Molecular Characterization of the Gallate Dioxygenase from *Pseudomonas putida* KT2440: the Prototype of a New Subgroup of Extradiol Dioxygenases*

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Running title: *Gallate Dioxygenase from P. putida KT2440*

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In this work we have characterized the galA gene product from *Pseudomonas putida* KT2440, a ring-cleavage dioxygenase that acts specifically on gallate to produce 4-oxalomesaconate. The protein is a trimer composed by three identical subunits of 47.6 kDa (419 aa) that uses Fe²⁺ as the main cofactor. The gallate dioxygenase showed maximum activity at pH 7.0, and the $K_m$ and $V_{max}$ values for gallate were 144 µM and 53.2 µmol/min/mg protein, respectively. A phylogenetic study suggests that the gallate dioxygenase from *P. putida* KT2440 is the prototype of a new subgroup of type II extradiol dioxygenases that share a common ancestor with protocatechuate 4,5-dioxygenases and whose two-domain architecture might have evolved from the fusion of the large and small subunits of the latter. A three-dimensional model for the N-terminal (residues 1-281) and C-terminal (residues 294-420) of the gallate dioxygenase from *P. putida* KT2440 was generated by comparison with the crystal structures of the large (LigB) and small (LigA) subunits of the protocatechuate 4,5-dioxygenase from *Sphingomonas paucimobilis* SYK-6. The expression of the galA gene was specifically induced when *P. putida* KT2440 cells grew in the presence of gallate. A *P. putida* KT2440 galA mutant strain was unable to use gallate as sole carbon source and it did not show gallate dioxygenase activity, suggesting that the GalA protein is the only dioxygenase involved in gallate cleavage in this bacterium. This work points to the existence of a new pathway that is devoted to the catabolism of gallic acid and that remained unknown in the paradigmatic *P. putida* KT2440 strain.

The variety and number of complete microbial genome sequences is increasing at an unprecedented rate. To date, more than 200 bacterial genomes have been successfully sequenced and nearly 1000 genomes are currently in progress, which provides an enormous amount of data for comparative genomic analysis and for identifying new enzymes, regulators, and pathways, including key aspects of genome evolution and bacterial adaptation (1). Among bacteria, *Pseudomonas* species constitute a large diverse group of ubiquitous γ-proteobacteria that are well known for their broad metabolic versatility and genetic plasticity. *Pseudomonas putida* is one of the best-studied species of the genus and many aspects of its biology have been unraveled (2, 3). *P. putida* strain KT2440 is a paradigm of a metabolically versatile bacterium used as workhorse for genetic and physiological studies as well as for the development of biotechnological applications (3). This strain is particularly renowned for its ability to metabolize aromatic compounds (4). The recent sequencing of the *P. putida* KT2440 genome (5) permitted a genomic analysis of the global catabolic potential of this bacterium towards aromatic compounds (4, 6). This search revealed the presence of...
three central pathways, that is, the ortho pathway for the catabolism of protocatechuate (pca genes) and catechol (cat genes), the phenylacetate pathway (pha genes) and the homogentisate pathway (hmg genes). A number of peripheral routes that channel different aromatic compounds to some of the three central pathways were also identified (4, 6).

A key enzyme in the aerobic catabolism of aromatic compounds is the ring-cleavage dioxygenase that opens the aromatic ring. There are two distinct classes of ring-cleavage dioxygenases: i) intradiol dioxygenases, which cleave the aromatic nucleus ortho to (between) the hydroxyl substituents, ii) extradiol dioxygenases, which cleave the aromatic ring meta (adjacent) to the hydroxyl substituents. Whereas intradiol dioxygenases only cleave substrates possessing vicinal hydroxyl groups (catecholic compounds), the extradiol dioxygenases may act both on catecholic and non-catecholic compounds (7). Extradiol dioxygenases belong to at least three evolutionary independent families. Type I extradiol dioxygenases belong to the vicinal oxygen chelate superfamily and include two-domain enzymes, such as the 2,3-dihydroxybiphenyl 1,2-dioxygenase from Burkholderia sp. LB400, and one-domain enzymes, such as the 2,3-dihydroxybiphenyl 1,2-dioxygenases II and III from Rhodococcus globerulus P6 (7), corresponding to Class II and Class I dioxygenases according to Spence et al. (8). Type III dioxygenases belong to the cupin superfamily and include enzymes such as the gentisate and homogentisate dioxygenases (7). Type II dioxygenases are multimers possessing one, e.g., the 2,3-dihydroxybenzylpropionate 1,2-dioxygenase (MhpB) from Escherichia coli, or two different subunit types (7). In the case of the protocatechuate 4,5-dioxygenase from Sphingomonas paucimobilis SYK-6, which has an αβ2 composition, the large (β) and small (α) subunits appear to be unrelated, being the β subunit similar to the protomers of the homooligomeric enzymes (7, 9). In contrast, the two subunits of the 2-aminophenol 1,6-dioxygenase share sequence similarity but it appears that only the β subunit contains an active site (10). Even though these three types of extradiol dioxygenases present different structural folds, they all share similar active sites and the two histidines and one glutamate structural motif for Fe2+ coordination (7).

In P. putida KT2440 we had identified three intradiol dioxygenases, the CatA and CatA2 catechol 1,2-dioxygenases and the PcaGH protocatechuate 3,4 dioxygenase, and one extradiol dioxygenase, the HmgA homogentisate dioxygenase (4, 6, 11). In this work we have characterized the galA gene of P. putida KT2440 that is the first one reported to code for a specific gallate dioxygenase. The characterization of the gallate dioxygenase from P. putida KT2440 revealed not only that this enzyme is a prototype of a new subgroup of type II extradiol dioxygenases, but also suggested the existence of a novel aromatic catabolic pathway involved in gallate mineralization in P. putida KT2440.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions-The** P. putida strain KT2440 (12, 13) was used throughout this study. E. coli DH10B [F−, mcrA Δ(mrr hsdRMS-mcrBC) Δ80dlaZ ΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK λ– rpsL endA1 mupG] (Life Technologies) and E. coli BL21(DE3) [F−ompT hsdS2(rpsLmB) gal dcm λDE3 (harboring the T7 RNA polymerase gene I under control of the lacUV5 promoter)] (14) were used as hosts for gene cloning and overexpression, respectively. Plasmid pET-29a(+) (Novagen) is an oriColE1 vector that confers kanamycin resistance and it allows gene cloning and expression under the control of the T7 promoter. pGEM-T Easy Vector (Promega) is an oriColE1 vector that confers ampicillin resistance and it allows direct cloning of PCR products. pK18mob (15) is a mobilizable (Mob+) oriColE1 plasmid that confers kanamycin resistance and it was used for directed insertional disruption in P. putida. E. coli cells were grown at 37 °C in Luria-Bertani (LB) medium (14) containing, where appropriate, ampicillin (100 µg/ml) or kanamycin (50 µg/ml). P. putida KT2440 cells were grown at 30 °C in LB medium or in MC minimal medium (16) containing 0.2%...
citrate. Where appropriate, 50 µg/ml of kanamycin was added to the culture medium. *P. putida* KT2440 cells were adapted to grow in MC minimal medium containing 1 mM L-cysteine, acting as a reducing agent to prevent gallic acid oxidation, and 5 mM gallic acid as sole carbon source.

**Molecular Biology Techniques**—Standard molecular biology techniques were performed as previously described (14). PCR products were purified with the High Pure plasmid isolation kit (Roche Applied Science). DNA fragments were purified with Gene-Clean Turbo (Q-BIOgene). Genomic DNA from *P. putida* KT2440 was isolated with GenomicPrep Cells and Tissue DNA Isolation kit (Amersham Biosciences). All cloned inserts and DNA fragments were confirmed by DNA sequencing with fluorescently labeled dideoxynucleotide terminators (17) and AmpliTaq FS DNA polymerase (Applied Biosystems Inc.) in an ABI Prism 377 automated DNA sequencer (Applied Biosystems Inc.). Transformation of *E. coli* cells was carried out by using the RbCl method (14).

**Sequence Data Analysis**—Nucleotide sequence analyses were done at the INFOBIOGEN server (http://www.infobiogen.fr/services/menuserv.html). A homology search with unfinished microbial genome data bases was performed with the TBLASTN algorithm (18) at the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Amino acid sequences retrieved from the protein data bases were aligned using the ClustalW program (19) at the INFOBIOGEN server. Phylogenetic analysis of the type II extradiol dioxygenases was carried out according to Kimura’s two-parameter method (20) and a tree was reconstructed using the neighbour-joining method (21) of the PHYLIP program (22).

**Modeling of Gallate Dioxygenase**—The three-dimensional models for the N- and C-terminal domains of the gallate dioxygenase from *P. putida* KT2440 were built using the automated SWISS-MODEL Protein Homology-Modeling Server (23). The crystal structure of the protocatechuate 4,5-dioxygenase from *S. paucimobilis* SYK-6 (Protein Data Bank entry 1BOU) was used as template. The gallate dioxygenase homology model was superimposed onto the crystal structure of the protocatechuate/protocatechuate 4,5-dioxygenase complex (Protein Data Bank entry 1B4U) by using the PyMOL viewer program (24).

**Overproduction of Gallate Dioxygenase**—The galA gene was PCR-amplified from *P. putida* KT2440 genomic DNA by using oligonucleotides GalA5´ (5’-CGCATATGGCTCGTATCATTGTTG-3’; the *galA* start codon is indicated in boldface type, and an engineered Ndel restriction site is underlined) and GalA3´ (5’-CGGAATTCTCCAGGGATGGGCAAAACGC-3’; it is located at 100-bp from the *galA* stop codon; an engineered EcoRI restriction site is underlined). The resulting 1.3-kb DNA fragment was cloned into the pGEM-T Easy vector and then subcloned as a Ndel/EcoRI DNA fragment into Ndel/EcoRI double-digested pET-29a(+) plasmid, giving rise to plasmid pETGalA (6.7 kb) that expresses the *galA* gene under control of the *T7lac* promoter and the ribosome-binding site from the pET-29a(+) plasmid.

*E. coli* BLB21(DE3) cells harboring plasmid pETGalA were grown until the cultures (1 l) reached an absorbance at 600 nm of 0.5. Overexpression of the cloned gene was then induced during 3 h by the addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested at 4 ºC, resuspended in 100 ml of extraction buffer (50 mM MOPS (morpholine propanesulfonic acid), pH 8.0, 10% glycerol, 0.2 mM ferrous ammonium sulfate, and 2 mM L-cysteine hydrochloride), and disrupted by two consecutive passages through a French press (Amino Corp.) operated at a pressure of 20,000 p.s.i. The cell lysate was centrifuged at 13,000 x g for 30 min at 4 ºC. The clear supernatant fluid was carefully decanted and used as the crude extract fraction.

**Purification of Gallate Dioxygenase**—The purification of gallate dioxygenase from *P. putida* KT2440 was performed at 4 ºC by a combination of ultracentrifugation and Phenyl-Septarose chromatography. The *E. coli* BLB21(DE3) (pETGalA) crude extract (100 ml, 490 mg protein) was ultracentrifuged at 80,000 x g for 60 min and the supernatant (step 2, ultracentrifugation fraction) was loaded onto a Phenyl-Sepharose CL-4B column (Amersham Biosciences) previously...
equilibrated with extraction buffer containing 1 M ammonium sulfate. Proteins were eluted from the column with a linear gradient (1 M to 0 M) of ammonium sulfate in 1 liter of extraction buffer, and protein concentrations and gallate dioxygenase activity were assayed. The gallate dioxygenase eluted around 0.1 M ammonium sulfate (step 3, Phenyl-Sepharose fraction). Proteins were analyzed by SDS-polyacrylamide gel electrophoresis as described previously (14). Protein concentration was determined by the method of Bradford (25) by using bovine serum albumin as the standard.

**N-Terminal Amino Acid Sequence Determination**-The N-terminal amino acid sequence of gallate dioxygenase from *P. putida* KT2440 was determined by Edman degradation with a 477A automated protein sequencer (Applied Biosystems Inc.). A crude extract of *E. coli* BLB21(DE3) (pETGalA) cells was loaded in a 12.5% SDS-polyacrylamide gel, and the gallate dioxygenase was electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad) as described previously (26).

**Analytical Ultracentrifugation**-Sedimentation velocity experiments of purified gallate dioxygenase solutions in extraction buffer were carried out at 40,000 rpm and 20 °C in an XL-A analytical ultracentrifuge (Beckman-Coulter Inc.) equipped with UV-VIS absorbance optics, using an An60Ti rotor and 12 mm double-sector centerpieces of Epon-charcoal. Absorbance scans were measured at 280 nm. Sedimentation coefficient distributions, c(s), were calculated by least-squares boundary modeling of sedimentation velocity data using the program SEDFIT (27). These coefficients were corrected to standard conditions (water and 20 °C) to obtain the corresponding *s*<sub>20,w</sub> values using the SEDNTERP program (28). The latter program was also used to calculate the partial specific volume of the protein (0.7326 ml/g) from its amino acid composition. Short-column (80 μl) sedimentation equilibrium experiments were performed at two successive speeds (10,000 and 13,000 rpm) on parallel protein samples. Afterwards, baselines were measured at high speed (40,000 rpm). The weight-average molar mass of the protein was calculated by fitting the experimental gradients to the equation that describes the radial concentration distribution of a solute at sedimentation equilibrium, as implemented in the EQASSOC program (29).

**Gallate Dioxygenase Assays**-The activity of the gallate dioxygenase was measured by monitoring the substrate-dependent oxygen consumption rate at 30 °C using an oxygen electrode (DW1 Hansa-Tech Oxygen Electrode, Hansa-Tech Oxygen Instrument Limited). The 1 ml assay mixture contained 100 mM Tris-HCl pH 7.0, gallate dioxygenase (15 μg of purified protein or 20 μg of gallate dioxygenase-containing crude extract) and 250 μM of the aromatic substrate (gallate, protocatechuate, catechol, resorcinolate, resorcinol, gentisate, hydroquinone, pyrogallol, methylgallate, syringate). The gallate dioxygenase activity in crude extracts was also measured spectrophotometrically by following the decrease in absorbance at 265 nm (ε<sub>265</sub> of gallic acid is 6,700 M<sup>-1</sup> cm<sup>-1</sup>). Kinetic parameters such as *K*<sub>m</sub> and *V*<sub>max</sub> were calculated with the oxygen electrode by changing gallate concentration from 0.0005 to 5 mM (7 out of the 10 data points covered the concentration range of 0.5-10X *K*<sub>m</sub>) in three independent experiments (with S.D. values of <10%) and directly fitting the Michaelis-Menten equation with a nonlinear regression program (GraphPad Software; www.graphpad.com). Temperature and pH optimal were calculated by assaying the gallate dioxygenase over a temperature range from 20 °C to 40 °C and a pH range from 6.0 to 9.0, respectively. The influence of different agents on the gallate dioxygenase activity was tested by incubating samples of the purified enzyme (1 mg/ml) with 1mM EDTA, 2 mM 2,2'-dipyridyl, or 1 mM hydrogen peroxide at 4 °C for 30 min in 100 mM Tris-HCl pH 7.0 buffer. The enzyme reaction was started by adding 250 μM of gallic acid. Whereas reactivation of the EDTA- or 2,2'-dipyridyl-inhibited enzyme was performed by adding 2 mM of different metal salts (FeSO<sub>4</sub>, MgCl<sub>2</sub>, MnSO<sub>4</sub>, ZnCl<sub>2</sub>, CoCl<sub>2</sub>, CuCl<sub>2</sub>, NiCl<sub>2</sub>) or ferric citrate, hydrogen peroxide-treated enzyme was reactivated by adding 10 mM sodium ascorbate. The samples were then incubated at 4 °C for 30 min and the reaction was started by adding 250 μM of gallic acid.

**Identification of the Reaction Product from Gallic Acid**-The reaction mixture (5 ml) consisted of 100 mM Tris-HCl pH 7.0, 100...
µg of gallate dioxygenase and 2 mM of gallic acid. The reaction was performed at 30 °C for 1 h with vigorous shaking. Where appropriate, the reaction mixture was acidified with 10 N hydrochloric acid to pH 1.0 and extracted with ethyl acetate. The spectra were acquired at 298 K on an AVANCE 500 MHz spectrometer, equipped with a broad-band z-gradient probe (Bruker). Heteronuclear single-quantum coherent spectroscopy (HSQC) (30) experiments were performed using the standard procedures provided by the manufacturer. Two different pHs, 8.0 and 3.0, were used.

Mass spectra were performed in a LCQ Deca XP plus mass spectrometer (Thermo Instruments) using an ion trap analyzer. The sample was analyzed by direct injection in the electrospray source, using a syringe pump with a 5 µl/min flux. The conditions used were as follows: voltage spray, 4000 V; input temperature, 250 ºC; Seath gas flow, 29 (arbitrary units); capillary voltage, 20 V. Additionally, three experiments were performed in negative and full scan mode, with zoom scan of the ions defined by m/z 201 and 183. Moreover, a MS/MS analysis was also performed on these key peaks and they gave rise to a loss of 44 units (decarboxylation) thus originating new peaks with [M-H] at m/z 139 and 157.1 units.

Construction of a P. putida KT2440ΔgalA Mutant Strain-To disrupt the galA gene in P. putida KT2440 by single homologous recombination, an internal fragment of galA was PCR-amplified by using oligonucleotides GalA5´int (5´-CGGAATTCGACATGAGCTTTTCCGCG-3´, the EcoRI restriction site is underlined) and GalA3´int (5´-CGGAATTCGACATTCCGGACATGAGCTTTTCCGCG-3´, the EcoRI restriction site is underlined). The resulting 0.7-kb DNA fragment was cloned into the EcoRI-digested pK18mob suicide vector (a mobilizable plasmid which does not replicate in Proteobacteria other than E. coli), giving rise to plasmid pK18GalAint (4.5 kb). To transfer the pK18GalAint plasmid into P. putida KT2440, triparental filter mating was performed as previously described (31) using E. coli DH10B (pK18GalAint) (donor strain), E. coli HB101 (pRK600) (helper strain) and P. putida KT2440 (recipient strain). A P. putida KT2440ΔgalA exconjugant harboring the galA disrupted gene was isolated on MC minimal medium plates containing citrate (which selected for the Pseudomonas recipient cells) and kanamycin (which selected for the insertion of the suicide vector) after incubation at 30 °C for 16 h. The mutant strain was analyzed by PCR to confirm the disruption of the galA gene.

Monitoring galA Expression in P. putida KT2440-P. putida KT2440 cells were grown in citrate-containing MC medium. When the cultures reached an absorbance at 600 nm of 0.5, samples were removed and 1 mM of different aromatic compounds, i.e., galate, protocatechuate, syringate, phloroglucinol, and pyrogallol, were added and the incubation was resumed for 2 h. The expression of the galA gene was checked by Reverse Transcription (RT)-PCR experiments. To this end, cells were collected by centrifugation and total RNAs were obtained with a High Pure RNA isolation kit (Roche). Any contamination by DNA was eliminated by the use of a DNase treatment and removal kit (Ambion). One microgram of purified total RNA was used to prepare cDNA by adding 3 U of avian myeloblastosis virus reverse transcriptase (Promega) and the oligonucleotide GalA3´int. PCRs were carried out with 2.5 U of AmpliTaq DNA polymerase (Roche) and the oligonucleotides GalA3´int and GalA5´int. Control reaction in which reverse transcriptase was omitted from the reaction mixture ensured that DNA products resulted from the amplification of cDNA rather than from DNA contamination.

To assay gallate dioxygenase activity in P. putida KT2440 cells, cultures were harvested at 4 ºC, resuspended in extraction buffer and disrupted by two consecutive passages through a French press (Aminco Corp.) operated at a pressure of 20,000 p.s.i. The clear supernatant fluid was used as the crude extract fraction.
RESULTS AND DISCUSSION

Cloning and Expression of the galA Gene from P. putida KT2440 - In the course of a genomic in silico search for novel aromatic ring-cleavage dioxygenases in P. putida KT2440, we have identified at position 2866 kb of the genome a 1263-bp gene (herein referred as galA) whose product, a putative 420-amino acids length protein, shared the last 340 residues with protein PP2518 (340 amino acids) of the annotated P. putida genome (http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gp). The deduced amino acid sequence of the galA gene product showed significant similarity to that of protocatechuate 4,5-dioxygenases (see below), suggesting that it could be involved in the meta cleavage of a catecholic compound, a type of reaction that has not been reported yet in P. putida KT2440.

To characterize the galA gene product, the galA gene was cloned into the pET-29a(+) expression vector and the resulting plasmid, pETGalA, was transferred to E. coli BLB21(DE3) cells to overexpress the cloned gene (see “Experimental Procedures”). SDS-PAGE analysis of crude lysates from E. coli BLB21(DE3) cells containing pETGalA revealed the presence of an intense band corresponding to a protein with an apparent molecular weight of 47 kDa (Fig. 1), in good agreement with that predicted for the product of the galA gene (47.6 kDa). Moreover, the amino-terminal sequence (Ala-Arg-Ile-Ile-Gly-Gly) of the overproduced protein was in agreement with that deduced from the galA gene, except for the first methionine which appeared to be processed. Remarkably, this analysis also confirmed that the PP2518 protein of P. putida KT2440 was erroneously annotated as a product 80 amino acids shorter than the wild-type one.

To determine whether the galA gene product was a ring-cleavage dioxygenase, crude extracts of E. coli BLB21DE3 (pETGalA) were assayed by measuring the oxygen consumption (see “Experimental Procedures”) in the presence of different substrates such as ortho-dihydroxylated (catecholic) aromatic compounds (e.g., protocatechuate and catechol), meta-dihydroxylated aromatic compounds (e.g., resorcinolate and resorcinol), para-dihydroxylated aromatic compounds (e.g., gentisate and hydroquinone), and trihydroxylated aromatic compounds (e.g., gallate). Whereas all dihydroxylated compounds did not cause oxygen consumption, gallate led to a significant oxygen consumption activity (19.1 µmol/min/mg protein) that was not observed with E. coli BLB21DE3 (pET-29a(+)) control extracts. Other gallate analogues such as pyrogallol (3,4,5-trihydroxybenzene), methylgallate (methyl ester of gallic acid) and syringate (3,5-dimethoxy-4-hydroxybenzoate) did not cause oxygen consumption, suggesting that the substrate specificity of the galA gene product was very restricted toward gallate. For 50, 100, and 250 nmol of gallate added to the enzymatic assay, 49, 99, and 244 nmol of O2 were consumed, respectively, indicating that one mol of O2 is consumed per mol of gallate. Enzyme activity was also monitored spectrophotometrically by following the decrease in absorbance at 265 nm due to gallate removal. Thus, whereas control extracts from E. coli BLB21DE3 (pET-29a(+)) cells did not show a decrease in absorbance at 265 nm in the presence of gallate, extracts from E. coli BLB21DE3 (pETGalA) cells caused a significant reduction of the gallate levels (19.2 µmol/min/mg protein). Thus, both substrate specificity and reaction stoichiometry suggested that the product of the galA gene could be a gallate ring-cleavage dioxygenase.

Cleavage of the aromatic ring of gallate has been reported previously to be carried out by the protocatechuate 4,5-dioxygenase and protocatechuate 3,4-dioxygenase, two enzymes that catalyze extradiol (at the 4,5 position) and intradiol cleavage of protocatechuate, respectively (32, 33). Moreover, the 3-O-methylgallate dioxygenase (DesZ) from S. paucimobilis SYK-6 was also shown to attack gallate (34). Although a specific gallate dioxygenase activity, which does not attack protocatechuate, has been reported previously in the catabolism of syringate in S. paucimobilis SYK-6 (34) and in some P. putida strains (33, 35), the gene and the enzyme responsible for such specific ring-cleavage activity have not been yet
characterized. Therefore, it was essential to demonstrate that *galA* certainly encodes a highly specific gallate dioxygenase.

**Purification and Characterization of the Gallate Dioxygenase**

The gallate dioxygenase was purified from *E. coli* BLB21(DE3) (pETGalA) cells by a combination of ultracentrifugation and Phenyl-Sepharose chromatography (see “Experimental Procedures”) (Table I, Fig. 1).

To identify the reaction product formed from gallate by the gallate dioxygenase, the reaction mixture was analyzed by NMR spectroscopy and mass spectrometry. The analysis of the 1D \(^1H\) and the 2D HSQC \(^1H\)-\(^13C\) NMR spectra of gallic acid showed one resonance signal at \(\delta 7.07\) ppm (Fig. 2A) and 110 ppm, respectively (H-C 2, 5 pairs). Reaction of gallic acid with the gallate dioxygenase revealed the complete transformation of this aromatic acid into a single opening product whose \(^1H\) NMR spectrum gives only a singlet at \(\delta 6.77\) ppm corresponding to an olefinic proton (Fig. 2B). According to the HSQC spectrum, the \(^13C\) chemical shift is 129.8 ppm, which corresponds, according to additivity rules, to the expected value (128.1 ppm) of the associated olefinic carbon (data not shown). These data are consistent with 4-oxalomesaconate (OMA) as a ring-cleavage product. Although OMA (keto form) has been shown to be in equilibrium with two enol forms (4-carboxy-2-hydroxymuconate) (33, 36) (Fig. 2B), these three isomers are detected as a single peak by \(^1H\) NMR because samples were prepared in D\(_2\)O and the keto-enol tautomerization will give rise to deuteration at the corresponding positions. On the other hand, negative mass spectrometry (electrospray) experiments revealed that the [M-H] \(^-\) peak at \(m/z\) value of 169.1 due to gallic acid completely disappeared upon treatment with the gallate dioxygenase, and two new peaks at \(m/z\) values of 201 and 183 were observed (Fig. 3). The molecular ion at \(m/z\) value of 201 corresponds to a ring-cleavage product of gallic acid by addition of two oxygen atoms. The molecular ion at \(m/z\) value of 183 is likely to be 2-pyrone-4,6-dicarboxylic acid (PDC) (Fig. 3) that arises by loss of water from the ring-cleavage product (37). Interestingly, PDC is not seen in the NMR spectra since the predicted olefinic proton and carbon signals, that should appear at 6.92 and 124 ppm, respectively, were not detected. Therefore, PDC formation is likely to be a consequence of the electrospray conditions rather than being a real ring-cleavage product. The peaks at \(m/z\) 139 (compound II) and 157.1 (compound I) units (Fig. 3) arise under the mass spectrometry conditions by decarboxylation of PDC and OMA, respectively.

As shown previously with crude extracts, purified gallate dioxygenase showed oxygen consumption activity only when gallate was used as substrate. Moreover, protocatechuate did not inhibit gallate cleavage at high concentrations (data not shown), suggesting that this aromatic compound cannot be accommodated in the active center of gallate dioxygenase.

The gallate dioxygenase was active in the pH range of 6.0 to 9.0, with maximum activity at pH 7.0. The optimal temperature for enzyme activity was around 30 °C. The \(K_m\) and \(V_{max}\) values for gallate were 144 \(\mu\)M and 53.2 \(\mu\)mol/min/mg protein, respectively. The high specific activity of the protein towards gallate (42.7 \(\mu\)mol/min/mg protein) is in agreement with the assumption that it has evolved as a gallate-specific dioxygenase.

The UV-visible spectrum of purified gallate dioxygenase exhibited a maximum at 280 nm. No long-wavelength absorbance characteristic of iron-sulfur clusters or organic cofactors, such as flavins or hemes, was observed. While the majority of aromatic ring-cleavage dioxygenases contain iron, Fe\(^{2+}\) for extradiol dioxygenases and Fe\(^{3+}\) for intradiol dioxygenases, as a catalytic metal center, there are a few reports on Mn(II)- and Mg(II)-dependent extradiol dioxygenases (7). To determine the metal dependency of gallate dioxygenase, a nonspecific chelator such as EDTA (1mM) was added to the purified enzyme, and a decrease in the gallate dioxygenase activity (<1% of untreated enzyme) was observed. Moreover, 2 mM of 2,2’-dipyridyl, a specific Fe\(^{2+}\) chelator, abolished the gallate dioxygenase activity completely. When 2 mM of different cations (e.g., Fe\(^{2+}\), Fe\(^{3+}\), Mg\(^{2+}\), Mn\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\) or Co\(^{2+}\)) were added to the gallate dioxygenase treated with the chelators, the activity was only fully recovered with Fe\(^{2+}\), Mn\(^{2+}\), Cu\(^{2+}\) and Co\(^{2+}\) recovered 8%, 9% and 5% activity of the untreated enzyme, respectively. The oxidizing...
agent hydrogen peroxide at 1 mM reduced the

gallate dioxygenase activity to 15%, and the

disactivity could be partially restored (50%

activity of the untreated enzyme) upon

addition of 10 mM ascorbate, as observed

with other extradiol dioxygenases (38, 39).

These results suggest that Fe²⁺ is the preferred

cofactor for gallate dioxygenase activity, and

they support the galA gene product as a meta-

cleavage (extradiol) dioxygenase.

Sedimentation velocity indicates

that the predominant sedimentation peak (ca.

75% of loading protein concentration)

corresponds to a protein species with a

corrected sedimentation coefficient of 7.29 ±

0.10 S. The molar mass of this main species

obtained by sedimentation equilibrium was

132,000 ± 12,000 Da. Because the molecular

mass of the monomer deduced from the

amino acid composition is 47,644 Da, these

combined analytical ultracentrifugation data

are compatible with the main gallate

dioxygenase holoenzyme species being a

globular trimer composed of identical

subunits. Although the quaternary structure of

aromatic ring-cleavage dioxygenases is

evolutionarily variable (7), the oligomeric

state of gallate dioxygenase from P. putida

KT2440 is rather uncommon since just a few

trimeric dioxygenases have been previously

reported (40-42).

All these data taken together

indicate that gallate dioxygenase is certainly

an extradiol dioxygenase that cleaves

specifically the aromatic ring of gallate to

form OMA. Interestingly, a similar reaction

product was observed when protocatechuate

4,5-dioxygenase acted on gallate (32). It is

tempting to speculate that gallate

dioxygenases and protocatechuate 4,5-
dioxygenases share a common ancestor that

might have evolved towards recognition of

gallate or either gallate and protocatechuate,

respectively. Comparative analysis of the

primary structure of gallate dioxygenase from

P. putida KT2440 could provide interesting

cles on this respect.

Gallate Dioxygenase is the

Prototype of a New Subgroup of Type II

Extradiol Dioxygenases—As indicated above,

gallate dioxygenase from P. putida KT2440

shows amino acid sequence similarity to

protocatechuate 4,5-dioxygenases (type II

extradiol dioxygenases). A more detailed

analysis of the primary structure of gallate

dioxygenase revealed that its N-terminal

region (residues 1-281) showed a significant

amino acid sequence identity with the large

subunit (β) of protocatechuate 4,5-
dioxygenases such as FldU from

Sphingomonas sp. LB126 (44%) (43), ProOb

from Pseudomonas ochraceae (straininaea)

(42%) (44), PmdB from Comamonas
testosteroni BR6020 (42%) (45), and LigB

from S. paucimobilis SYK-6 (39%) (46). On

the other hand, the C-terminal region of

gallate dioxygenase (residues 294-420)
presented similarity to the corresponding

small subunit (α) of protocatechuate 4,5-
dioxygenases, i.e., FldV (43), ProOa (44),
PmdA (45) and LigA (46), although this

region was less conserved than the N-terminal

one as revealed by the lower degree of amino

acid sequence identity observed (about 24-

28%). These data suggest, therefore, that

gallate dioxygenase from P. putida is a two-
domain protein with a linker region spanning

residues 282-293 (Fig. 4A).

Since the three-dimensional

structure of the protocatechuate 4,5-
dioxygenase from S. paucimobilis SYK-6

(LigAB) is known (9), an homology modeling

for the N-terminal and C-terminal domains of

gallate dioxygenase from P. putida KT2440

was accomplished (Fig. 4B). According to

this model, the N-terminal domain of gallate

dioxygenase forms a globular α/β

structure where His12, His59 and Glu240 would be the

Fe²⁺ ligands, and His193 would act as the
catalytic base. A potential weak Fe²⁺ ligand,

Asn59 in the β subunit of protocatechuate

4,5-dioxygenase, is also conserved in gallate

dioxygenase (Asn57) (Fig. 4C). In the

protocatechuate 4,5-dioxygenase from S.
paucimobilis SYK-6, the substrate is

accommodated in a cavity located in the

upper part of the β subunit (LigB), with the

α subunit (LigA) forming a lid that closes the

open end of the binding pocket to avoid

interactions of the bound protocatechuate

with the solvent (9). To mimic the role of the

α subunit in the protocatechuate 4,5-
dioxygenase, the C-terminal domain of gallate
dioxygenase must be folded and situated on
top of the binding pocket (Fig. 4B). The C-
terminal domain of gallate dioxygenase has a
predicted plate-like shape composed by nine
α-helices, resulting in a large surface area that
might contribute to the interaction with the N-
terminal domain (Fig. 4B). Remarkably, other critical residues for the function of the protocatechuate 4,5-dioxygenase (9) are also conserved in gallate dioxygenase. Thus, His125, which corresponds to His127 in the LigB subunit of protocatechuate 4,5-dioxygenase, would stabilize the coordination of the substrate with the iron ion, and Pro14, Val195 and Thr268, which correspond to Pro14, Leu197 and Thr271 in LigB, would make contacts with the aromatic ring of the substrate (Fig. 4C). Interestingly, residue Tyr90 in the LigA subunit of protocatechuate 4,5-dioxygenase, that occupies the O₂-binding site in the absence of the substrate (protocatechuate) (9), is substituted by residue Phe374 in the C-terminal domain of gallate dioxygenase (Fig. 4C). Nevertheless, the model should be viewed as predictive and as a guide to future experimental tests to confirm, for instance, the amino acid residues responsible for the different substrate specificity of gallate dioxygenase and protocatechuate 4,5-dioxygenase.

Sequence comparison analyses suggest, therefore, that the gallate dioxygenase from P. putida KT2440 has a two-domain architecture that might have evolved from the fusion of the genes encoding the β and α subunits of the protocatechuate 4,5-dioxygenase. In agreement with this hypothesis, a domain-swapping event might have also occurred in the protocatechuate 4,5-dioxygenase (PcmA) of Arthrobacter keyseri 12B, a protein that shows a significant similarity to other protocatechuate 4,5-dioxygenases but whose primary structure (αβ domain architecture) corresponds to the fusion of the α and β subunits of the latter (47). It is worth noting that gallate dioxygenase showed the highest amino acid sequence identity (64%-76%) with several hypothetical proteins present in the databanks whose size and domain structure were similar to that proposed for gallate dioxygenase and that most probably act on gallate derivatives such as 3-O-methylgallate (DesZ) (34) and 5,5’-dehydrovanillinate (LigZ) (48).

Physiological Role of Gallate Dioxygenase in P. putida KT2440-To gain some insights on the physiological role of galA, we have analyzed by reverse transcription (RT)-PCR experiments the expression of this gene when P. putida KT2440 cells were grown in citrate-containing minimal medium in the presence of different aromatic compounds such as gallate, syringate, protocatechuate, phloroglucinol, and pyrogallol. As shown in Fig. 6, a specific induction of galA was only observed in the presence of gallate. Therefore, these data indicate that gallate is not only a substrate of the gallate dioxygenase but also a specific inducer of the galA gene in P. putida KT2440. In this sense, it could be assumed that P. putida KT2440 might have the ability to mineralize gallate. In agreement with this assumption, we have been able to adapt P. putida KT2440 cells to grow on 5 mM gallate as sole carbon source, a catabolic ability that was not reported so far in this bacterium. To confirm that the galA gene was involved in the catabolism of gallate in P. putida KT2440, we have constructed a mutant strain, P. putida KT2440dgalA, that harbors a disrupted galA gene (see “Experimental Procedures”). As expected, whereas the parental strain was able to grow on gallate and crude extracts from cells growing in citrate plus gallate showed a significant gallate dioxygenase activity in the spectrophotometric assay (1.0 μmol/min/mg of protein), the P. putida KT2440dgalA strain was unable to grow on gallate and it did not show gallate dioxygenase activity when growing in citrate plus gallate, which suggests that the galA gene product is the only dioxygenase of P. putida KT2440 involved in gallate cleavage.

Interestingly, gallate did not induce the expression of genes involved in central aromatic catabolic pathways that have been previously identified in P. putida KT2440, e.g., pca, pha, and hmg genes (4, 6, 11), as revealed by RT-PCR experiments (data not shown). Thus, the identification of the galA gene suggests the existence of a new and distinct catabolic pathway that surprisingly remained unknown in the well studied P. putida KT2440 strain.
In summary, this work illustrates how the combination of in silico, in vitro, and in vivo approaches is a useful strategy to identify new archetypical enzymes, such as the gallate dioxygenase, that, in turn, pave the way to discover novel and unexpected metabolic pathways in model bacteria like P. putida KT2440.

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REFERENCES

FOOTNOTES

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1 The abbreviations used are: LB, Luria-Bertani; OMA, 4-oxalomesaconate; PDC, 2-pyrone-4,6-dicarboxylic acid; RT, reverse transcription.

FIGURE LEGENDS

FIG. 1. SDS-PAGE analysis of the overproduction and purification of the gallate dioxygenase. Proteins were separated in a SDS-12.5% polyacrylamide gel and stained with Coomasie brilliant blue. Lane 1, molecular mass markers (in kDa); lane 2, soluble fraction of the control crude extract from E. coli BL21(DE3) (pET-29a(+) ); lane 3, soluble fraction of the crude extract from E. coli BL21(DE3) (pETGalA); lane 4, protein fraction after the ultracentrifugation step; lane 5, purified gallate dioxygenase after Phenyl-Sepharose chromatography. The gallate dioxygenase is indicated with an arrow.

FIG. 2. $^1$H-NMR signals of the gallate dioxygenase reaction assay. A, $^1$H-NMR signals at time 0. B, $^1$H-NMR signals at time 60 min. The reaction assay and the NMR analyses were performed as described under “Experimental Procedures”. The structures of gallic acid and the predicted ring cleavage products (OMA) are indicated. The olefinic proton of OMA detected at δ 6.77 ppm is shown in bold.

FIG. 3. Mass spectrum analysis of the gallate ring-cleavage products. The products generated by the gallate dioxygenase acting on gallate were extracted and analyzed by mass spectrometry as indicated under “Experimental Procedures”. Structures of OMA and PDC are shown. Compounds I and II, which are generated by decarboxylation of OMA and PDC, respectively, are also indicated.

FIG. 4. Amino acid sequence analysis and three-dimensional model of gallate dioxygenase from P. putida KT2440. A, amino acid sequence alignment of gallate dioxygenase with the LigA and LigB subunits of the protocatechuate 4,5-dioxygenase from S. paucimobilis SYK-6. The amino acid residues of each sequence, indicated by their standard one-letter code, are numbered on the right. Sequences were aligned using the sequence alignment program ClustalW (19). Gray shadow shows identical residues. Residues that are discussed in text are indicated by a black shadow. Secondary structure elements predicted from the three-dimensional model of gallate dioxygenase (panel B) are drawn. The α-helices (red cylinders) and β-strands (yellow arrows) of the N-terminal domain of gallate dioxygenase are indicated at the top of the alignment. The α-helices (blue cylinders) of the C-terminal domain of gallate dioxygenase are indicated at the bottom of the alignment. The 12-amino acids length linker region is underlined. B, ribbon diagram of the three-dimensional model for the N-terminal and C-terminal domains of gallate dioxygenase. The overall fold of the N-terminal domain (residues 2 to 281) contains ten α-helices (red ribbons) and eight β-strands (yellow arrows). Loops are indicated in green. The overall fold of the C-terminal domain (residues 294 to 420) consists of nine α-helices (α11 to α19) (blue ribbons). C, Comparison of the active site of the
protocatechuate 4,5-dioxygenase (LigAB)-protocatechuate complex (9) and the predicted active site for the gallate dioxygenase. Residues of the N-terminal and C-terminal domains of gallate dioxygenase are indicated in red and blue colors, respectively. Residues of the LigA and LigB subunits are shown in yellow and gray colors, respectively. The iron coordination sphere (Fe$^{2+}$) and the protocatechuate (PCA) are indicated in green. Panels B and C have been prepared with PyMOL (24).

FIG. 5. Phylogenetic tree of gallate dioxygenase with type II extradiol dioxygenases. The bar indicates one inferred nucleotide substitution per ten nucleotides. The enzymes are: AmnA_CNB-1 and AmnB_CNB-1, α and β subunits of 2-aminophenol 1,6-dioxygenase from Comamonas sp. CNB-1 (AAT35227, AAT35226); AmnA JS45 and AmnB JS45, α and β subunits of 2-aminophenol 1,6-dioxygenase from Pseudomonas pseudoalcaligenes JS45 (AAB71525, AAB71524); AmnA_AP3 and AmnB_AP3, α and β subunits of 2-aminophenol 1,6-dioxygenase from Pseudomonas sp. AP-3 (BAB03532, BAB03531); CarBb_CA10, β-subunit of the 2′-aminobiphenyl-2,3-diol 1,2-dioxygenase from S. paucimobilis SYK-6 (BAC79261); EdD_I1, extradiol dioxygenase from Rhodococcus sp. I1 (CAO06875); FldU_LB126, β subunit of putative protocatechuate dioxygenase from Sphingomonas sp. LB126; GalA_KT2440, gallate dioxygenase from P. putida KT2440; GalA_Avinelandii, putative gallate dioxygenase from Azotobacter vinelandii (ZP_00090231); GalA_CH34, putative gallate dioxygenase from Ralstonia metallidurans CH34 (ZP_00273634); GalA_R18194, putative gallate dioxygenase from Burkholderia cepacia R18194 (ZP_00216311); HpaD_W, homoprotocatechuate 2,3-dioxygenase from E. coli W (CAA86042); HppB_PWD1, 2,3-dihydroxyphenylpropionate 1,2-dioxygenase from Rhodococcus globarulus PWD1 (AAB81314); MhpB_K12, 2,3-dihydroxyphenylpropionate 1,2-dioxygenase from E. coli K-12 (CA70748); MhpB_TA441, 2,3-dihydroxyphenylpropionate 1,2-dioxygenase from Comamonas testosteroni TA441 (BAA82879); LigB_SYK6, β subunit of protocatechuate 4,5-dioxygenase of S. paucimobilis SYK-6 (BAA97118); LigZ_SYK6, 5,5′-dehydrovanillate dioxygenase from S. paucimobilis SYK-6 (BAA75884); MpcI_JMP222, catechol 2,3-dioxygenase I from Ralstonia eutropha JMP222 (S10154); OhpD_V49, 2,3-dihydroxynaphthalene 1,2-dioxygenase from Rhodococcus sp. V49 (AA81826); PhnC_RP007, extradiol dioxygenase of Burkholderia sp. RP007 (AAD09870); PcmA_12B, protocatechuate 4,5-dioxygenase of Arthrobacter keyseri 12B (AAK16524); PmdB_BR6020, β subunit of protocatechuate 4,5-dioxygenase of C. testosteroni BR6020 (AAM09637); ProOb_NGJ1, β subunit of protocatechuate 4,5-dioxygenase of Pseudomonas ochraceae NGJ1.

FIG. 6. Gene expression analysis of galA in P. putida KT2440. The expression of galA was analyzed by RT-PCR experiments with P. putida KT2440 cells grown on citrate-containing minimal medium in the absence (lane 2) or in the presence of 1 mM syringate (lane 3), protocatechuate (lane 4), phloroglucinol (lane 5), pyrogallol (lane 6), or gallate (lane 7). RT-PCRs were performed as described under “Experimental Procedures”. PCR of the galA gene with genomic DNA as a positive control is also indicated (lane 8). Numbers on the right represent the sizes (in base pairs) of the markers (lanes 1 and 9). The 0.7-kb galA internal fragment is indicated with an arrow.
TABLE I

Purification of gallate dioxygenase from E. coli BL21(DE3) (pETGalA) cells

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (µmol/min)</th>
<th>Specific activity (µmol/min/mg)</th>
<th>Yield (%)</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>490</td>
<td>10,497</td>
<td>21.4</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>2. Ultracentrifugation</td>
<td>385</td>
<td>9,673</td>
<td>25.1</td>
<td>92.1</td>
<td>1.17</td>
</tr>
<tr>
<td>3. Phenyl-Sepharose</td>
<td>150</td>
<td>6,412</td>
<td>42.7</td>
<td>61</td>
<td>2.0</td>
</tr>
</tbody>
</table>

\(^a\) The cultivation of cells and the preparation of the crude extract were carried out as indicated under “Experimental Procedures”.


Fig. 1
Fig. 3
Fig. 4
Molecular characterization of the gallate dioxygenase from Pseudomonas putida KT2440: The prototype of a new subgroup of extradiol dioxygenases
Juan Nogales, Ángeles Canales, Jesús Jiménez-Barbero, José Luis García and Eduardo Díaz

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