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2-Aminophenol 1,6-Dioxygenase: a Novel Aromatic Ring Cleavage Enzyme Purified from *Pseudomonas pseudoalcaligenes* JS45

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Most bacterial pathways for the degradation of aromatic compounds involve introduction of two hydroxyl groups either *ortho* or *para* to each other. Ring fission then occurs at the bond adjacent to one of the hydroxyl groups. In contrast, 2-aminophenol is cleaved to 2-aminomuconic acid semialdehyde in the nitrobenzene-degrading strain *Pseudomonas pseudoalcaligenes* JS45. To examine the relationship between this enzyme and other dioxygenases, 2-aminophenol 1,6-dioxygenase has been purified by ethanol precipitation, gel filtration, and ion exchange chromatography. The molecular mass determined by gel filtration was 140,000 Da. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed two subunits of 35,000 and 39,000 Da, which suggested an $\alpha_2\beta_2$ subunit structure. Studies with inhibitors indicated that ferrous iron was the sole cofactor. The K_m values for 2-aminophenol and oxygen were 4.2 and 710 μM, respectively. The enzyme catalyzed the oxidation of catechol, 6-amino-*m*-cresol, 2-amino-*m*-cresol, and 2-amino-4-chlorophenol. 3-Hydroxyanthranilate, protocatechuate, gentisate, and 3- and 4-methylcatechol were not substrates. The substrate range and the subunit structure are unique among those of the known ring cleavage dioxygenases.

Ring cleavage is a key reaction in microbial degradation of aromatic compounds. In aerobic bacteria, it is usually catalyzed by dioxygenases (17). Typically, substrates of ring cleavage dioxygenases feature an aromatic ring substituted with two hydroxyl groups oriented either *ortho* or *para* to each other (26). Indeed, nearly all bacterial catabolic pathways transform aromatic substrates to catechol or gentisate and their derivatives, which subsequently undergo ring cleavage (10).

Recently, we reported the discovery of 2-aminophenol 1,6-dioxygenase, a new ring cleavage enzyme that catalyzes the direct conversion of 2-aminophenol to 2-aminomuconic acid semialdehyde. The enzyme is induced during the growth of *Pseudomonas pseudoalcaligenes* JS45 on nitrobenzene. The degradation pathway (Fig. 1A) starts with reduction of nitrobenzene to hydroxylaminobenzene followed by rearrangement to 2-aminophenol (19).

Two enzymes have been reported to catalyze the ring cleavage of 2-aminophenol at very low rates. Catechol 1,2-dioxygenase from *Pseudomonas arvilla* C-1 catalyzes extradiol ring fission of 2-aminophenol at a rate that is 0.1% that of the intradiol cleavage of catechol (27). 2-Aminophenol was oxidized by catechol 2,3-dioxygenase from *P. arvilla* at a rate less than 0.01% of the rate with catechol (14, 22). These low turnover rates indicate that 2-aminophenol is not a physiological substrate of catechol 1,2- or 2,3-dioxygenase. To our knowledge, this is the first report of an enzyme that cleaves 2-aminophenol as its primary substrate.

We have purified and characterized 2-aminophenol 1,6-dioxygenase from *P. pseudoalcaligenes* JS45 to determine how it is related to previously known ring cleavage enzymes. The tertiary structure, substrate range, and cofactor requirement were compared with that of other ring fission enzymes.

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MATERIALS AND METHODS

Chemicals. Protocatechuate was from Pfaltz and Bauer, Inc. (Waterbury, Conn.). 3-Chlorocatechol and 4-chlorocatechol were from Helix Biotech Corp. (Richmond, British Columbia, Canada). 3-Methylpicolinic acid and 4-chloropicolinic acid were obtained from TCI America Inc. (Portland, Oreg.). All other chemicals were from either Sigma (St. Louis, Mo.) or Aldrich (Milwaukee, Wis.).

Enzyme assays. The activity of 2-aminophenol 1,6-dioxygenase was determined either by measuring oxygen depletion with a Clark-type oxygen electrode or spectrophotometrically by observing the decrease of A_{282} (ε_{282} = 3,220 $M^{-1}cm^{-1}$) concomitant with the disappearance of 2-aminophenol. The spectral changes during ring cleavage have been described previously (19). 2-Aminomuconic acid semialdehyde absorbs light between 300 and 450 nm with a maximum at 380 nm. Picolinic acid, which is spontaneously formed from 2-aminomuconic acid semialdehyde, shows little UV A_{282} (ε_{282} = 450 $M^{-1}cm^{-1}$). The formation of picolinic acid was slow and therefore did not affect the measurement of the initial rate of 2-aminophenol transformation. The K_m for 2-aminophenol was measured by monitoring the increase in A_{380} (ε_{380} = 15,100 $M^{-1}cm^{-1}$), indicating the production of 2-aminomuconic acid semialdehyde. Unless stated otherwise, assays were carried out in 50 mM Tris (pH 8.0) at 25°C. Appropriate amounts of protein were mixed with the reaction buffer, and then the assay was started by adding substrate (10 mM in water acidified to pH 2) at an initial concentration of 50 μM.

Growth of bacteria. Cultures of *P. pseudoalcaligenes* JS45 were grown in a 20-liter carboy containing 15 liters of mineral salt broth (31) at an initial nitrobenzene concentration of 100 μM. The temperature was approximately 25°C. Air was supplied at a rate of 40 ml · min^{-1}, and the culture was agitated with a magnetic stirrer at 250 rpm. Nitrobenzene was added continuously with a syringe pump at an initial rate of 0.1 ml · h^{-1}. With increasing culture density, the nitrobenzene addition rate was raised in several steps to a rate of 1 ml · h^{-1} until a total of 25 ml of nitrobenzene was added. Prior to harvesting, the culture was immediately cooled by adding ice to the cell suspension. The cells were washed with phosphate buffer (pH 7.0, 20 mM) and concentrated with a Pellicon filtration unit (Millipore, Bedford, Mass.) fitted with a 0.45-μm-pore-size filter. The resulting 2 liters of cell suspension was centrifuged at 10,000 × *g* for 10 min. Cells could be stored frozen at −80°C for 6 months without significant loss of activity.

Enzyme purification. Unless otherwise indicated, purification steps were carried out at 4°C. The cell pellets were resuspended in equal volumes of lysis buffer, resulting in 60 ml of cell suspension. The lysis buffer consisted of MOPS (morpholinepropanesulfonic acid; 50 mM) at pH 7.3 (all pH values measured at 25°C), ascorbic acid (2 mM), ferrous sulfate (100 μM), and glycerol (10% [vol/vol]). Cells were lysed with two passages through a French pressure cell at 20,000 lb · in^{-2}. The resulting lysate was centrifuged at 100,000 × *g* for 1 h, and the pellet was discarded. Cold ethanol (−20°C) was slowly added to 6 ml of cell extract to a concentration of 50% (vol/vol). After 30 min of incubation at 0°C with stirring, the solution was centrifuged at 40,000 × *g* for 20 min and the pellet was discarded. Ethanol was added to the supernatant to a final concentration of 70% (vol/vol) and again allowed to equilibrate for 30 min. After centrifugation, the supernatant was discarded and the precipitate was dissolved in approximately 2 ml of lysis buffer. Insoluble protein was removed by centrifugation at 40,000 ×

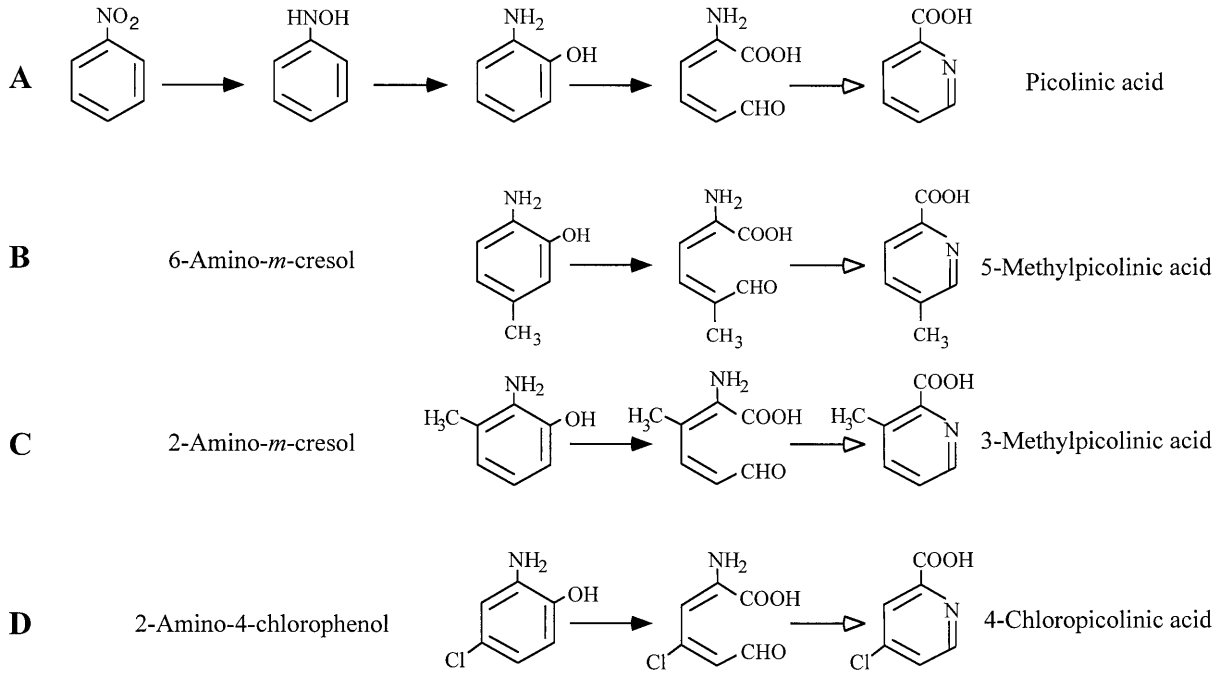

FIG. 1. (A) Pathway of nitrobenzene degradation in *P. pseudoalcaligenes* JS45 and formation of picolinic acid from ring cleavage product; (B, C, and D) ring cleavage of alternate substrates and formation of corresponding picolinates. Reactions marked with open arrows are nonenzymatic.

g for 20 min. This 50 to 70% ethanol fraction was applied to a Sephacryl S-300 HR column (1.6 by 96 cm; Pharmacia Biotech, Piscataway, N.J.). The protein was eluted at a flow rate of 1 ml · min^{-1} with a buffer containing 50 mM MOPS (pH 7.3) and ethanol (10% [vol/vol]). The buffer in the reservoir bottle was continuously sparged with argon. Fractions containing 2-aminophenol 1,6-dioxygenase activity were combined and loaded onto a MonoQ 10/10 ion-exchange column (1.0 by 10 cm; Pharmacia). The column was washed with a buffer containing 50 mM MOPS (pH 7.3), ethanol (10% [vol/vol]), L-cysteine (2 mM), ferrous sulfate (100 μM), and NaCl (100 mM). 2-Aminophenol 1,6-dioxygenase was eluted at a flow rate of 2 ml · min^{-1} with a linear NaCl gradient (100 to 300 mM) over a period of 1 h. The chromatography buffers were kept under argon. The collected fractions were desalted by passage through a PD-10 column (Pharmacia) equilibrated with buffer containing MOPS (50 mM) and ethanol (10% [vol/vol]).

Protein concentrations. Protein concentrations during the course of purification were determined with the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, Ill.). Alternatively, the dry weight concentration of the purified enzyme was determined by transferring the enzyme into deionized water by a passage through a Pharmacia PD-10 desalting column. The samples were then transferred into preweighed 4-ml glass vials. Dry weight was measured after incubation at 90°C for 16 h.

Molecular weight determination. The molecular weight of the native protein was estimated by gel filtration with a Sephacryl S-200 HR column (1.6 by 95 cm; Pharmacia) at a flow rate of 0.5 ml · min^{-1} with MOPS buffer (50 mM, pH 7.3)-NaCl (0.10 M). Thyroglobulin, ferritin, catalase, aldolase, albumin, and ovalbumin (all from Pharmacia) served as calibration standards. The molecular masses of the subunits were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% resolving gel and a 3% stacking gel (16). Protein molecular weight standards for SDS-PAGE were purchased from Bio-Rad (Hercules, Calif.).

Sequence data. The subunits of 2-aminophenol 1,6-dioxygenase were separated by SDS-PAGE, and the gel was electroblotted onto an electrophoresis-grade polyvinylidene difluoride membrane (Sigma) by using a transfer buffer containing 10 mM MES (morpholineethanesulfonic acid [pH 6.0]) and SDS (0.01% [wt/vol]). After the filter was stained with Coomassie blue (0.01%), the bands of the two subunits were cut out. The N-terminal amino acid sequence was determined by the Protein Core Facility of the University of Florida, Gainesville.

Iron analysis. The ferrous iron cofactor was quantified as described by Percival (25).

Kinetics. The optimum pH for enzyme activity was measured spectrophotometrically by using the buffers MES (pH 5 to 7), MOPS (pH 6 to 8), Tris (pH 7 to 9), TAPS (pH 8 to 10), and CAPS (pH 9 to 11), all at a concentration of 50 mM. For K_m determinations, the initial rate of 2-aminophenol oxidation was measured for concentrations between 0.1 and 100 μM. The K_m for oxygen was measured polarographically at oxygen concentrations between 0 and 1,200 μM. The partial O_2 pressure was adjusted by sparging oxygen-nitrogen mixtures through the buffer. K_m and V_{max} values were estimated by nonlinear parameter estimation.

Substrate specificity. The substrate range of purified 2-aminophenol 1,6-dioxygenase was determined by measuring the rate of oxygen consumption with various aromatic compounds at initial concentrations of 50 μM. 2-Aminophenol was added 60 s later to test the inhibition of 2-aminophenol turnover by the alternate substrate.

Identification of picolinic acids. Picolinic acid, 3-methylpicolinic acid, and 4-chloropicolinic acid were produced from the corresponding aminophenols and identified by comparison with authentic standards by high-performance liquid chromatography (HPLC) with a diode array detector (Hewlett-Packard, Wilmington, Del.). Reaction mixtures contained 0.4 mM 2-aminophenol, 2-amino-*m*-cresol, or 2-amino-4-chlorophenol and 110 μg of partially purified enzyme (Table 1, ethanol precipitate) in 500 μl of Tris buffer (50 mM, pH 8.0). Chromatography of picolinic acid and 3-methylpicolinic acid was carried out on a Spherisorb hexyl (C_6) column (Alltech, Deerfield, Ill.) at a flow rate of 1.0 ml · min^{-1}. Tris (20 mM, pH 8.0) containing 5 mM PIC A Low UV Reagent (Waters-Millipore, Milford, Mass.) was used as the eluent. For chromatography of 4-chloropicolinic acid, methanol (20% [vol/vol]) was added to the same eluent. No standard for 5-methylpicolinic acid was available. It was tentatively identified by comparison of the UV spectrum of the reaction product with a spectrum published for 5-methylpicolinic acid at pH 7.0 (4).

The identities of the picolinic acids were confirmed by gas chromatography-mass spectrometry (GC-MS) analysis. Reaction mixtures were the same as those described above but contained 2.0 mM 2-aminophenol, 2-amino-*m*-cresol, 6-amino-*m*-cresol, or 2-amino-4-chlorophenol and 300 μg of partially purified enzyme. The mixture was aerated until no further activity was observed. Protein was precipitated with trifluoroacetic acid (2% [vol/vol]) and removed by centrifugation. The supernatant was transferred into 1-ml reaction tubes, and the water was evaporated. The methyl ester derivatives of the picolinic acids were obtained by derivatization with diazomethane-ether. Methyl ester standards were obtained by derivatization of 200 μg of picolinic acid, 3-methylpicolinic acid, or 4-chloropicolinic acid. After evaporating the diazomethane-ether, the products were dissolved in 200 μl of chloroform for GC-MS analysis (HP5890 GC with HP5970 mass selective detector [Hewlett-Packard]). Separation was achieved with a DB-5 column (20 m by 0.1 mm by 0.4 μm; J&W Scientific, Folsom, Calif.).

Inhibition and activation experiments. For inhibition experiments, samples of purified enzyme were incubated with metal ions (2 mM), chelators (2 mM), and oxidizing reagents (1 mM) for 1 h, and the remaining activity was measured spectrophotometrically. In the case of oxidizing reagents, samples were incubated for a second hour with ascorbate (5 mM) to measure reactivation.

TABLE 1. Purification of 2-aminophenol 1,6-dioxygenase

Purification step	Total protein (mg)	Total activity (μmol · min^{-1})	Sp act (μmol · min^{-1} · mg^{-1})	Yield (%)
Cell extract	186.3	285	1.5	100
Ethanol precipitation	35.0	201	5.7	71
S-300 HR gel filtration	9.9	117	11.8	41
MonoQ chromatography	2.5	53	21.6	19

RESULTS

Purification. The scheme summarized in Table 1 resulted in a homogeneous 2-aminophenol dioxygenase preparation. Gel electrophoresis of the purified enzyme under nondenaturing conditions (7% gel) at 10°C exhibited a single band. An identical gel run at room temperature showed the same major band along with two diffuse bands migrating about twice the distance that indicated breakdown products of the enzyme, possibly the subunits. Similar results have been observed previously for protocatechuate 4,5-dioxygenase (1). Preliminary experiments carried out with cell extract revealed that the activity was most stable in 50 mM MOPS at pH 7.3 in the presence of glycerol (10% [vol/vol]). The absence of ferrous iron and L-cysteine in the buffer during MonoQ ion-exchange chromatography resulted in an almost complete loss of the enzyme activity, which could not be restored later by incubation with ferrous iron (100 μM) and ascorbate (2 mM). This effect has been widely observed for extradiol ring cleavage dioxygenases (17). However, the presence of ferrous sulfate was detrimental to long-term storage of the purified enzyme. Dialysis against 50 mM MOPS (pH 7.3) containing ethanol (10% [vol/vol]) for 24 h abolished 95% of the activity of 2-aminophenol 1,6-dioxygenase. Incubation of the dialyzed enzyme with ferrous iron and ascorbate could restore only 10% of the initial activity. Loss of activity during dialysis is another common phenomenon among extradiol dioxygenases (18). The enzyme lost 50% of its activity upon incubation at 60°C for 5 min. It could be stored frozen without significant loss of activity over a period of several months.

Typically, the specific activity of 2-aminophenol 1,6-dioxygenase increased approximately 15-fold during the course of purification. There are two reasons for the low increase of the specific activity. First, the enzyme was unstable as a result of the removal of the iron cofactor during protein liquid chromatography. Second, 2-aminophenol 1,6-dioxygenase accounted for 3 to 4% of the protein in the cell extract. In SDS gels of cell extracts, the two bands of the enzyme were already visible as strong bands.

Molecular mass. The native enzyme had a molecular weight of 140,000 Da as measured by gel filtration. SDS-PAGE showed comparable amounts of two types of subunits. Subunits α and β had molecular weights of approximately 35,000 and 39,000 Da, respectively. Therefore, the enzyme appears to be a heterotetramer of the structure $\alpha_2\beta_2$. Treatment with mercaptoethanol did not change the migration pattern during SDS-PAGE, indicating that no disulfide bonds are involved in stabilization of the tertiary structure.

N-terminal sequence. The first 30 amino acids of the α and β subunits were determined. The N-terminal sequence of the α subunit was found to be as follows: Thr Ile Val Ser Ala Phe Leu Val Pro Gly Ser Pro Leu Pro Gln Leu Arg Ala Asp Val Pro Gly [Trp] His Asn Phe Gln Gln Ala Met. The N-terminal sequence of subunit β was the following: Ala Thr Gly Glu Ile Ile Ser Gly Phe Leu Ala Pro His Pro Pro His Met Leu Tyr Ala Glu Asn Pro Pro Gln Asn Glu Pro Arg [Ser]. The identity of the amino acids in brackets is uncertain.

Kinetic properties of 2-aminophenol turnover. The enzyme exhibited activity between pH 5.5 and 10.5. The pH optimum was very broad, ranging from 7 to 9. At pH values of 10 and higher, spontaneous oxidation of 2-aminophenol was observed and the turnover rate had to be corrected by subtracting a blank value measured without enzyme in the assay. The spontaneous oxidation rate at pH 10 and higher never exceeded 5% of the enzymatic turnover rate at the pH optimum. Measured at air saturation (~258 μM O_2), the K_m for 2-aminophenol was 4.2 $\pm$ 0.3 μM and the V_{max} was 0.063 $\pm$ 0.001 μmol · μg^{-1} · min^{-1} (mean $\pm$ standard deviation). This corresponds to a specific maximal turnover rate of 147 s^{-1}. However, the K_m for oxygen was 710 $\pm$ 93 μM, indicating that K_m and V_{max} for 2-aminophenol were not measured under saturating conditions with respect to the oxygen concentration. Additionally, the K_m value for oxygen is only approximate, because the solubility of oxygen in the buffer at atmospheric pressure (~1,400 μM) did not allow for oxygen saturation of the enzymatic reaction.

Substrate specificity. 2-Aminophenol 1,6-dioxygenase exhibited a narrow substrate range. Three groups of compounds could be identified. Substrates that were transformed and compounds that were not transformed but inhibited 2-aminophenol ring cleavage are listed in Table 2. 3-Aminosalicylic acid, 5-aminosalicylic acid, gentisate, homogentisate, hydroquinone, *o*-cresol, *p*-cresol, 2-chlorophenol, *o*-nitrophenol, *p*-nitrophenol, salicylic acid, and phenol were not transformed and did not affect the activity of the enzyme toward 2-aminophenol. With the exception of catechol, only some *o*-aminophenols

TABLE 2. Substrate specificity of 2-aminophenol 1,6-dioxygenase[a]

Substrate	Activity for alternate substrate (%)	Substrate left after 1 min (μM)	Inhibition of 2-aminophenol turnover (%)
2-Aminophenol	100	0	NA[b]
6-Amino-*m*-cresol	25	36	90
2-Amino-*m*-cresol	1	46	90
2-Amino-4-chlorophenol	15	42	90
Catechol	13	42	100[c]
3-Methylcatechol	0	50	100
4-Methylcatechol	0	50	100
3-Chlorocatechol	0	50	100
4-Chlorocatechol	0	50	100
1,2,3-Trihydroxybenzene	0	50	100
1,2,4-Trihydroxybenzene	0	50	100
4-Aminoresorcinol	0	50	100
2-Amino-*p*-cresol	0	50	90
2,3-Dihydroxybenzoic acid	0	50	20
Protocatechuate	0	50	20
2-Methoxyphenol	0	50	30
4-Amino-3-hydroxybenzoic acid	0	50	30
3-Amino-4-hydroxybenzoic acid	0	50	20
3-Hydroxyanthranilic acid	0	50	20
4-Amino-*m*-cresol	0	50	10

[a] The activity of 2-aminophenol 1,6-dioxygenase for various substrates was tested polarographically. The compounds were added to the reaction mixture to a concentration of 50 μM, and oxygen depletion was measured. After 60 s, 2-aminophenol (50 μM) was added to the mixture to provide a measure of inhibition of 2-aminophenol turnover by the test compound. The concentration of the alternate substrate remaining after 1 min was estimated from the oxygen consumed by assuming a 1:1 stoichiometry of oxygen consumption to substrate transformed. Duplicate measurements deviated less than 10%.

[b] NA, not applicable.

[c] The enzyme was inactivated by catechol. After 1 min, neither additional catechol nor 2-aminophenol caused any oxygen consumption.

substituted either with a methyl or a chloro group served as substrates for the enzyme. A carboxyl group was not accepted as a substituent in any position. Substituted catechols were not oxidized but abolished or strongly reduced 2-aminophenol turnover. Aromatic compounds not having either two vicinal hydroxyl groups or an amino group adjacent to a hydroxyl group were not transformed and, with the exception of guaiacol, had no inhibitory effect on 2-aminophenol oxidation. Catechol was cleaved in an extradiol manner as indicated by the increase of A_{375} due to the accumulation of 2-hydroxymuconic acid semialdehyde during catalysis (6). However, 2-aminophenol 1,6-dioxygenase lost activity when approximately 200 mol of catechol was oxidized per mol of enzyme. In comparison, 1 mol of enzyme could transform up to 30,000 mol of 2-aminophenol before activity was lost. Ring cleavage of 6-amino-*m*-cresol, 2-amino-*m*-cresol, and 2-amino-4-chlorophenol resulted in products exhibiting an absorption maximum at 380 nm, which suggested extradiol cleavage. However, these compounds were unstable as observed by the decrease of A_{380} along with an increase in absorbance between 260 and 270 nm. These spectral changes were similar to those observed for 2-aminomuconic acid semialdehyde, which condenses intramolecularly to picolinic acid (19). Accordingly, extradiol ring fission of 6-amino-*m*-cresol would yield 2-amino-5-methylmuconic acid semialdehyde, which spontaneously forms 5-methylpicolinic acid (Fig. 1B). The spectrum of the compound produced from 6-amino-*m*-cresol (data not shown) matched a UV spectrum previously published for 5-methylpicolinic acid (4). 2-Amino-*m*-cresol would yield 2-amino-3-methylmuconic acid semialdehyde, which condenses to 3-methylpicolinic acid (Fig. 1C), and 2-amino-4-chlorophenol would yield 2-amino-4-chloromuconic acid semialdehyde, which forms 4-chloropicolinic acid (Fig. 1D).

The compounds formed from 2-aminophenol, 2-amino-*m*-cresol, and 2-amino-4-chlorophenol were identified as picolinic acid, 3-methylpicolinic acid, and 4-chloropicolinic acid, respectively, by comparison of HPLC retention times and UV spectra with those of authentic standards. The yields in the reaction mixture were 43% for picolinic acid, 25% for 3-methylpicolinic acid, and 66% for 4-chloropicolinic acid. No attempts have been made to optimize the product yields. The identities of the picolinic acids were confirmed by GC-MS analysis. The retention time and mass spectrum of the methyl ester derivatives of the compounds produced from 2-aminophenol, 2-amino-*m*-cresol, and 2-amino-4-chlorophenol were identical to those of a picolinic acid methyl ester [*m/z* 137 (relative intensity, 7%), 107 (44), 79 (100), 78 (81), 52 (20), 51 (33)], 3-methylpicolinic acid methyl ester [*m/z* 151 (40), 136 (14), 121 (11), 120 (17), 119 (22), 93 (100), 92 (78), 91 (67), 65 (52), 39 (43)], and 4-chloropicolinic acid methyl ester [*m/z* 171 (4), 141 (30), 115 (33), 114 (25), 113 (100), 112 (57), 76 (38), 50 (18)], respectively. The mass spectrum of the compound formed from 6-amino-*m*-cresol [*m/z* 151 (8), 121 (27), 93 (100), 92 (51), 65 (32), 39 (22)] showed the same fragmentation pattern as a spectrum published for 5-methylpicolinic acid methyl ester (3). These results clearly identify the picolinic acids spontaneously formed from the ring cleavage products, which confirms that 2-aminophenol 1,6-dioxygenase opens the ring of 2-aminophenol, 2-amino-*m*-cresol, 6-amino-*m*-cresol, and 2-amino-4-chlorophenol in an extradiol manner.

Inhibition and activation. Only ferrous iron in combination with ascorbate increased the activity of the enzyme (Table 3). Most bivalent cations at a concentration of 2 mM reduced the activity of 2-aminophenol 1,6-dioxygenase. Copper ($CuSO_4$) and zinc ($ZnCl_2$) abolished the activity completely. Ferric iron reduced the activity significantly. The nonspecific chelators nitrilotriacetic acid and EDTA (2 mM) slightly reduced activity after 1 h. The Fe^{2+} chelators *o*-phenanthroline and 2,2′-dipyridyl abolished activity completely. The oxidizing agents hydrogen peroxide and potassium ferricyanide abolished the activity, which could be restored upon addition of excess ascorbate (Table 3). These properties, which are characteristic of extradiol cleavage enzymes (17, 21), provide circumstantial evidence that ferrous iron is the sole cofactor required for the activity of 2-aminophenol 1,6-dioxygenase.

TABLE 3. Effects of inhibitors and activators on 2-aminophenol 1,6-dioxygenase[a]

Addition (concn)	Reagent(s) added	Activity (% of untreated enzyme)
None		100
Metal ions (2 mM)	Fe^{2+}, ascorbate[d]	115
	Fe^{2+}	100
	Mo^{2+}	96
	Mg^{2+}	85
	Ca^{2+}	84
	Fe^{3+}	78
	Ni^{2+}	50
	Co^{2+}	47
	Mn^{2+}	31
	Cd^{2+}	5
	Cu^{2+}	0
	Zn^{2+}	0
Complexing agents (2 mM)	NTA[b]	86
	EDTA	70
	o-Phenanthroline	1
	2,2′-Dipyridyl	0
Oxidizing agents (1 mM)	H_2O_2	0 (42)[c]
	$K_3Fe(CN)_6$	17 (101)

[a] Eight micrograms of 2-aminophenol 1,6-dioxygenase was incubated with each of the reagents listed.
[b] NTA, nitrilotriacetic acid.
[c] The percentage of activity restored upon addition of ascorbate to a concentration of 5 mM is given in parentheses. Duplicate measurements deviated less than 10%.
[d] Ascorbate concentration, 10 mM.

Iron analysis. The iron/enzyme ratio was 2.2 ± 0.2 mol of ferrous iron per mol of enzyme when the protein concentration was measured with Coomassie blue. The molecular mass of the enzyme was assumed to be 140,000 Da as determined by gel filtration. Determination of protein dry weight resulted in (1.3 ± 0.2)-fold-higher protein concentrations resulting in an iron/enzyme ratio of 1.7 ± 0.2. Passage of the enzyme through a Chelex 100 column did not reduce the iron content of the enzyme preparation. Because ferrous iron and either ascorbate or L-cysteine had to be included at several steps during the purification to prevent loss of activity, the iron content of the enzyme in vivo is unknown.

DISCUSSION

2-Aminophenol 1,6-dioxygenase is a colorless protein with an $\alpha_2\beta_2$ subunit structure that employs ferrous iron as cofactor. Hence, it is a typical member of the nonheme iron dioxygenases (21) or the aromatic ring cleavage dioxygenases (10). In contrast to the most prominent enzymes of this group, the catechol and protocatechuate dioxygenases, this enzyme has a much higher activity towards *o*-aminophenolic substrates than towards catecholic substrates. Nevertheless, 2-aminophenol 1,6-dioxygenase shares several structural (Table 4) and biochemical properties with aromatic ring cleavage enzymes.

Ring cleavage dioxygenases are classified by their mode of ring fission. Intradiol enzymes cleave the aromatic ring be-

TABLE 4. Structures of selected nonheme iron ring cleavage dioxygenases

Enzyme	Source	No. of subunits (size [kDa])	Molecular mass (kDa)	Reference
2-Aminophenol 1,6-dioxygenase	*Pseudomonas pseudoalcaligenes* JS45	2 (35.0), 2 (39.0)	140	This study
Gentisate 1,2-dioxygenase	*Pseudomonas testosteroni*	4 (40.8)	158	11
Catechol 2,3-dioxygenase	*Pseudomonas putida*[a]	4 (35.2)	140	14
Protocatechuate 2,3-dioxygenase	*Bacillus macerans* JJ1b	4 (35.0)	143	35
2,3-Dihydroxybiphenyl 1,2-dioxygenase	*Pseudomonas aeruginosa*	8 (31.0)	250	7
2,3-Dihydroxybiphenyl 1,2-dioxygenase	*Pseudomonas putida* BS893	2 (34.0), 2 (22.5)	135	28
Protocatechuate 4,5-dioxygenase	*Pseudomonas testosteroni*	2 (33.3), 2 (17.7)	142	2
3-Hydroxyanthranilate 2,3-dioxygenase	Rat liver	1 (37.0)	37	23

[a] *xylE* cloned and expressed in *Escherichia coli.*

tween two hydroxyl groups, whereas extradiol enzymes cleave the aromatic ring between one hydroxylated carbon and another adjacent nonhydroxylated carbon (21). Typically, intradiol and extradiol dioxygenases use ferric and ferrous iron as cofactors, respectively (10). Accordingly, 2-aminophenol 1,6-dioxygenase seems more similar to extradiol ring cleavage enzymes.

The only known enzyme catalyzing ring cleavage vicinal to a hydroxyl and an amino group is 3-hydroxyanthranilate 3,4-dioxygenase. To date, this enzyme has been found only in mammals, where it is involved in tryptophan metabolism and synthesis of quinolinic acid (20). It is specific for 3-hydroxyanthranilate and is noncompetitively inhibited by 2-aminophenol (33). In contrast, 2-aminophenol 1,6-dioxygenase is only slightly inhibited by 3-hydroxyanthranilate (Table 2). Additionally, 3-hydroxyanthranilate dioxygenase is a monomer (15), whereas 2-aminophenol 1,6-dioxygenase is a heterotetramer. Hence, the differences in origin, substrate specificities, and structures suggest that the catalytic sites of these two enzymes may differ considerably and that a close evolutionary relationship is unlikely.

Protocatechuate 4,5-dioxygenase (1, 2) and 2,3-dihydroxybiphenyl 1,2-dioxygenase (28) have $\alpha_2\beta_2$ tertiary structures as well. Their large subunits, along with the small subunit of 2-aminophenol 1,6-dioxygenase, are well within the usual size of most extradiol ring cleavage dioxygenases (Table 4). In contrast, the large subunit of 2-aminophenol 1,6-dioxygenase has twice the molecular mass of the small subunit of protocatechuate 4,5- and dihydroxybiphenyl 1,2-dioxygenase. Indeed, the large subunit of 2-aminophenol 1,6-dioxygenase is similar in size to the subunit of gentisate dioxygenase. 2-Aminophenol 1,6-dioxygenase cleaved neither protocatechuate nor gentisate, indicating that the active site must be considerably different from that of protocatechuate 4,5- and gentisate 1,2-dioxygenase. Hence, the subunit structure and the substrate specificity of 2-aminophenol 1,6-dioxygenase are unique among those of the known ring fission dioxygenases.

Comparison of the N termini of the two subunits of 2-aminophenol 1,6-dioxygenase with published sequences of ring cleavage dioxygenases revealed only limited similarity. The N-terminal sequence of the α subunit has about 20% identity with that of the catechol 2,3-dioxygenase encoded by *nahH* (8), and the β subunit has about 30% sequence identity with the 15 known N-terminal amino acids of gentisate 1,2-dioxygenase of *Pseudomonas testosteroni* (11). Further sequence analysis is required to establish whether the small and large subunits of 2-aminophenol 1,6-dioxygenase are related to catechol 2,3- or gentisate 1,2-dioxygenase.

Most mechanisms for extradiol ring cleavage dioxygenases propose that two of the six coordination sites of the active-site Fe(II) are occupied by the substrate. For catechol 2,3-, protocatechuate 2,3-, and protocatechuate 4,5-dioxygenase, the two vicinal hydroxyl groups serve as ligands (17, 29). Theoretically, an amino group can also serve as an iron ligand. However, the mechanisms proposed for protocatechuate 2,3- or 4,5-dioxygenase (17) and catechol 2,3-dioxygenase (29) differ slightly. In the first, the distal hydroxyl group serves only as a ligand of the active-site Fe(II), whereas in catechol 2,3-dioxygenase, it is proposed to undergo intermediate transformation to an oxo group. An amino group can form an imino intermediate as well. Therefore, either mechanism could apply for substrates where one hydroxyl group is replaced with an amino group. The fact that catechol 2,3-dioxygenase cleaved 2-aminophenol at an extremely low rate (13, 14, 22) provides support for this hypothesis. By analogy, protocatechuate 2,3-dioxygenase cleaved 4-amino-3-hydroxybenzoic acid at a rate of 0.5% of that of protocatechuate (35). Another enzyme allowing substitution of one hydroxyl group with an amino group is gentisate 1,2-dioxygenase, which oxidized 5-aminosalicylic acid at a rate that is 7% of that for gentisate (12). An enzyme that cleaves 5-aminosalicylic acid as its primary substrate has also been described (32). Hence, the aromatic rings of *o*-aminophenols appear to be opened in a similar manner to the extradiol cleavage of catecholic substrates.

Reduction of the nitro group of nitroaromatic compounds has been studied extensively (9) and occurs under both aerobic (30) and anaerobic conditions (5). The reduction intermediates, i.e., nitrosoaromatic, hydroxylaminoaromatic, and aminoaromatic compounds, easily form azo (24) and azoxy compounds (34), which are mostly end products of biotransformation. The capability to convert hydroxylaminobenzene to 2-aminophenol and to open the ring of 2-aminophenol with the ring fission enzyme described in this paper enables *P. pseudoalcaligenes* JS45 to avoid forming toxic end products and to use nitrobenzene as a source of carbon, nitrogen, and energy. The enzymes of this pathway can potentially be applied for the biotechnological synthesis of substituted picolinates from nitroaromatic compounds.

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