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3-Hydroxyaminophenol Mutase from *Ralstonia eutropha* JMP134 Catalyzes a Bamberger Rearrangement

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3-Hydroxyaminophenol mutase from *Ralstonia eutropha* JMP134 is involved in the degradative pathway of 3-nitrophenol, in which it catalyzes the conversion of 3-hydroxyaminophenol to aminohydroquinone. To show that the reaction was really catalyzed by a single enzyme without the release of intermediates, the corresponding protein was purified to apparent homogeneity from an extract of cells grown on 3-nitrophenol as the nitrogen source and succinate as the carbon and energy source. 3-Hydroxyaminophenol mutase appears to be a relatively hydrophilic but soluble and colorless protein consisting of a single 62-kDa polypeptide. The pI was determined to be at pH 4.5. In a database search, the NH$_2$-terminal amino acid sequence of the undigested protein and of two internal sequences of 3-hydroxyaminophenol mutase were found to be most similar to those of glutamine synthetases from different species. Hydroxyaminobenzene, 4-hydroxyaminotoluene, and 2-chloro-5-hydroxyaminophenol, but not 4-hydroxyaminobenzoate, can also serve as substrates for the enzyme. The enzyme requires no oxygen or added cofactors for its reaction, which suggests an enzymatic mechanism analogous to the acid-catalyzed Bamberger rearrangement.

The recognition that synthetic nitroaromatic compounds are environmental hazards has led to a considerable amount of research on their biodegradation (for reviews, see references 18, 21, 32, 47, and 48). As a result, a variety of novel enzymatic mechanisms for the degradation or transformation of nitroarenes have been discovered. Oxidative elimination of the nitro group as nitrite seems to be a key reaction in the catabolism of many mononitroaromatic and some dinitroaromatic compounds (49). In general, the electron-withdrawing character of the nitro group favors biological reduction, giving rise to ring hydrogenation (28, 29, 54, 55) or transformation of the nitro group to either nitroso, hydroxylamine, or amino derivatives (47, 48). Each of these products can be subjected to further transformation or mineralization (47).

Recent evidence suggests that the hydroxyaminoaromatic compounds are key intermediates in a variety of metabolic pathways of mononitroaromatic compounds. For example, an enzymatic rearrangement of aromatic hydroxylamines and hydroxamic acids to their corresponding ortho-aminophenol derivatives was observed in rabbit liver (6) and rat liver homogenates (51). In the latter report, a hepatic isomerase-catalyzed mechanism that corresponded to the acid-catalyzed chemical rearrangement of hydroxylaminobenzene to 2-aminopheno! was proposed (3). The latter reaction proceeded in cell extracts of induced *R. eutropha* JMP134 in the absence of oxygen and without any cofactors; therefore, a rearrangement analogous to that observed for hydroxyaminobenzene in *P. pseudoalcaligenes* JS45 (37) was postulated. Although *R. eutropha* JMP134 was not able to mineralize nitrobenzene, enzymes in extracts of 3NP-grown cells catalyzed the rearrangement of hydroxymamino benzene. However, whereas the mutase enzyme(s) from *P. pseudoalcaligenes* JS45 produced ortho-aminophenol almost exclusively, the enzyme(s) from *R. eutropha* JMP134 produced both the ortho and para isomers (43).

Several authors proposed that the mechanism of the enzymatic reaction corresponds to that of the Bamberger reaction (Fig. 1) but provided no experimental evidence (14, 49, 51). To date, none of the enzymes whose physiological role is the rearrangement of an aromatic hydroxylamine to an aminophenol has been isolated or characterized. To gain insight into the
reaction mechanism and to determine whether the enzymatic isomerization of 3-hydroxylaminophenol to aminohydroquinone in _R. eutropha_ JMP134 was catalyzed by a single enzyme, we have purified and characterized the 3HAP mutase.

**Materials and Methods**

Culture conditions. _R. eutropha_ JMP134 (39) was grown in a 20-liter bioreactor (Bioengineering) containing 2 liters of nitrogen-free mineral salt medium (10). Two 1-liter cultures pregrown on 3NP (0.5 mM) at the nitrogen source and succinate (10 mM) as the carbon and energy source served as the inoculum. During fed-batch fermentation, addition of 3NP was controlled by use of a syringe pump, so that the concentration of the nitroaromatic compound never exceeded 0.5 mM. The culture was stirred at 150 rpm and aerated with 150 liters of air/h. After 37.5 mmol of 3NP was consumed (absorbance at 546 nm = 1.6) the cells were harvested by centrifugation and washed twice in 50 mM phosphate buffer (pH 7.4). The wet cell paste (19.5 g) was stored frozen at -20°C overnight.

Enzyme purification. The protein was purified by ultracentrifugation, anion-exchange chromatography, and hydrophobic interaction chromatography. All steps were performed in 50 mM sodium-potassium phosphate buffer (pH 7.5) at 4°C unless stated otherwise. The thawed cells were resuspended in 55 ml of buffer and lysed by two passages through a French pressure cell at 20,000 lb-in². The resulting lysate was centrifuged at 100,000 × g for 1 h, and the pellet was discarded. The supernatant was applied to a DEAE CL-6B (weak anion-exchange resin) column (HK 16/30; bed volume, 7.5 ml; diameter, 16 mm [Phar- macia, Uppsala, Sweden]) equilibrated with buffer. After the column was washed with 55 ml of buffer, proteins were eluted with 275 ml of a linear NaCl gradient (0 to 0.5 M) at a flow rate of 0.3 ml/min as follows. A linear gradient with 10 ml of ammonium sulfate (0.5 to 0 M) eluted the protein containing 3HAP mutase activity at 0.2 M ammonium sulfate. The butyl group of the resin (Sigma, Deisenhofen, Germany) was preequilibrated with buffer containing 1 M ammonium sulfate. Then a gradient with 37.5 ml of ammonium sulfate (0.5 to 0 M) eluted the 3HAP mutase activity at 0.2 M ammonium sulfate. Fractions (3 ml) containing mutase were tested for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (27) with a 10% ready-to-use polyacrylamide gel (Bio-Rad, Munich, Germany). Proteins were made visible by silver staining (Silver Stain Plus kit; Bio-Rad). For extended storage, aliquots of purified mutase were kept frozen at -70°C. The protein contents of lysates and enzyme fractions were determined by the method of Bradford (9).

**Molecular weight determination.** The mass of subunits was determined by SDS-PAGE (see above). Protein molecular weight standards for SDS-PAGE were purchased from Pharmacia. Proteins were stained as described above. The number of subunits forming the native 3HAP mutase was determined by ultrafiltration with a Centriprep-100 filter unit (Amicon, Witten, Germany), which excludes proteins with a molecular mass of >100 kDa from passing through the membrane. The filter unit loaded with a sample of 3HAP mutase was centrifuged at 500 × g and 4°C for 45 min.

**Determination of 3HAP mutase activity.** The activity of the 3HAP mutase was estimated spectrophotometrically by measuring the increase of the absorption at 300 nm concomitant with the accumulation of aminohydroquinone. 3HAP does not absorb at this wavelength. The standard assay mixture contained 0.4 mM 3HAP in 100 mM phosphate buffer (pH 7). Since both the product and substrate of the enzymatic reaction were autooxidizable, the phosphate buffer was made anaerobic by sparging with argon. Spontaneous 3HAP decomposition led to slow formation of products absorbing at 300 nm, but aminohydroquinone was not found. The rate of spontaneous decomposition was subtracted from the rate of the enzyme-catalyzed reaction. Some assay mixtures included 1 mM 3HAP and 2 mM diethanol (DTE) to stabilize 3HAP. The stoichiometry of the reaction was estimated by relating the decrease of 3HAP to the increase in the absorption at 300 nm due to accumulation of aminohydroquinone. Various concentrations of 3HAP were used in the standard mutase assay (see above), and after the reaction was complete (<2 min), samples of the reaction mixtures were analyzed by high-pressure liquid chromatography (HPLC). The increase in the absorption at 300 nm was proportional to the concentrations of aminohydroquinone formed and 3HAP consumed. Aminohydroquinone was the only product of the reaction detectable by HPLC. The increase of 0.48 absorption unit at 300 nm corresponded to the formation of 0.1 mM aminohydroquinone. One unit of enzyme activity was defined as the production of 1 μmol of aminohydroquinone per min.

**Inactivation-activation experiments.** For testing the activity of 3HAP mutase with potential effectors, portions of 3HAP mutase were preincubated with different reagents at 1 mM (unless stated otherwise) for at least 1 h. Some reagents were added directly to the assay buffer (final concentration, 1 mM). Standard assays with 2 mM DTT and 16.5 μg of 3HAP mutase (3.8 U/mg) were performed as described above. Instead of purified enzyme, some of the measurements were performed with cell extract (0.12 mg of protein; 0.52 U/mg). The activity without the addition of effectors was set to 100%.

**Kinetics and ρH determination.** The optimum ρH for enzyme activity was determined by using the standard assay with 100 mM phosphate buffer in the range from ρH 5.5 to 8 and 200 mM succinate buffer in the range from ρH 4 to 6.

The ρHmax and Km were measured spectrophotometrically as described above, with 0.05, 0.1, 0.25, 0.5, and 1 mM 3HAP and 2 mM DTT in 100 mM phosphate buffer (pH 7). For this purpose, 20 mM 3HAP was freshly prepared as described below and diluted appropriately in aqueous HCl (1:100 [vol/vol]), which stabilized 3HAP. The dilutions were stored on ice, and no spontaneous Bamberger rearrangement was observed under these conditions. The apparent ρHmax and Kν were estimated by linear regression in a Lineweaver-Burk plot (5).
The pl of 3HAP mutase was determined by chromatofocusing. A partially purified sample of mutase was applied to a Mono P HR 5/20 column (Pharmacia) after it was equilibrated with 25 mM bis-Tris HCl buffer (pH 6.3). Then the column was changed to Polybuffer 74 (1.10 mM Tris-HCl buffer [pH 3.5]). Protein was eluted by the pH gradient (pH 6.1 to 3.8, measured at room temperature) in 36 ml at a flow rate of 1 ml/min.

Turnover experiments. Turnover experiments of nitro and hydroxylamino amines in the absence of oxygen were conducted as described previously (43). Conversion of 1 mM 3HAP by 0.6 U (11.4 μg) of 3HAP mutase was performed in 100 mM phosphate buffer (pH 7). After an appropriate incubation period, samples were treated with concentrated HCl (approximately 1:50 [vol/vol]) to stop the enzymatic reaction and to stabilize the substrate and product. Samples were stored on ice until analyzed. 3HAP was stable under these conditions. Hydroxylaminobenzene (1 mM) conversion was carried out in the same way, except that more 3HAP mutase (3.8 U [157 μg]) was added. The concentrations of substrate and product were measured as described previously (43).

Anaerobic conversion of 4-nitrothrene was carried out with an extract from 3NP-grown cells (2.8 mg of protein) in 20 ml of 50 mM phosphate buffer (pH 7) containing 2 mM NADPH and 0.5 mM nitroaromatic compound. The substrate and products were detected by HPLC (43) with 50% methanol and 50% water, each containing hexane sulfonate (Pic B6; Waters) at the solvent. The same conditions were used to convert 4-nitrosoate, except that 2.5 mg of cell extract protein was added to the medium. Conversion was monitored using an HPLC gradient method as described previously (43), but 0.34% (vol/vol) phosphoric acid instead of hexane sulfonate was added to the solvents.

Deamination of amines. Amino acid sequences. A partial tryptic digestion of the purified enzyme was carried out by the method of Stone and Williams (52). The peptides were separated on a Smart system (Pharmacia) with a reversed-phase Hypersil 5 μ OD5 column (50 by 2.1 mm). The liquid phase consisted of solvent A (H2O, 0.1% trifluoroacetic acid) and solvent B (70% acetonitrile, 30% H2O, 0.1% trifluoroacetic acid). The peptides were eluted by a gradient which started from 100% solvent A and changed linearly over 10 min to 25% solvent A–75% solvent B and then changed over 2 min to 100% solvent B at a flow rate of 0.3 ml/min. For NH2-terminal amino acid sequencing of undigested protein and of isolated peptides, the preparations were directly spotted onto a BioPrene membrane (ABI, Foster City, Calif.) and sequenced with a model 473A protein-sequencing system (ABI). The sequences were compared with those in the nonredundant GenBank CDS translations-PDB-SwissProt-Supdata-PIR database (as of 15 August 1998) by using the BlastP program (2).

Chemical Bamberger reaction of 3HAP. Rearrangement of phenylhydroxylamine to aminophenol was observed (15 mg) was added. The solutions (40). To find which products are formed from 3HAP by the chemical reaction, a solution of 3HAP (18 μl of approximately 36 mM 3HAP) was synthesized under argon (43) and treated with concentrated HCl (96%) to add the aqueous solution. The reaction mixture was placed in a serum bottle closed with a gas-tight rubber septum. Then the reaction was evacuated and flushed with argon before an overpressure of 0.5 × 105 Pa was set with argon. The bottle was incubated overnight in a shaking water bath at 30°C. The next day, samples were analyzed by HPLC (43) with 5% methanol and 95% water each containing hexane sulfonate as the solvent.

Chemicals. 3HAP was synthesized as described previously (43), except that the 3HAP concentration in the reaction mixture was 20 or 50 mM. Frozen stock solutions, which were acidified with HCl (1:100 [vol/vol]), could be stored for approximately 4 weeks at 70°C under an argon atmosphere, and thawed solutions were used for longer than 2 h. Hydroxylaminobenzene was provided by Shirley Nishino (Tyndall Air Force Base, Fla.), and 4-aminomethylcatechol was provided by Andreas Stolz (Universität Stuttgart, Stuttgart, Germany).

RESULTS

Purification and molecular mass of 3HAP mutase. Table 1 summarizes how the 3HAP mutase was purified to homogeneity. The specific activity increased only 12-fold, either because of inactivation during the course of purification or because the enzyme may already make up a substantial part of the protein in the cell extract. On the assumption that no inactivation occurred, the mutase could have made up more than 8% of the cell protein. As shown in Fig. 2, lane 5, the SDS-PAGE gel exhibited a single band demonstrating that the enzyme consists of a single type of subunit with a molecular mass of 62 kDa. Attempts to determine the molecular mass of the native enzyme by gel filtration chromatography failed, although different resins and buffer systems were tested. The results were not reproducible, and the activity eluted mostly in several overlapping protein bands. Gel electrophoresis under nondenaturing conditions revealed inconsistent results concerning the size of the native enzyme. However, ultrafiltration of the purified enzyme showed that it passed through a membrane that had an exclusion limit of 100 kDa. This indicates, together with the result from SDS-PAGE, that the native 3HAP mutase consists of a single 62-kDa polypeptide. Gel electrophoresis under non-denaturing conditions gave inconsistent results concerning the size of the native enzyme.

Amino acid sequences. Amino acid sequences of the NH2-terminal of 3HAP mutase and of three internal peptides obtained by trypic digestion of the enzyme were determined. The NH2-terminal and those from 10 different glutamine synthetases (glutamate-ammonia ligase; EC 6.3.1.2). Figure 3 shows an alignment of sequences from 3HAP mutase and those from 10 different glutamine synthetases.

Characteristics of the enzyme. The purified 3HAP mutase stored on ice retained 87% of its initial activity after 14 days and 63% after 38 days. After 30 days of storage at −70°C, the enzyme lost 72% of its activity. Since the enzyme was remarkably stable, no additional attempts at optimizing the storage conditions were made. Heating of cell extract to 60°C for 1 min abolished 72% of the original mutase activity, and no activity remained after 8 min.

TABLE 1. Purification of the 3HAP mutase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total amount of protein (μg)</th>
<th>Total activity (U)</th>
<th>Sp act (U/μg)</th>
<th>Yield (%)</th>
</tr>
</thead>
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<td>Cell extract</td>
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<td>452</td>
<td>0.41</td>
<td>100</td>
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<tr>
<td>Lysate after centrifugation</td>
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<td>420</td>
<td>0.44</td>
<td>92</td>
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<td>259</td>
<td>1.6</td>
<td>57</td>
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<tr>
<td>Butyl agarose chromatography</td>
<td>11.4</td>
<td>54</td>
<td>4.7</td>
<td>12</td>
</tr>
</tbody>
</table>

* One unit is defined as the production of 1 μmol of aminohydroquinone per min.

FIG. 2. SDS-PAGE analysis during purification of the 3HAP mutase from R. eutropha JMP134. Lanes 3 and 8 contained the following molecular mass standards (from top to bottom): bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20 kDa). All other lanes contained 0.5 μg of protein from the respective preparations of the mutase. Lane 1, cell extract after ultracentrifugation; lane 2, pooled fractions from DEAE anion-exchange chromatography; lanes 4, 5, and 7, pooled fractions from butyl agarose hydrophobic interaction chromatography; each containing 3HAP mutase with minor contaminations; lane 5, pooled fraction from butyl agarose hydrophobic interaction chromatography containing purified 3HAP mutase.
fore, the presence of flavins or other visible-light-absorbing prosthetic groups attached to the enzyme could be excluded. The isoelectric point of the protein was 4.5 as shown by chromato-focusing. The pH optimum of the purified enzyme was determined to be 6.5. The temperature optimum could not be determined, since the substrate was too unstable at temperatures higher than 30°C. However, the highest transformation rate was measured at 30°C.

Kinetics and inhibition by decomposition products of 3HAP. 3HAP mutase had a \( V_{\text{max}} \) of approximately 4.8 \( \mu \text{mol} \text{min}^{-1} \text{mg}^{-1} \) and an apparent \( K_{\text{m}} \) of approximately 0.1 mM. Experiments to determine the exact kinetic data of the enzyme failed because 3HAP stock solutions rapidly developed colored decomposition products. Depending on the decomposition state, Lineweaver-Burk plots showed parallel curves, indicating that there was not a competitive effect. Under optimized conditions, however, the measured rates were reproducible. Inhibition could be partly reversed by the addition of 2 mM DTT and/or 10 mM hydroxylamine to the assay buffer. This indicated that spontaneous autoxidation products of 3HAP, e.g., 3-nitrosophenol, could have been formed and interfered with 3HAP mutase. Hydroxylamine reacts rapidly with nitrosaromatic compounds to form benzenediazonium salts (17), whereas DTT would reduce the nitroso group. Additionally, azoxy compounds could have been formed from 3HAP and nitrosophenol (24). However, it could not be clarified which autoxidation products present in 3HAP stock solutions interacted with the enzyme.

Influence of possible activators or inhibitors on the enzyme. To obtain information about the mechanism of the enzymatic reaction, the influence of reagents having a possible effect on the 3HAP mutase was investigated. 1,10-Phenanthroline inhibited the activity slightly (25%). Due to the relationship of the protein with glutamate synthetases, glutamate and glutamine were tested as inhibitors. Neither preincubation of the compounds with the enzyme nor their addition to the assay buffer affected 3HAP mutase activity.

3HAP conversion by the purified enzyme and its substrate specificity. Preliminary studies (43) indicated that cell extracts from induced cells of *E. coli* JMP134 formed aminohydroquinone from 3HAP during 3NP degradation. It was not clear whether a single enzyme was responsible for the complex conversion of 3HAP to aminohydroquinone. A reduction of 3HAP to 3-aminophenol and subsequent hydroxylation of 3-aminophenol by a monooxygenase could be an alternative mechanism for the formation of aminohydroquinone. Therefore, the enzyme was incubated with 3HAP under anaerobic conditions (Fig. 4). The fast decrease in the concentration of 3HAP was concomitant with the production of aminohydroquinone. 3HAP mutase required neither a cofactor nor oxygen for the enzymatic reaction. This indicated that the enzyme catalyzes a Bamberger-type rearrangement. Three isomeric dihydroxylanilines (aminohydroquinone, 4-aminocatechol, and 3-aminocatechol) can theoretically be formed from 3HAP based on the mechanism of a Bamberger rearrangement (Fig. 1). The chemical reaction of 3HAP in dilute sulfuric acid formed 4-aminocatechol, which could be clearly identified by comparison of the UV spectrum and the chromatographic patterns with those of an authentic standard. The yield of 4-aminocatechol was low (approximately 10%), and the reaction mixture adopted a deep black coloration. Because no products other than 4-aminocatechol were detected in relevant amounts by HPLC analysis, formation of aminohydroquinone and 3-aminocatechol from 3HAP by the nonenzymatic reaction seems unlikely. The conversion of 3HAP by 3HAP mutase yielded exclusively aminohydroquinone instead of aminocatechol, which clearly supported the enzymatic nature of the reaction.

Extracts from induced cells of *R. eutropha* JMP134 converted hydroxylaminobenzene to a mixture of 2- and 4-aminophenol (43). The purified enzyme gave similar results (data not shown).

![FIG. 3. Comparison of amino acid sequences from 3HAP mutase and from different bacterial glutamine synthetases. Amino acid residues from glutamine synthetases that are identical to those of 3HAP mutase (*R. eutropha* 3HAP mutase) are shown in boldface. A dash means that no corresponding amino acid sequence was found. X means an unidentified amino acid.](image-url)
solution, which suggested that the metabolite was 4-hydroxy- 
aminobenzoate (not available as authentic compound). The 
accumulation of 4-nitrobenzoate could be excluded, since it 
would have been spontaneously reduced to 4-hydroxyama-

Bacteriol.

The reaction rate with hydroxylaminobenzene was 44-
fold lower than that with 3HAP. Additionally, extracts of R. 
eutropha JMP134 containing 3NP nitroreductase and 3HAP 
mutase in the presence of excess NADPH converted 4-nitro-
toluene anaerobically to two metabolites. One was identified as 
6-amino-m-cresol by comparing its chromatographic patterns 
and its UV spectrum with those of an authentic standard. The 
second metabolite could be reduced to 4-aminotoluene by 
treatment with zinc in HCl solution. The results indicated that 
the reaction involved partial reduction of 4-nitrotoluene to 
4-hydroxylaminotoluene, which then underwent rearrange-
ment to 6-amino-m-cresol by the cell extract containing 3HAP 
mutase.

In contrast, the transformation of 4-nitrobenzene under the 
same conditions did not lead to production of 3-hydroxy-4- 
aminobenzoate, although 4-nitrobenzene was completely con-
verted. The only metabolite which accumulated could be re-
duced to 4-aminobenzoate by treatment with zinc in HCl 
solution, which suggested that the metabolite was 4-hydroxy-
aminobenzoate (not available as authentic compound). The 
accumulation of 4-nitrobenzoate could be excluded, since it 
would have been spontaneously reduced to 4-hydroxyamino-
benzoate by NADPH (4, 30).

In Fig. 5, the above-described reactions catalyzed by 3HAP 
mutase are summarized. The conversion of 2-chloro-5-hy-
droxyaminophenol to 2-amino-5-chlorohydroquinone has been 
reported previously (44). Here, the reaction rate was 1.6-fold 
higher than that with 3HAP.

**DISCUSSION**

Purification and characterization of 3HAP mutase revealed 
clearly that the conversion of 3HAP to aminohydroquinone is 
catalyzed by a single 62-kDa enzyme. Since oxygen or cofactors 
were not required, it was confirmed that the enzymatic trans-
formation corresponds to the acid-catalyzed Bamberger rear-

P. pseudoalcalig-

COOH

\[ \text{3-Hydroxylaminophenol} \rightarrow \text{2-Chloro-5-hydroxylaminophenol} \]

\[ \text{Hydroxylaminebenzene} \rightarrow \text{4-Hydroxyaminotoluene} \rightarrow \text{4-Hydroxyaminobenzoic acid} \]

\[ \text{Aminohydroquinone} \rightarrow \text{2-Amino-5-chloro-hydroquinone} \rightarrow \text{2-Aminophenol} \rightarrow \text{6-Amino-m-cresol} \]

FIG. 4. Conversion of 3HAP (■) to aminohydroquinone (□) by 3HAP mutase from R. eutropha JMP134. The enzyme (0.6 U) and 3HAP (1 mM) were 
icubated in 100 mM phosphate buffer (pH 7) under an argon atmosphere at 30°C. The conversion was analyzed by HPLC.

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\[ \text{Aminohydroquinone} \rightarrow \text{2-Amino-5-chloro-hydroquinone} \rightarrow \text{2-Aminophenol} \rightarrow \text{6-Amino-m-cresol} \]

FIG. 5. Conversion of hydroxylaminoaromatic compounds by 3HAP mutase from R. eutropha JMP134.
concomitant release of ammonia by a lyase reaction. The same reaction has been described by Groenewegen and de Bont (19) for 4-nitrobenzoate degradation by *Comamonas acidovorans* NBA-10. The corresponding enzyme was partially purified and had a narrow substrate specificity (34). Although the mechanism of the reaction was unknown, a mechanism similar to that of a Bamberger rearrangement was discussed (34). 4-Hydroxylaminobenzoate lyase had a molecular mass of 45 kDa, and reducing agents were required to restore activity. The different chemoselectivity, together with the failure of 3HP mutase to attack 4-hydroxybenzoate, clearly demonstrates that the 4-hydroxylaminobenzoate lyase from *C. acidovorans* NBA-10 and the 3HP mutase from *R. eutropha* JMP134 are different enzymes. In contrast to our results with *R. eutropha* JMP134, *Pseudomonas putida* B2 also degrades 3NP via 3HP (35), but a subsequent lyase reaction converts 3HP to 1,2,4-trihydroxybenzene and ammonia. In contrast, 3NP-grown cells of *R. eutropha* JMP134 release ammonia only when oxygen is present (43).

Inhibition studies performed with 3HP mutase did not elucidate the enzymatic reaction mechanism. Inhibition of the enzyme was observed by finding that 3HP stock solutions contained impurities resulting from spontaneous and rapid decomposition of the compound in the presence of oxygen, but the inhibitory compound(s) could not be identified. Inactivation of 3HP mutase by high concentrations of H₂O₂ indicated the presence of structurally important sulfhydryl groups in the enzyme. Inhibition by cysteine, which was dependent on the preincubation time, cannot be explained, since other reducing agents did not affect the enzyme. All other compounds tested had no significant effect on the mutase activity.

A striking similarity of the amino acid sequence of the purified enzyme to that of glutamine synthetases exists. Glutamine synthetase catalyzes an ATP-dependent amidation of glutamate to glutamine plus ammonia (36). A mechanistic analogy between the mutase and the amidase reaction is difficult to identify, particularly since neither glutamate nor glutamine inhibited the 3HP mutase, so that a potential phylogenetic relationship among these enzymes would require a complete analysis of the amino acid sequence of the 3HP mutase.

Arylhydroxylamines are highly reactive and hence cytotoxic, mutagenic, and carcinogetic. Interestingly, the formation of mutagenic agents from hydroxylaminocarbanions plays an important role in these effects, because the reactive cations and radicals are also susceptible to nucleophilic attack by nucleic acid bases and other nucleophiles (7, 8, 38). Enzymes that convert arylhydroxylamines to harmless products are useful detoxification tools and are essential to survival for a biological system. In the case of bacterial metabolism of nitroaromatics, such enzymes allow the conversion of a highly reactive intermediate to compounds that can serve as growth substrates. Several recent reports indicate that enzymes catalyzing Bamberger-type rearrangements can play key roles in the bacterial metabolism of nitroaromatics via the highly reactive hydroxyaminodiazonium and arylnitronium intermediates (22, 37, 43, 44, 50). 3HP mutase from *R. eutropha* JMP134 is the first of these enzymes to be purified and characterized. The reported characteristics of the enzyme may be useful for comparison with enzymes from other organisms which catalyze analogous reactions.

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**REFERENCES**


21. Hughes, J. Personal communication.


