A Novel 2-Aminomuconate Deaminase in the Nitrobenzene Degradation Pathway of *Pseudomonas pseudoalcaligenes* JS45

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2-Aminomuconate, an intermediate in the metabolism of tryptophan in mammals, is also an intermediate in the biodegradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* JS45. Strain JS45 hydrolyzes 2-aminomuconate to 4-oxalocrotonic acid, with the release of ammonia, which serves as the nitrogen source for the microorganism. As an initial step in studying the novel deamination mechanism, we report here the purification and some properties of 2-aminomuconate deaminase. The purified enzyme migrates as a single band with a molecular mass of 16.6 kDa in 15% polyacrylamide gel electrophoresis under denaturing conditions. The estimated molecular mass of the native enzyme was 100 kDa by gel filtration and 4 to 20% gradient nondenaturing polyacrylamide gel electrophoresis, suggesting that the enzyme consists of six identical subunits. The enzyme was stable at room temperature and exhibited optimal activity at pH 6.6. The *Km* for 2-aminomuconate was approximately 67 μM, and the *Vmax* was 125 μmol·min⁻¹·mg⁻¹. The N-terminal amino acid sequence of the enzyme did not show any significant similarity to any sequence in the databases. The purified enzyme converted 2-aminomuconate directly to 4-oxalocrotonate, rather than 2-hydroxymuconate, which suggests that the deamination was carried out via an imine intermediate.

**MATERIALS AND METHODS**

**Growth of bacteria.** *P. pseudoalcaligenes* JS45 was maintained and grown with nitrobenzene (12). Cells were harvested by centrifugation and washed with 25 mM potassium phosphate (pH 7.0), and the cell pellets were stored at −70°C until use.

**Protein purification.** All purification procedures were carried out at 4°C in 25 mM potassium phosphate buffer (pH 7.0). Cells (9.5 g [wet weight]) were suspended in 50 ml of buffer and were broken by two passages through a French pressure cell at 135,000 kPa. The resulting suspension was centrifuged at 100,000 × g for 60 min, and the pellet was discarded. The supernatant (crude extract) was stored at −70°C until use.

Half of the crude extract (25 ml) was thawed and diluted to 200 ml with phosphate buffer and loaded onto a DEAE-Sepharose column (Pharmacia; 2.6 by 10 cm). The column was washed with 200 ml of buffer, and proteins were eluted with a linear NaCl gradient (0 to 0.4 M in buffer; 400 ml at 2 ml/min). The fractions (6 ml each) containing 2-aminophenol 1,6-dioxygenase or 2-aminomuconate semialdehyde dehydrogenase activities were used to prepare 2-aminomuconate deaminase activity. The fractions (3 ml each) containing 2-aminomuconate deaminase activity were pooled and loaded onto a Hitrap Cu(II)-chelating column (Pharmacia; 2 by 5 ml). The column was washed with 30 ml of 0.5 M NaCl in buffer, and proteins were eluted with a linear EDTA gradient (0 to 50 mM in buffer; 400 ml at 2 ml/min). The fractions (6 ml each) containing either 2-aminophenol 1,6-dioxygenase or 2-aminomuconate semialdehyde dehydrogenase activities were used to prepare 2-aminomuconate (see below). The fractions containing 2-aminomuconate deaminase activity were pooled and loaded onto a Hitrap Cu(II)-chelating column (Pharmacia; 2 by 5 ml). The column was washed with 30 ml of 0.5 M NaCl in buffer, and proteins were eluted with a linear NaCl gradient (0 to 50 mM in buffer; 60 ml at 1 ml/min). The fractions (3 ml each) containing 2-aminomuconate deaminase activity were pooled and concentrated in a Centriprep-10 tube (Amicon, Beverly, Mass.) to a final volume of 2.4 ml. The concentrated preparation was applied to a Sepharose S-300 gel filtration column (Pharmacia; 1.6 by 100 cm) and eluted with phosphate buffer (1 ml/min). The active fractions (2 ml each) were pooled, the protein was loaded onto a Hitrap-Q column (Pharmacia; 5 ml). The column was washed with 40 ml of buffer, and proteins were eluted with an NaCl gradient (0 to 0.25 M NaCl in buffer; 60 ml at 1 ml/min). The active fractions (2 ml each) were pooled, the protein was loaded onto a Hitrap-Q column (Pharmacia; 5 ml). The column was washed with 40 ml of buffer, and proteins were eluted with a linear NaCl gradient (0.25 to 0.5 M NaCl in buffer; 150 ml at 1 ml/min). The active fractions (3 ml each) were pooled and concentrated in a Centriprep-10 tube and used for characterization studies.

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Enzyme assays. 2-Aminomuconate deaminase activity was determined spectrophotometrically by monitoring the decrease in absorbance at 326 nm concomitant with the disappearance of 2-aminomuconate (ε = 16,500 M⁻¹ cm⁻¹) (7, 8, 13). The reaction was started by the addition of the enzyme preparation (5 to 10 µl) to 2-aminomuconate solution (0.03 mM, 750 µl) in potassium phosphate buffer (25 mM, pH 8.0) containing 0.12 M NaCl, unless stated otherwise. The initial rate of deamination (less than 1 min) was recorded. In inhibition experiments, the enzyme was incubated with various additives for 10 min in potassium phosphate buffer (200 mM, pH 7.0) prior to the addition of 2-aminomuconate solution. Tris-HCl buffer (100 mM, pH 7.0) was used in testing the effects of metal ions on the enzyme activity. Specific activities are expressed as micromoles of substrate transformed per minute per milligram of protein. For experiments to determine substrate specificity, the activity was measured by determining the release of ammonia with test kit 171-C from Sigma (St. Louis, Mo.) in order to measure the oxidation of NADPH in the presence of 2-ketogluurate and glutamate dehydrogenase. The appearance of an absorbance peak at 375 nm (2-hydroxymuconic semialdehyde) was used to determine the deamination of 2-aminomuconic semialdehyde.

The purity and the molecular mass of 2-aminomuconate deaminase were examined by native-gradient (4 to 20%) polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE (4). The molecular mass of the enzyme was determined by comparison with protein molecular mass standards. The molecular mass standards used in SDS-PAGE were bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa), bovine milk β-lactalbumin (14.2 kDa). The molecular mass standards used in native-gradient PAGE were jack bean urease (hexamer, 545 kDa; trimer, 272 kDa), and bovine serum albumin (dimer, 132 kDa; monomer, 66 kDa). The native molecular mass was also measured by gel filtration on a Sephacryl S-300 column (Pharmacia; 1.6 × 60 cm) with a flow rate of 1 ml of 25 mM potassium phosphate–0.1 M NaCl, phosphorylase a (94 kDa), yeast alcohol dehydrogenase (150 kDa), and bovine serum albumin (66 kDa).

N-terminal sequence. Subunits of 2-aminomuconate deaminase were obtained on an SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Trans-Blot; Bio-Rad). The blotted membrane was stained with Coomassie blue R-250. The N-terminal amino acid sequence was determined by the Protein Core Facility of the University of Florida, Gainesville.

Preparation of 2-aminomuconate. 2-Aminomuconone was prepared as described previously (7), but pooled fractions containing partially purified 2-aminomuconate 1,6-dioxynogense and 2-aminomuconate semialdehyde dehydrogenase from the DEAE-Sepharose chromatography were used instead of crude extracts. The isolation column used was Hitrap-Q (2 by 5 ml). Generally, 2-aminomuconone was prepared daily. If the preparation of 2-aminomuconone was frozen at −70°C, the absorbance at 326 nm decreased about 50% after thawing. The compound was more stable under alkaline conditions (pH 13).

Chemicals. 2-Hydroxymuconic acid was prepared by the method of Lapworth (9) from the potassium salt of diethyl 2,4-hexadiene-5-hydroxy-1,6-dioate, which was obtained from condensation of diethyl oxalate and ethyl crotonate in the presence of potassium metal in toluene, as described by Wiley and Hart (23). All other chemicals were from Sigma or Aldrich (Milwaukee, Wis.), unless stated otherwise.

RESULTS

Purification of 2-aminomuconate deaminase. A typical purification (Table 1) yielded a 222-fold purification with a recovery of 36% of the 2-aminomuconate deaminase activity. The preparation of the enzyme is colorless and does not have an absorbance peak above 300 nm.

An analysis of the purified protein by SDS–15% PAGE revealed a single band corresponding to a molecular mass of 16.6 kDa (Fig. 1), which is bigger than that of 4-oxalocrotonate tautomerase (3.6 kDa) (2). Nondenaturing 4 to 20% gradient PAGE showed a single band at 100 kDa. The 2-aminomuconate deaminase activity was found in the band on the gel when the appropriate area of the gel was cut out and transferred to potassium phosphate buffer (100 mM, pH 7.5) prior to staining. Gel filtration chromatography also revealed a native molecular mass of 100 kDa. Therefore, the enzyme is apparently composed of six identical subunits, each with a molecular mass of 16.6 kDa.

Catalytic properties of the enzyme. The 2-aminomuconate deaminase was stable when stored at room temperature for 3 days. However, 77% of the activity was lost when the enzyme was heat treated for 5 min at 60°C. 2-Aminomuconate deaminase exhibited optimal activity at pH 6.6 and was stable for at least 3 h between pH 5.7 and 8.8 at room temperature without significant loss of activity. For 2-aminomuconate, the Kᵣ was approximately 67 µM, the V_max was 125 µmol·min⁻¹·mg⁻¹, and the k_cat was 208 s⁻¹ at pH 6.6 (80 mM potassium phosphate–0.1 M NaCl) and 25°C. No absorbance at 326 nm was observed when ammonia (0.8 mM) and 4-oxalocrotonate (0.14 mM) were incubated with 2-aminomuconate deaminase (0.01 mg/ml) in 50 mM potas-

![Figure 1](http://jb.asm.org/Downloaded from http://jb.asm.org/ on June 20, 2017 by guest)

**FIG. 1.** SDS–15% PAGE of 2-aminomuconate deaminase. Purified 2-aminomuconate deaminase (lane 2; 2 µg) was compared with the crude extract (lane 7; 40 µg), the DEAE fraction (lane 6; 20 µg), the Cu(II)-chelating fraction (lane 5; 20 µg), the gel filtration fraction (lane 4; 20 µg), the Hitrap-Q fraction (lane 3; 7 µg), and protein molecular mass standards (lane 1).
sium phosphate (pH 7.0) for 1 h. Therefore, the deamination reaction appears to be irreversible under the assay conditions.

Substrate specificity and inhibition. 2-Aminomuconate deaminase did not act on 2-aminomuconic semialdehyde, the precursor of 2-aminomuconate. It did not deaminate saturated α-amino acids, including glycine, alanine, aspartic acid, glutaric acid, and 2-aminoadipic acid (a saturated analog of 2-aminomuconate). On the other hand, the deamination of 2-aminomuconate was not inhibited by the presence of these amino acids (2 mM). The enzyme activity was not affected by changes in potassium phosphate buffer concentration from 12 to 260 mM. EDTA (25 mM) did not inhibit the enzyme activity, which indicated that divalent cations are not required for the enzyme activity. MgSO₄ (2 mM) did not affect the enzyme activity. Both CuSO₄ and MnCl₂ inhibited the activity by 20%; ZnCl₂ inhibited it by 70%. Phenylhydrazine (10 mM) decreased the activity to 20% of the activity with no inhibitors. Diethyl pyrocarbonate (2 mM) completely destroyed the activity, which indicated that a histidine residue may be involved in the catalytic mechanism.

Although the crude extracts of JS45 catalyzed the tautomeration of 2-hydroxymuconate to 4-oxalocrotonate (the half-life of 2-hydroxymuconate decreased from 7 min in spontaneous tautomeration to 2 min in tautomeration catalyzed by crude extracts of JS45 [0.1 mg of protein/ml]), the purified deaminase did not change the rate of spontaneous tautomeration. This result indicated that 2-aminomuconate deaminase is distinct from 4-oxalocrotonate tautomerase.

**True products of deamination.** 2-Hydroxymuconate and 4-oxalocrotonate are spectrally distinguishable. 2-Hydroxymuconate exhibits maximum absorbance at 296 nm, and 4-oxalocrotonate exhibits maximum absorbance at 237 nm (6, 15, 22). 2-Hydroxymuconate spontaneously converts to 4-oxalocrotonate in an aqueous solution, but the process is slow (about a 7-min half-life under the experimental conditions) (Fig. 2A). When 2-aminomuconate was deaminated by excess purified 2-aminomuconate deaminase, the absorbance at 326 nm decreased from 0.35 to about 0.1 in 5 s, concomitant with an increase in absorbance at 237 nm and with no increase in absorbance at 296 nm (Fig. 2B). These results clearly indicate that the true product of enzymatic deamination of 2-aminomuconate is 4-oxalocrotonate rather than 2-hydroxymuconate.

**N-terminal sequence.** The N-terminal amino acid sequence for the first 25 residues of the subunit was STLSS NDAKV VDGKA TPLGS FPHVK. A search of the sequence databases (nonredundant GenBank CDS translations, PDB, SwissProt, Spupdate, and PIR) through the National Center for Biotechnology Information with BLAST software revealed no significant similarity between the sequence of 2-aminomuconate deaminase and any other known amino acid sequence.

**DISCUSSION**

The novel aminohydrolytic enzyme 2-aminomuconate deaminase from *P. pseudoalcaligenes* JS45 is the first purified deaminase found which acts on an unsaturated linear amino acid. A similar enzymatic deamination of trans-4-amino-6-carboxy-2-oxo-hexa-3,5-dienoate was reported for bacterial metabolism of 5-aminosalicylic acid (17), but the purification and the properties of the enzyme were not reported.

2-Aminomuconate deaminase acted specifically on the unsaturated α-amino acid 2-aminomuconate. The fact that the enzyme did not act on saturated α-amino acids and 2-aminomuconic 6-semialdehyde indicated that the double bonds and the distal carboxylate group are essential for enzyme activity. The absence of absorbance above 300 nm suggests that the enzyme does not contain a pyridoxal 5'-phosphate group, which would be characteristic of a classical aminotransferase.

On the basis of the general catalytic mechanism, the hydrolysis of 2-aminomuconate to 2-hydroxymuconate (Fig. 3A) and the deamination of 2-aminomuconate to 4-oxalocrotonate (Fig. 3B) can be considered as typical aminohydrolytic reactions.
lytic deamination of 2-aminomuconate could be base or acid catalyzed (Fig. 3). The fact that 2-aminomuconate hydrolizes spontaneously to 4-oxalocrotonate at low pH suggests that the nonenzymatic reaction occurs by the acid-catalyzed mechanism. The two different direct products of hydrolis of 2-aminomuconate are spectrally distinguishable. Our result (Fig. 2) clearly indicated that the direct product of enzymatic deamination is 4-oxalocrotonate, which provides strong evidence for the acid-catalyzed mechanism. In this mechanism, an active imine intermediate is formed and hydrolysis of the imine produces 4-oxalocrotonate. Imine bond formation (Schiff base) is a common mechanism of deamination or transamination (20). The imine can be formed by oxidation, as in the deamination of D-amino acids by D-amino acid oxidase in the presence of flavin NAD or NADP (16) and as in the deamination of various D-amino acids by a D-amino acid oxidase in the presence of flavin adenine dinucleotide (19). The imine bond can also be formed with the help of pyridoxal 5'-phosphate, as for 1-aminocyclopropane 1-carboxylate deaminase (10) and as in the transamination catalyzed by aminotransferases (20). Aminoacylate, an unsaturated α-amino acid, tautomizes nonenzymatically to its imine form, which hydrolizes spontaneously to pyruvate and ammonia (20). 2-Aminomuconate contains the conjugated double bonds which enable spontaneous tautomerization to the imine form, and it hydrolizes to release ammonia as aminoacylate does; however, the nonenzymatic process is slow. In the enzyme-catalyzed reaction, a proton donor could initiate and facilitate the tautomerization. The mechanism of tautomerization would be analogous to the conversion of 2-hydroxymuconic semialdehyde to 4-oxalocrotonate, which is catalyzed by 4-oxalocrotonate tautomerase (11, 14, 18, 22). The evidence for the involvement of a histidine in the active site of 2-aminomuconate deaminase would be consistent with a mechanism involving donation of a proton by the enzyme. The tautomerization of 2-aminomuconate to form an imine intermediate would provide an explanation for why a cofactor is not required for the 2-aminomuconate deaminase activity.

The direct conversion of 2-aminomuconate to 4-oxalocrotonate by 2-aminomuconate deaminase without the formation of the enol intermediate of 2-hydroxymuconic semialdehyde is catalyzed by 4-oxalocrotonate tautomerase (11, 14, 18, 22). The evidence for the involvement of a histidine in the active site of 2-aminomuconate deaminase would be consistent with a mechanism involving donation of a proton by the enzyme. The tautomerization of 2-aminomuconate to form an imine intermediate would provide an explanation for why a cofactor is not required for the 2-aminomuconate deaminase activity.

FIG. 4. Comparison of the meta cleavage pathway of catechol (A) with the pathway for degradation of 2-aminophenol by JS45 (B).


