Novel Genes Encoding 2-Aminophenol 1,6-Dioxygenase from *Pseudomonas* Species AP-3 Growing on 2-Aminophenol and Catalytic Properties of the Purified Enzyme*

(Received for publication, February 26, 1997, and in revised form, April 4, 1997)

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2-Aminophenol 1,6-dioxygenase was purified from the cell extracts of *Pseudomonas* sp. AP-3 grown on 2-aminophenol. The product from 2-aminophenol by catalysis of the purified enzyme was identified as 2-aminomuconic 6-semialdehyde by gas chromatographic and mass spectrometric analyses. The molecular mass of the native enzyme was 140 kDa based on gel filtration. It was dissociated into molecular mass subunits of 32 (α-subunit) and 40 kDa (β-subunit) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, indicating that the di-oxygenase was a heterotetramer of αβ. The genes coding for the α- and β-subunits of the enzyme were cloned and sequenced. Open reading frames of the genes (*amnA* and *amnB*) were 816 and 918 base pairs in length, respectively. The amino acid sequences predicted from the open reading frames of *amnA* and *amnB* corresponded to the NH₂-terminal amino acid sequences of the α-subunit (*AmnA*) and β-subunit (*AmnB*), respectively. The deduced amino acid sequences of AmnB showed identities to some extent with HpaD (25.4%) and HpcB (24.4%) that are homoprotocatechuate 2,3-dioxygenases from *Escherichia coli* W and C, respectively, belonging to class III in the extradiol dioxygenases. On the other hand, AmnA had identity (23.3%) with only AmnB among the enzymes examined.

Dioxygenases catalyzing the fission of benzene rings are key enzymes in the metabolic pathways of aromatic compounds by microorganisms. Most of these kinds of previously reported dioxygenases attack monocylic aromatic compounds with two adjacent hydroxyl groups such as catechol and protocatechuic acid and open the benzene rings through the intradiol or extradiol fission reaction (1, 2). However, some bacteria have been reported to synthesize dioxygenases that cleave the benzene rings of hydroquinone (3–5) and gentisic acid (6, 7).

In our investigations on the microbial metabolism of anilines, we isolated several microorganisms capable of growing on 2-aminophenol as the sole carbon, nitrogen, and energy source. When one isolate, *Pseudomonas* sp. AP-3, grew with this substrate, it synthesizes an enzyme acting on 2-aminophenol. This enzyme was partially purified with a 103-fold increase in the specific activity from its cell extracts. We proposed that the enzyme is a dioxygenase catalyzing the ring fission of 2-aminophenol with the consumption of 1 mol of O₂ per mol of substrate (8).

Our aim was to advance the purification of 2-aminophenol 1,6-dioxygenase from *Pseudomonas* sp. AP-3 and elucidate the molecular and catalytic properties of the purified enzyme. Because the product from 2-aminophenol by catalysis of the enzyme is rapidly and nonenzymatically converted into picolinate (8, 9), the real product has remained unverified. Furthermore, we attempted the cloning and sequencing of the gene of the dioxygenase, which would determine the category of this enzyme in the dioxygenase groups.

Recently, Lendenmann and Spain (10) reported the purification and characterization of the 2-aminophenol 1,6-dioxygenase from nitrobenzene-degrading *Pseudomonas pseudoalcaligenes JS45*, although they did not refer to the cloning and sequencing of its gene. In this report, the comparison of the dioxygenases from the two *pseudomonads* growing on 2-aminophenol or nitrobenzene is also described.

MATERIALS AND METHODS

**Chemicals**—The chemicals used in this study and their sources are as follows. Polypeptone, 2-aminophenol, catechol, 4-methylcatechol, methyl chlorocarbonate, and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide were from Wako Pure Chemicals, Osaka; 2-aminop-cresol, 6-amino-p-cresol, 2-amino-4-chlorophenol, 3-methylcatechol, 3-chlorocatechol, 4-chlorocatechol, and 3-fluorocatechol were from Tokyo Kasei, Tokyo; pentafluorophenylhydrazine, 2-amino-p-cresol, and 2-amino-4,5-di-methylphenol were from Aldrich; DE52 cellulose was from Whatman; DEAE-Cellulofine A-500 was from Seikagaku Co., Tokyo; and restriction endonucleases were from Takara Shuzo, Otsu.

**Bacterial Strains, Plasmids, and Bacteriophages**—*Pseudomonas* sp. AP-3 was used throughout this study as a producer of 2-aminophenol 1,6-dioxygenase and a donor of its gene. *Escherichia coli* JM109 and *E. coli* P2292 were used as hosts for the recombinant plasmids and bacteriophages, respectively. A lambda FIX I/pXhol partial fill-in vector (Stratagene, La Jolla) was used for the construction of a gene library. pGEM-T (Promega, Madison) and pBluescript II SK (+) vectors (Stratagene) were used for cloning of the PCR (polymerase chain reaction) products and subcloning of the DNA fragments, respectively.

**Media and Cultural Conditions**—*Pseudomonas* sp. AP-3 was cultured in the 2-aminophenol medium (8) containing 0.12% (w/v) of Polypeptone. *Pseudomonas* sp. AP-3 used for isolating its total DNA and *E. coli* strains were cultured in Luria broth (11) with shaking at 30 and 37 °C, respectively.

**Enzyme Assays**—The activity of 2-aminophenol 1,6-dioxygenase was measured by monitoring the decrease in absorbance at 262 nm according to a previous paper (8). The activities for the 2-aminophenol analogs were measured by scanning changes in the absorbance of each reaction mixture, because all substrates tested had absorption bands in the UV range. Molar extinction coefficients of the substrates attacked by the enzyme were determined in this study as follows: 3100 at 287 nm for 2-aminop-cresol, 2700 at 287 nm for 6-amino-p-cresol, 3100 at 291 nm.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) D89855.

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for 2-aminophenol-1,6-dioxygenase—All operations for enzyme purification were done at 0–4 °C, and centrifugation was carried out at 20,000 × g for 10 min. The frozen cells (37 g, wet weight) of Pseudomonas sp. AP-3 were used for the purification. The preparation of the cell extracts (step 1, fraction 1), streptomycin sulfate treatment (step 2, fraction 2), and ammonium sulfate fractionation (step 3, fraction 3) were essentially carried out by the same methods as described previously (8).

Step 4. Acetone Fractionation—After the protein concentration of fraction 3 was adjusted to 7 mg ml⁻¹ by adding buffer A (20 mM Tri-HCl (pH 8.0) containing 10% (v/v) ethanol, 1 mM dithiothreitol, and 0.5 mM L-ascorbate), acetone was added to the diluted solution to give 55% (v/v). The precipitate was removed by centrifugation and then acetone was added to the supernatant to give 65% (v/v). The precipitate was obtained by centrifugation and then dissolved in buffer A. The enzyme solution was dialyzed against buffer A (fraction 4).

Step 5. Chromatography on DE52 Cellulose—Fraction 4 was applied to a column (2.2 × 27 cm) of DE52 cellulose equilibrated with buffer A. Proteins were eluted with a linear gradient (0 to 0.4 M) of NaCl in 1.4 liters of buffer A, and then the protein concentrations and 2-aminophenol-1,6-dioxygenase activities were assayed. Fractions with the specific activity higher than 2.7 units mg⁻¹ were pooled (fraction 5).

Step 6. Chromatography on DEAE-Cellulofine A-500—Fraction 5 was dialyzed against buffer A. The dialyzed solution was applied to a column (2 × 26 cm) of DEAE-Cellulofine A-500 equilibrated with buffer A. The enzyme was eluted with a linear gradient (0 to 0.35 M) of NaCl in 1.4 liters of buffer A. The enzyme in each fraction was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15). Fractions showing two distinct protein bands (α- and β-subunits) on the gel were pooled (fraction 6).

Determination of Molecular Masses—The molecular mass of the native enzyme was measured by the two methods of gel filtration and PAGE (16). Those of the enzyme subunits were measured by SDS-PAGE (15).

Identification of Reaction Product (Compound I) from 2-Aminophenol—The enzyme reaction mixture consisted of 5 mM 2-aminophenol, 12 ml; 0.05 mM 2-amino-4-chlorophenol, 2100 at 279 for 2-amino-4-chlorophenol, 2100 at 279 for 2-amino-4-chlorophenol.

RESULTS

Purification of 2-aminophenol 1,6-dioxygenase—Table I shows a summary of a typical enzyme purification. The specific activity of the final preparation of 2-aminophenol 1,6-dioxygenase was 4.8 units mg⁻¹ with an overall recovery of 36%. A 120-fold increase in the specific activity was observed at the final step of the purification procedure. The final enzyme preparation showed one major protein band and two indistinct bands on a polyacrylamide gel without SDS. The molecular mass of the major band was 146 kDa on the gel (16). Those of other two bands were lower than 85 kDa. However, on a SDS-polyacrylamide gel, the final preparation showed two distinct protein bands with molecular masses of 32 kDa (α-subunit) and 40 kDa (β-subunit) (Fig. 1).

Denisomeric analyses of the bands revealed that the molar ratio of the two subunits was one to one on the basis of these molecular sizes. In addition, the molecular mass of the native enzyme was 140 kDa by gel filtration. These findings indicate that the enzyme was made up of four heterogeneous subunits with the structure of α₂β₂.

The purified enzyme was stable in buffer A at 4 °C for a week without any decrease in activity. However, it lost activity within 24 h in the absence of ethanol, dithiothreitol, and L-ascorbate.

Identification of Product I—Fig. 2 shows the mass spectra of

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; AminA and B, α- and β-subunits of 2-aminophenol 1,6-dioxygenase, respectively; kb, kilo base pair(s); MOPS, morpholinepropanesulfonate.

| Fraction | Volume | Total Protein | Total Activity | Specific Activity | Recovery |
|----------|--------|---------------|----------------|------------------|---------|
| 1. Cell extract | 460 | 160 | 4300 | 0.04 | 100 |
| 2. Streptomycin sulfate | 463 | 150 | 4300 | 0.03 | 94 |
| 3. Ammonium sulfate | 97 | 230 | 1500 | 0.15 | 140 |
| 4. Acetone | 58 | 260 | 500 | 0.52 | 160 |
| 5. DE52 | 49 | 74 | 26 | 2.8 | 48 |
| 6. DEAE-cellulofine | 25 | 58 | 12 | 4.8 | 36 |
the N-acylated and trimethylsilylated hydrazone derivative of compound I. There was a molecular ion at \( m/z \ 451 \) (M+), which agreed with the empirical formula of \( C_{11}H_{18}F_{2}N_{4}O_{6}Si \). Major fragment ions appeared at \( m/z \ 436 \) (M+ − CH3), 419 (M+ − OCH3 − H), 404 (M+ − OCH3 − CH3 − H), 391 (M+ − COOCH3 − H), 377 (M+ − COOCH3 − NH), 361 (M+ − COOCH2 − CH2 − H), 302 (M+ − COOCH3 − H − COOCH3 − CH2 − H), 274 (M+ − COOCH3 − H − COOCH3 − CH3 − H), 255 (M+ − C6F5 − NH − N − CH), 242 (M+ − C6F5 − NH − N − CH − H), 229 (M+ − C6F5 − NH − N − 2CH), 195 ([C6F5 − N3]+), and 73 ([Si(CH3)3]+). These data showed that compound I was 2-aminomuconic 6-semialdehyde.

**Absorption Spectrum**—The enzyme did not have any absorption band in the visible range. However, it had an absorption peak at 280 nm and a small shoulder at 287–290 nm: \( \epsilon_{280} = 13.7 \times 10^4 \) at 280 nm. The \( F_{280}/F_{600} \) ratio of the enzyme was 1.8.

**Iron Content**—The enzyme contained 0.98 mol of Fe2+ per mol of protein on the basis of the molecular mass of 140 kDa.

**Substrate Specificity**—The substrate specificity of 2-aminophenol 1,6-dioxygenase was examined with 39 aromatic compounds consisting of catechol, phenol, and aniline compounds (Table II). Besides 2-aminophenol, the enzyme was active toward 2-amino-p-cresol, 6-amino-m-cresol, 2-amino-m-cresol, 2-amino-4,5-dimethylphenol, 2-amino-4-chlorophenol, and catechol. However, it did not act on 2-aminophenol analogs that were substituted by a carboxyl or nitro group at the 3-, 4-, and 5-positions. In addition, catechol compounds except catechol were not substrates of the enzyme.

**Kinetic Properties**—The \( K_m \) and \( V_{max} \) values for 2-aminophenol of 2-aminophenol 1,6-dioxygenase were 46.7 \( \mu M \) and 0.10 \( \mu M \cdot s^{-1} \cdot mg^{-1} \), respectively. The enzyme for 2-aminophenol as the substrate was inhibited by the catechols and 4-aminoresorcinal listed in Table II. In addition, the 2-aminophenol analogs that could be degraded by the enzyme also inhibited it from the action on 2-aminophenol, although their types of inhibition were unmeasured. The activity of the enzyme for 2-aminophenol was not affected by the 2-aminophenol analogs such as phenols and anilines that were not substrates of this enzyme.

**Inhibition**—The effects of metal salts, chelating and sulphydryl agents on the enzyme activity were tested using 2-aminophenol as the substrate (Table III). Among the metal ions tested, the enzyme was strongly inhibited by CuSO4, FeCl3, K3Fe(CN)6, AgNO3, HgCl2, or MnCl2. FeSO4 and MgSO4 did not inhibit the enzyme very much, and FeSO4(2NH4)SO4 slightly increased the activity for 2-aminophenol. Chelating...
Eco probe for the hybridization to about 3000 plaques was performed using the sp. AP-3 was constructed in a lambda FIXII phage vector. The recognition sites for sp. AP-3. Pseudomonas and their corresponding primer sequences. The primers of α1 and β1 were synthesized on the basis of coding strands, and the α2 and β2 primers were anticoding strands. I, Y, S, R, and W in primer sequences indicate inosine, CT, GC, AG, and AT, respectively.

![Image](image1)

**FIG. 3.** NH₂-terminal amino acid sequences of the α- and β-subunits and their corresponding primer sequences. The primers of α1 and β1 were synthesized on the basis of coding strands, and the α2 and β2 primers were anticoding strands. I, Y, S, R, and W in primer sequences indicate inosine, CT, GC, AG, and AT, respectively.

![Image](image2)

**FIG. 4.** Southern blot hybridization of genomic DNA from Pseudomonas sp. AP-3. PCR products labeled with ³²P were used as a probe. Lanes 2–10 show autoradiograms of genomic DNA digested with various restriction endonucleases. Lane 1, markers (HindIII-digested lambda DNA); lane 2, Apal; lane 3, BamHI; lane 4, BanIII; lane 5, EcoRI; lane 6, EcoRV; lane 7, KpnI; lane 8, SacI; lane 9, SacII; lane 10, XhoI; lane 11, markers. Sizes of markers and hybridized fragments are indicated in the left and right margins, respectively.

agents and NaN₃, completely repressed the enzyme activity.

**NH₂-terminal Amino Acid Sequences**—The amino acid sequences of 30 and 20 residues of the α- and β-subunits, respectively, of the enzyme were determined. On the basis of the two sequences, the four primers α1, β1, α2, and β2 were synthesized (Fig. 3).

**Amplification of amn Gene by PCR**—When the β1 and α2 primers and DNA purified from Pseudomonas sp. AP-3 as a template were incubated, a 1-kb DNA fragment was amplified. The sequencing of both termini of the amplified fragment showed that this fragment encoded a large portion of the β-subunit and an NH₂-terminal region of the α-subunit. The sequenced fragment was labeled with [α-³²P]dCTP and was used as a probe for Southern hybridization. This DNA probe hybridized to the DNA fragments from the AP-3 strain digested with several restriction endonucleases (Fig. 4). These results showed that the PCR product was amplified on the basis of the DNA sequence from the AP-3 strain. The appearance of two positive bands for the KpnI (lane 7) and SacI (lane 8) digested DNAs suggests that the PCR product contained recognition sites for KpnI and SacI in its sequence.

**Cloning of amn Gene**—The genomic library of Pseudomonas sp. AP-3 was constructed in a lambda FIXII phage vector. The hybridization to about 3000 plaques was performed using the probe for the amn gene mentioned above. After screening twice, we obtained five positive clones, p3-1, p4-3, p5-2, p12-2, and p12-7. The DNAs purified from these phage clones were digested with Apal, BanIII, EcoRI, EcoRV, KpnI, and SacI. Agarose gel electrophoretic analyses revealed that restriction fragments obtained from these DNAs contained several fragments with the same size as the positive bands detected in the Southern hybridization (Fig. 4). The p4-3 DNA was selected for subcloning of the amn gene, because it was recovered with the greatest yield of the obtained fragments. SacI (1.7-kb) and EcoRI (1.4-kb) fragments encoding whole α- and β-subunit genes, respectively, were separated from each other and ligated into a pBluescript II SK(+) vector. The obtained pS1 and pE1 plasmids carried 1.7-kb SacI and 1.4-kb EcoRI fragments, respectively.

**DNA Sequences of amn Gene and Its Deduced Amino Acid Sequences**—The DNA fragments inserted into pS1 and pE1 were sequenced. Fig. 5a shows that 884 base pairs of the two fragments overlapped. Two open reading frames containing each primer sequence used in the PCR were found in the connected sequence and were designated as amnB for the first open reading frame and amnA for the second one. Fig. 5b shows a DNA sequence connected with pE1 and pS1, and amino acid sequences deduced from amnB and amnA. The amnB encoded 305 amino acid residues. The deduced amino acid sequences from positions 2 to 31 completely agreed with the NH₂-terminal

![Image](image3)

**FIG. 5.** Cloning of amnA and amnB. a, location of amnA and amnB. PCR products were used as a probe to select DNA fragments carrying amnA and amnB. The region constructed by pE1 and pS1 contains whole amnA and amnB. The numbers indicate the positions of the nucleotides in the sequenced and connected region. Abbreviations used for the restriction endonucleases are: E, EcoRI; S, SacI; K, KpnI.

b, the nucleotide sequence of the region covered with pE1 and pS1 and deduced amino acid sequences of AmnA and AmnB.
amino acid sequences of the \( \beta \)-subunit determined from the purified enzyme. The initiation codon (ATG) of \( \alpha \)-subunit of the \( amnB \) gene was used as a primer for PCR amplification of the \( amnB \) gene. In addition, the NH\(_2\) terminal amino acid sequences of the \( \alpha \)-subunit were homologous to each other in all sequences. The amino acid sequences of the \( \beta \)-subunit of the \( amnB \) gene were used as a primer for PCR amplification of the \( amnB \) gene. The \( amnB \) gene consists of 271 amino acid residues and was located at the 32 base pairs downstream from the termination codon (TAA). Therefore, we consider that the final preparation for sequencing exhibited only one amino acid residue at the NH\(_2\) terminus. Each protein band electroblotted onto the transfer membrane for purification. The initiation codon (ATG) of the \( amnA \) gene was located at the 32 base pairs downstream from the termination codon (TAA). Therefore, we consider that the final preparation for sequencing exhibited only one amino acid residue at the NH\(_2\) terminus. Each protein band electroblotted onto the transfer membrane for purification. The \( amnA \) gene was made up of an equal molar ratio on the basis of the molecular masses of 32 and 40 kDa. In addition, each protein band was detected by colloidal Coomassie Blue staining. The two components were made up of an equal molar ratio on the basis of the molecular masses of 32 and 40 kDa. In addition, each protein band was detected by colloidal Coomassie Blue staining. The final preparation of the \( amnA \) gene was shown to be a heterotetramer of \( amnA \) and \( amnB \) subunits. In addition, the enzyme had 0.98 mol of Fe\(^{2+}\) per mol of enzyme.

**Fig. 6. Alignment of amino acid sequences of \( amnA \) and \( amnB \) with those of the class III enzymes in extradiol dioxygenases.**

![Alignment of amino acid sequences of \( amnA \) and \( amnB \) with those of the class III enzymes in extradiol dioxygenases.](http://www.jbc.org/)

The 2-aminophenol 1,6-dioxygenase from \( P. \) paucimobilis was purified with a 120-fold increase in the specific activity. Although the final preparation of the enzyme did not show one band on PAGE under non-denaturing conditions, these showed distinct two protein masses of 32 and 40 kDa. In addition, each protein band was detected by colloidal Coomassie Blue staining. The two components were made up of an equal molar ratio on the basis of the molecular masses of 32 and 40 kDa. In addition, each protein band was detected by colloidal Coomassie Blue staining. The final preparation of the \( amnA \) gene was shown to be a heterotetramer of \( amnA \) and \( amnB \) subunits. In addition, the enzyme had 0.98 mol of Fe\(^{2+}\) per mol of enzyme.

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ble residues (83% identity); AmnB (β-subunit) from the AP-3 strain and the β-subunit from the JS45 strain showed 21 identical amino acid residues of the 30 comparable residues (70% identity). The comparison of the amino acid sequences of AmnB with those deduced from DNA data bases revealed that it had amino acid sequence identities of 25.5 and 24.4% with HpaD (23) and HpcB (24), respectively, which are homoprotocatechuate 2,3-dioxygenases from *E. coli* strains (Fig. 6). However, AmnB did not show any identity with other extradiol dioxygenases such as XylE (catechol 2,3-dioxygenase) (25) encoded on the TOL plasmid and several 2,3-dihydroxybiphenyl 1,2-dioxygenases (26, 27).

HpaD and HpcB belong to class III in the extradiol dioxygenases proposed by Spence et al. (28). They described that the enzymes of class III possess an NH$_2$-terminal domain containing the active center consisting of a Fe$^{2+}$ cofactor and four histidine residues that are conserved in these enzymes (shaded area in Fig. 6). Multiple alignments of AmnB and the class III enzymes revealed that AmnB retains three histidine residues, His-14, His-63, and His-196, of the four histidine residues conserved in the class III enzymes; one residual histidine residue is replaced by an arginine residue at the corresponding position in AmnB (Fig. 6). At the same position in human 3-hydroxyanthranilic-acid dioxygenase belonging to class III, the histidine residue conserved in the class III enzymes was replaced by a threonine residue (28). In addition, we found the identical amino acid residues with those in the class III enzymes, which are located in the vicinity of the three conserved histidine residues. They were Pro-16, Glu-51, Leu-57, Ser-191, His-14, His-63, and His-196 of the four histidine residues conserved in every sequence of the enzymes listed in Fig. 6, although they are not catalytically active histidine residues (marked by † in Fig. 6). The similarity of AmnA with AmnB and the existence of the conserved amino acid residues in AmnA may support the fact that AmnA also belongs to class III in extradiol dioxygenases. Because AmnA lacks the functional histidine residues conserved in other class III enzymes, it may be independent of the catalytic process of 2-aminophenol 1,6-dioxygenase and therefore play other roles, such as the stabilization of AmnB in the enzymatic reaction.

Among the class III enzymes whose amino acid sequences have been reported, only protocatechuate 4,5-dioxygenase from *Pseudomonas paucimobilis* SYK6 consists of two distinct subunits, LigA and LigB (29). However, it is noted that LigA, the small subunit of protocatechuate 4,5-dioxygenase, showed no identity with AmnA, the small subunit of 2-aminophenol 1,6-dioxygenase, although LigB aligned with AmnB. This fact suggests that LigA and AmnA are different in the process of molecular evolution and in the function of enzymatic catalysis.

Further efforts are now in progress to identify other genes responsible for the 2-aminophenol metabolism using the cloned DNA fragments from *Pseudomonas* sp. AP-3.

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Novel Genes Encoding 2-Aminophenol 1,6-Dioxygenase from *Pseudomonas* Species AP-3 Growing on 2-Aminophenol and Catalytic Properties of the Purified Enzyme

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*J. Biol. Chem.* 1997, 272:14727-14732.

doi: 10.1074/jbc.272.23.14727

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