subunit interacting with α4 and α5 from the second PaaF subunit and forming a four-helix bundle. In turn, the CTD from the second PaaF subunit interacts with the α4 and α5 of the third PaaF, and so on, as observed for other crotonase fold proteins (Fig. 4B and supplemental Fig. S3) (17). The contact area between each two subunits in the homotrimer is ~1900 Å²/subunit.

The two trimeric PaaF discs pack head-to-head and interact through the equivalent surfaces (Figs. 3 and 4C). In the observed arrangement, each monomer of the top trimer is situated opposite to the monomer of the bottom trimer. The α2 of the bottom monomer interacts with α2 of the upper monomer (Fig. 4C). Additionally, the CTD domain of the bottom monomer interacts with the CDT of a second monomer in the upper disc (supplemental Fig. S3). The short α-helical turn before α2 provides additional interactions.

The PaaG monomer is structurally very similar to PaaF and also contains two β-sheets surrounded by six α-helices and the C-terminal helical domain. The PaaG homotrimer contains interactions similar to those observed for PaaF, namely the contacts between the CTD of one subunit with helices α4 and α5 of another subunit (supplemental Fig. S4). Rather than forming homohexamers like PaaF, PaaG homotrimers bind to the PaaF homohexamer in a head to tail fashion, forming a sandwich with PaaG homotrimers outside and the PaaF homohexamer inside. The contact area between one PaaF and one PaaG subunit is 280 Å²/subunit, making the overall interaction surface between PaaG and PaaF discs about 850 Å²/disc (Fig. 4D). PaaG CTD domain interacts with the loop preceding α7 in PaaF; the Tyr-234PaaG, Leu-230PaaG, and Leu-237PaaG form hydrophobic interactions with Pro-179PaaF and Leu-182PaaF; Arg-234PaaG, and Arg-240PaaG make salt bridges with Glu-185PaaF, whereas Arg-232PaaG, Arg-236PaaG, and Asp-233PaaG form hydrogen bonds to the hydroxyl of Tyr-186PaaF (Fig. 4D). The trimeric discs are stacked on top of each other with the openings in their centers forming one long tunnel that extends through the entire PaaFG complex. This open channel has a diameter of 9 Å

![FIGURE 2. Purification of PaaF-His$_{6}$PaaG complexes. Size exclusion chromatography profile on a Superdex 200 column. The inset shows the SDS-PAGE of the peak fraction containing both proteins (left lane) and the Western blot (right lane), showing only one band, corresponding to His-tagged PaaG.](image)

**TABLE 2**

X-ray data collection and refinement statistics

| Data set | PaaF-His$_{6}$G |
|----------|-----------------|
| Space group | P21 |
| $a$, $b$, $c$ (Å) | 132.0, 132.0, 154.0 |
| α, β, γ (deg) | 90.0, 90.0, 120.0 |
| Wavelength (Å) | 0.97949 |
| Resolution (Å) | 50.0–2.55 |
| Last shell of resolution (Å) | 2.59–2.55 |
| Observed $hkl$ | 412,674 |
| Unique $hkl$ | 51,177 |
| Completeness (%) | 99.8 (100.0) |
| Redundancy | 8.0 (7.3) |
| $R_{\text{sym}}$ | 0.11 (0.488) |
| $I/(\sigma(I))$ | 22.0 (3.9) |
| $R_{\text{work}}$ | 0.280 |
| $R_{\text{free}}$ (%) | 0.321 (4%) |
| Wilson B (Å²) | 48.0 |
| B-factor Å² (no. of atoms) |
| Protein | 49.5 (7474) |
| Solvent | 42.9 (121) |

**Ramachandran plot**

| Allowed (%) | 95.8 |
| Generous (%) | 4.0 |
| Disallowed (%) | 0.2 |

**Root mean square deviation**

| Bonds (Å) | 0.010 |
| Angles (°) | 1.21 |

**Protein Data Bank code**

4FZW
and is lined with three short loops from each monomer. Two of those loops contain conserved Gly residues located in the turns of the polypeptide backbone. One of them is in a conformation (phi/psi angles) accessible only to Gly residues; the second is in conformation corresponding to a left-handed $\alpha$-helix.

That the two PaaG discs do not associate into a hexamer like PaaF agrees with the structure of \textit{T. thermophilus} PaaG (24) (45% identity, entire length); PaaG forms trimeric discs, which in the crystal, pack with their centers offset by about 20 Å along the disc plane, significantly diminishing the contact surface area for hexamer formation. In solution the functional unit of \textit{T. thermophilus} PaaG was proven to be a homotrimer as well, as judged by gel filtration (24).

**Single Particle Electron Microscopy**—We chose electron microscopy to determine whether the PaaFG complex indeed adopts the same four-layered sandwich architecture in solution as observed in the crystal. The images show a homogeneous particle population with most of the particles laying in a side orientation, revealing an assembly of four layers of densities aligned to a central axis (Fig. 3B, panels a1–a3, a5–a7, a9, and a10). Some of the images show the particle in a top view or represent individual trimers (Fig. 3B, panels a4 and a7). The class average obtained from the side view images as the three-dimensional reconstruction of the complex confirms the four-layer arrangement observed on the crystal structure (Fig. 3B).

The reconstruction shows the two central trimers (corresponding to the PaaF hexamer) in close proximity. The distance between the centers of the two central electron densities is ~33 Å. The peripheral densities (attributed to PaaG) are slightly more separated, and the distance between the centers of the two peripheral electron densities is ~38 Å. This arrangement is in very good agreement with the crystallographic model (Fig. 3B).

**Structural Comparison with Homologs**—Three enzymes with the crotonase fold occur in the fatty acid $\beta$-oxidation pathway: ECH, ECI, and DCI. The PaaF protein is the most similar to ECH both in sequence and in structure; the two structures can be superimposed with a root mean square deviation of 0.95 Å for 234 common C\textsubscript{a} atoms (48% identity). PaaF and ECH have the same relative arrangement of the CTD (supplemental Fig. S5), in which helices $\alpha$9 and $\alpha$10 protrude away from the rest of the molecule and cover the active site of the adjacent subunit (17, 18). Not surprisingly, both enzymes perform the same type of reaction: hydration of the double bond.

Although the PaaG is an isomerase, its structure is also most similar to ECH rather than ECI or DCI, with a root mean square deviation of 1.4 Å for 212 C\textsubscript{a} atoms (33% identity). PaaF and ECH have the same relative arrangement of the CTD (supplemental Fig. S5), in which helix $\alpha$9 covers the active site of its own subunit (13, 14).

The formation of a homotrimer by both PaaF and PaaG is similar to what was observed for ECH. The formation of a
homohexamer as seen in PaaF is typical for crotonase enzymes from fatty acid metabolism, observed in ECH (17, 18), ypECI (14), and DCI (15). However, there is no evidence of homohexamer formation for human mitochondrial ECI (13). The arrangement of homotrimeric discs in a hexamer differs between the enzymes and has been described by two parameters: the distance between the centers of the discs and the angle of rotation of the discs relative to each other (supplemental Table S2 and Fig. S7) (12). For ypECI two different arrangements were found, with the two trimers being either close or more distant from each other (ypECI tight and loose forms) (12). The two discs forming the PaaF homohexamer are very close to each other, similar to the tight form of ypECI. The individual PaaF subunits in the opposite discs are placed against each other, whereas in ypECI the two discs are rotated by ~60°, such that the monomeric subunit in one disc is situated in between two subunits of the other disc.

Substrate Binding and Catalysis—Despite structural similarity, crotonase fold enzymes from fatty acid catabolism catalyze a variety of reactions: a hydration of the double bond (ECH), an isomerization of a double bond (ECI), and an isomerization of a conjugated double bond system in fatty acids (DCI). These reactions are related in that they all involve the abstraction of a proton α to the thioester and likely proceed through an enolic intermediate. The type of catalyzed reaction is determined by the constellation of negatively charged residues within the active site. ECI, ECH, and DCI contain one, two, and three catalytically important acidic residues, respectively. They are placed in three conserved positions within the crotonase fold. Even for the same reaction type, the position of the catalytic residue may vary, as can be seen for the two isomerases: hmECI and ypECI. In positions 1, 2, and 3, hmECI contains, respectively (where catalytic residues are indicated in bold) Leu-114, Glu-136, and Pro-144; ypECI contains Ala-129, Phe-150, and Glu-158; ECH contains Glu-144, Glu-164, and Gly-172; DCI contains Asp-176, Glu-196, and Asp-204; PaaG contains Thr-116, Phe-136, and Asp-144; and PaaF contains Glu-109, Glu-129, and Gly-137. The oxyanion of the enolate formed during the reaction is stabilized by an oxyanion hole (35).

The residues Glu-144 and Glu-164 in ECH were shown to be involved in catalysis (36, 37). The structurally equivalent residues in PaaF are Glu-109 and Glu-129, which suggests that the catalytic mechanism of PaaF is similar to ECH. Glu-109 would act as a general base, activating the nucleophilic water molecule.
for addition to the $\beta$-carbon of the substrate. The resulting enolate is protonated at the $\alpha$-carbon by Glu-129. (Fig. 5A and supplemental Fig. S5). The transient negative charge on the thioester carbonyl group is stabilized in the oxyanion hole formed by the amino groups of Ala-63 and Ala-106. Our attempts to visualize benzoyl-CoA bound to PaaFG either by co-crystallization or by soaking native crystals in 10 mM benzoyl-CoA were unsuccessful. Nevertheless, the position of the ligand could be reliably predicted based on the known structures of human mitochondrial ECI and rat liver mitochondrial ECH, both in complex with octanoyl-CoA (13, 18). The position of the CoA moiety in these structures is very similar, and it was modeled in PaaF based on their superposition with ECI and ECH. However, the conformations of the octanoyl moieties in ECI and ECH differ significantly. Therefore the acyl moiety of the PaaF substrate was manually built, and the torsion angles were manually adjusted to maximize the fit to the specific shape of the substrate-binding site in PaaF (supplemental Fig. S5). The substrate-binding surface in PaaF is very similar to that in ECH with a closed tunnel that accommodates the acyl moiety. This tunnel is wider in PaaF because of the replacement of a bulky Phe-267 with an Ala-232. The bottom of the tunnel in PaaF is lined with positively charged residues: Lys-71, Arg-81, and Arg-225' (from the neighboring molecule in the homotrimer). The substrate for PaaF is 2,3-dehydroadipyl-CoA, an extended aliphatic moiety with a carboxyl group at the end. In our model, which follows the conformation of the octanoyl moiety in ECH, the 2,3-dehydroadipyl fits well in the active site with the carboxylic group being within the hydrogen bond distance of these positively charged residues. PaaG catalyzes two different reactions within this pathway: (a) the isomerization of 1,2-epoxyphenylacetyl-CoA to oxepin-CoA and (b) the isomerization of $\Delta^2$-dehydroadipyl-CoA to $\Delta^2$-dehydroadipyl-CoA. The PaaG active site contains the catalytic Asp-144, which is placed in a spatially equivalent position to the catalytically important Glu-158 in ypECI, enabling isomerization reactions (supplemental Fig. S6). The first substrate, 1,2-epoxyphenylacetyl-CoA, was modeled within the PaaG-binding site the same way as described above for PaaF. The carboxyl group of Asp-144 is within 3 Å of the $\alpha$-carbon atom of the 1,2-epoxyphenylacetyl moiety in the enzyme-substrate model, the site of initial catalytic attack, according to the mechanism proposed by Teufel et al. (6) for conversion of 1,2-epoxyphenylacetyl-CoA to oxepin-CoA (Fig. 5B and supplemental Fig. S6). The substrate binding surface of PaaG forms a deep cleft rather than a tunnel like PaaF. However, the unstructured segment 67–77 may serve as a lid to close the cleft and surround the substrate completely. This site has a hydrophobic character being lined with Phe-136, Leu-66, Leu-80, Leu-139, Leu-141, Tyr-88, and Tyr-244' (other subunit). Modeling of the 1,2-epoxyphenylacetyl moiety in the active site of PaaG indicates that the binding site can accommodate this large substrate. The active site of PaaG can also accommodate the second substrate, dehydroadipyl-CoA. The available structures of hmECI and ECH, both in complex with octanoyl-CoA, show...
that the octanoyl moiety is bound in an extended conformation, but differently in each case (13, 18). Interestingly, these conformations of an octanoyl moiety (after C?) are not possible in PaaG because of steric hindrance (supplemental Fig. S8). Thus the conformation of the dehydroadipyl moiety in PaaG will most probably differ from the conformations of the octanoyl moiety seen in hmECI and ECH.

The modeling shows that the dehydroadipyl moiety is likely to bind in an extended conformation albeit different from that previously observed. However, PaaG unlike PaaF has no positively charged residues to bind the carboxyl group of the substrate. Instead, the carboxyl group is likely to form hydrogen bonds with the Gln-235 and Ser-83 in the hydrophobic environment of the PaaG active site (supplemental Fig. S6).

The catalysis of the second reaction performed by PaaG, isomerization of the double bond in dehydroadipyl-CoA, should be similar to that of ECI (14) and involves the abstraction of a proton from the α-carbon by negatively charged Asp-144, followed by reprotonation at the γ-carbon. The oxyanion hole in PaaG is formed by main chain NHs of Gln-64 and Ala-113 (Fig. 5C).

What Is the Advantage of Complex Formation?—The reason for the formation of a complex between PaaF and PaaG is not immediately clear, nor is the reason for homohexamer formation in enzymes of fatty acid β-oxidation pathway or the reason for diverse quaternary structures of those homohexamers (12). In the arrangement of PaaFG complex, the active sites of PaaG are located on the external surfaces of the double disc structure and face the flat bottom of the trimeric PaaG disc (Fig. 3A). The distance between the closest substrate-binding sites on PaaF and PaaG is ~45 Å, and there is no direct connection/tunnel within the heterohexamer between these two sites. In this respect PaaFG complex differs from the fatty acid β-oxidation complex (16) and the multifunctional enzyme from peroxisomes (22).

PaaG and PaaF catalyze consecutive steps along the phenylacetate degradation pathway: isomerization and hydration. The complex contains 12 active sites brought together by this architecture. The proximity of the active sites results in an increased local concentration of the enzymes themselves and of the product of PaaG (outer discs) that is available for processing by PaaF (inner discs). Such an arrangement must provide a sufficient fitness advantage to be retained by the bacteria.

Recently it was found that the phenylacetate degradation pathway is capable of detoxification of a dangerous epoxide (Fig. 1A, compound III), formed by PaaABC monooxygenase. A novel activity was described for this monooxygenase, which is the deoxygenation of the epoxide-CoA back to the PA-CoA (38). This reverse reaction is also beneficial because it does not allow the unstable epoxide to form spontaneously the dead end product 2-hydroxyphenylacetate, which cannot be metabolized (38). Thus the PaaFG complex may be an evolutionary adaptation of the lower part of the pathway for speeding up subsequent reactions after aromatic ring oxygenation, thereby preventing accumulation of the epoxide.

Acknowledgments—We thank Dr. J. Lee for comments on the manuscript and Dr. D. R. Palmer for comments and help with Figs. 1 and 5.

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