Purification and Characterization of Two Muconate Cycloisomerase Isozymes from Aniline-assimilating Frateuria Species ANA-18

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Two muconate cycloisomerases (MC I and MC II, EC 5.5.1.1) were purified to homogeneity from an aniline-grown Frateuria sp. ANA-18. MC I and MC II were similar in molecular mass, optimal pH, and pH stability but different in thermostability, and some other enzymatic properties. NH2-terminal amino acid sequences were different between the two isozymes, indicated that these are encoded by different genes. Different inducible production of MC I and MC II suggested that two catechol branches involved in the β-ketoadipate pathway function in Frateuria sp. ANA-18.

Key words: Frateuria; β-ketoadipate pathway; muconate cycloisomerase; catechol 1,2-dioxygenase; aniline degradation

A β-ketoadipate pathway is one of the main routes for metabolism of aromatic compounds by microorganisms.1-6 This pathway contains a few branches such as catechol or chlorocatechol branches (Fig. 1).5-8 The catechol branch produces cis,cis-muconate from catechol by catechol 1,2-dioxygenase (catechol 1,2-dioxygenase I, EC 3.1.11.1) in the first step and subsequently 4-carboxymethylbut-2-en-4-olide (muconolactone) by muconate cycloisomerase (muconate lactonizing enzyme, EC 5.5.1.1).5,8 On the other hand, chlorocatechol 1,2-dioxygenase (catechol 1,2-dioxygenase II, EC 3.1.11.1) and chloromuconate cycloisomerase (chloromuconate lactonizing enzyme, EC 5.5.1.7), constructing the chlorocatechol branch, convert chlorocatechols and chloromuconates to corresponding chloromuconate and chlorodiene-lactone (or dienelactone), respectively.5-8

Catechol 1,2-dioxygenase and chlorocatechol 1,2-dioxygenase have been purified from many bacteria.5,10,12-15 Dorn and Knackmuss have found both enzymes and chloromuconate cycloisomerase from the cell extracts of 3-chlorobenzoate-grown Pseudomonas sp. B13.5 On the other hand, muconate cycloisomerase was found in benzoate-grown cells.5 These observations show that though catechol 1,2-dioxygenase is produced in 3-chlorobenzoate-grown cells, the expression of the catechol branch is independent of the chlorocatechol branch. Both muconate cycloisomerase and chloromuconate cycloisomerase were found in some organisms.5,6 But in any cases, muconate cycloisomerase and chloromuconate cycloisomerase were also produced when bacteria were grown on non-chlorinated and chlorinated aromatic compounds, respectively. It has been reported that Trichosporon cutaneum produces two kinds of muconate cycloisomerase isozymes.6 But this organism produced no catechol 1,2-dioxygenase isozymes.7

In previous studies, we purified two catechol 1,2-dioxygenase isozymes (CD I and CD II) from Frateuria sp. ANA-18,10 which uses aniline as a sole carbon and nitrogen source.10 CD I and CD II are involved in the catechol branch because both enzymes show high activities for catechol only.10 We were interested in the catechol branch, starting from two isozymes, and tried to purify muconate cycloisomerases from this microorganism. In this study, we found two muconate

Fig. 1. Catechol Branch (A) and Chlorocatechol Branch (B) in the β-Ketoadipate Pathway.

The catechol branch that is functional in Frateuria sp. ANA-18 is shown.10 The chlorocatechol branch that is functional in Alcaligenes eutrophus JMP134 is described.11

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Abbreviations: Sm, streptomycin sulfate; PAGE, polyacrylamide gel electrophoresis.
cycloisomerase isozymes, MC I and MC II, and purified them to homogeneity. This paper deals with purification of the enzymes and shows differences in some enzymatic properties. Furthermore, we examined induction of CD I, CD II, MC I, and MC II.

Materials and Methods
Microorganisms and growth conditions. Fratereuria sp. ANA-18 was cultured in 4.5 liters of aniline medium containing 0.3% aniline hydrochloride as a sole carbon and nitrogen source\(^9\) at 30°C with shaking. In place of aniline hydrochloride, 0.93% sodium succinate hydrate and 0.15% ammonium sulfate were used for induction experiments.

Escherichia coli DH5α carrying a plasmid, pK26,\(^9\) with the catechol 1,2-dioxygenase gene of Rhodococcus erythropolis, was cultured in Luria-Bertani medium\(^29\) with ampicillin (200 μg/ml) and 0.5 mM of isopropyl-β-D-thiogalactopyranoside.

Enzymatic assays. A substrate, cis,cis-muconate, was prepared from catechol enzymatically as follows. E. coli cells, suspended in 1.5 ml of 20 mM Tris-HCl (pH 8.0) buffer (buffer A), were disrupted with a Kubota 201 M ultrasonic oscillator (Kubota Shoji, Tokyo) and centrifuged at 10,000 × g for 10 min. The resulting supernatant was used as crude extract. The catechol 1,2-dioxygenase fraction was recovered from the supernatant after addition of streptomycin sulfate (Sm) at a final concentration of 1% and centrifugation. The catechol 1,2-dioxygenase solution (20 μl) was added to 2.78 ml of 33.3 mM Tris-HCl (pH 8.0) containing 0.11 mM catechol, and catechol was converted perfectly into cis,cis-muconate by incubation at 10 min at 24°C. Furthermore, 0.1 ml of 20 mM MnCl\(_2\)·4H\(_2\)O was added into the reaction mixture.

Muconate cycloisomerase activity was measured by the decrease of absorbance at 260 nm after adding enzyme solution (0.1 ml) to the prepared reaction mixture. One unit of enzyme activity was defined as described by Ornston.\(^9\) Protein was measured by the method of Lowry et al.\(^{21}\)

Catechol 1,2-dioxygenase activity was assayed by a modification of the method of Aoki et al.\(^{12}\)

Enzyme purification. The enzyme was purified at zero to 4°C. Stored cells (14.9 g, wet weight) were suspended in 150 ml of buffer A and were disrupted with a Kubota 201 M ultrasonic oscillator at 180 W for 8 min in a 15-ml batch. After centrifugation, the supernatant was used as a cell extract. To the cell extracts, Sm was added at a final concentration of 1% and the supernatant was recovered by centrifugation. The supernatant was fractionated using ammonium sulfate at 30 to 45% saturation. The protein was precipitated by centrifugation and dissolved in 57 ml of buffer A. The solution was dialyzed against 1 liter of buffer A with two changes of the buffer and put on a column (1.6 × 27 cm) of DE52 (Whatman Chem. Sepn., Clifton). Enzymes were eluted with 800 ml of buffer A containing NaCl in a linear gradient from zero to 0.2 M. The collected enzymes were dialyzed and put separately on columns (1.6 × 14 cm) of DEAE-Toyopearl 650S (Toyo Soda MG, Tokyo). Each enzyme was eluted with a linear gradient between zero and 0.2 M NaCl in 400 ml of buffer A and concentrated with a colloid bag (Model 25, Sartorius GmbH, Goettingen), and then put on the top of column (3 × 58 cm) of Toyopearl HW-55 (Toyo Soda MG) superfine. Enzymes were eluted with buffer A containing 0.2 M NaCl. After addition of ammonium sulfate to a final concentration of 15% (weight/weight), enzyme was put on a column (1.6 × 7 cm) of ether-Toyopearl (Toyo Soda MG) and eluted with 260 ml of buffer A containing ammonium sulfate in a linear gradient from 15% (weight/weight) to zero. Purity of enzymes was examined by 7.5% PAGE.\(^{22}\)

Molecular mass measurement. The molecular masses of MC I and MC II were measured by gel filtration on Toyopearl HW-55 superfine calibrated with egg albumin (45 kDa), bovine serum albumin (67 kDa), γ-globulin (158 kDa), catalase (232 kDa), and ferritin (440 kDa) as standard reference proteins. The molecular mass of subunits was measured by SDS-PAGE\(^{23}\) with a LMW electrophoresis calibration kit (Pharmacia Biotech).

NH\(_2\)-terminal amino acid sequencing. SDS-PAGE was done for MC I and MC II (each 4 μg) and proteins were electroblotted onto a Durapore membrane (Millipore, Tokyo) with Mini Trans-Blot (Bio-Rad, Richmond).\(^{20}\) The membrane was stained with Coomasie brilliant blue R-250 and protein bands were cut out. Both enzyme proteins were sequenced with a Shimadzu protein sequencer PPSQ-10 (Shimadzu, Kyoto).

Induction of isozymes and its analysis. Fratereuria sp. ANA-18 cells were suspended in reaction solutions containing 2 mM MgSO\(_4\)·7H\(_2\)O, 20 μM FeSO\(_4\)·7H\(_2\)O, and 10 mM catechol or aniline hydrochloride in 0.1 M potassium-sodium phosphate buffer (pH 5.5) and the turbidity of cell suspensions was adjusted to 8 at 660 nm. In induction by cis,cis-muconate, a reaction solution containing 10 mM cis,cis-muconate converted from catechol by catechol 1,2-dioxygenase in 0.1 M potassium-sodium phosphate buffer (pH 7.5), adjusted to pH 5.5, was used. Fratereuria cells were incubated at 30°C with shaking, harvested by centrifugation, and then disrupted in buffer A. Catechol 1,2-dioxygenase and muconate cycloisomerase activities and protein concentrations were measured in cell extracts recovered by centrifugation. To the cell extracts, Sm was added at a concentration of 1% and supernatants were recovered by centrifugation, then dialyzed against buffer A. The dialyzed sample was put on a column (1.6 × 13 cm) of DE52, and eluted with 500 ml of buffer A containing NaCl in a linear gradient from zero to 0.35 M. Catechol 1,2-dioxygenase and muconate cycloisomerase activities of collected fractions were assayed and the total activities of CD I, CD II, MC I, and MC II were estimated by summing up the activities of their fractions.
Results

Enzyme purification

Muconate cycloisomerase was separated into two fractions by DE52 column chromatography; the first and second peaks were named MC II and MC I, respectively (Fig. 2). MC I and MC II were purified to a single band on the polyacrylamide gel (Fig. 3). The purification procedures are summarized in Table I. MC I and MC II were purified 240- and 120-fold, respectively, and their recovery was 1.2% for MC I and 7.1% for MC II at the final steps. Specific activities of the purified MC I and MC II were 84 and 42 units/mg. The molecular masses of MC I and MC II were calculated to be 400 kDa by gel filtration on Toyopearl HW-55 superfine, and SDS-PAGE showed that the molecular masses of subunits of both enzymes are 44 kDa.

pH and heat stabilities of MC I and MC II

Both MC I and MC II showed pH optima at 8.0 and retained more than 70% of their activities in the pH range of 6.0 to 10.0 after incubation for 24 h at 4°C.

Figure 4 shows the thermostability of MC I and MC II after treatment for 10 min at various temperatures. MC I heated at 70°C lost 65% of the activity of the untreated enzyme, but MC II retained 81% activity after the treatment at 75°C, showing that MC II has higher thermostability than MC I.

Other enzymatic properties of MC I and MC II

Both enzymes showed no activity for 2- and 3-methylmuconates produced from 3- and 4-methylcatechols, respectively.

| Table I. Summary of Purification of Muconate Cycloisomerase Isozymes |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Fraction        | Total activity (units) | Total protein (mg) | Specific activity (units/mg) | Recovery (%) |
| MC I | MC II | MC I | MC II | MC I | MC II | MC I | MC II |
| Cell extract   | 780           | 2,200          | 810            | 2,300          | 0.35 | 0.35 | 100 | 104 |
| Streptomycin sulfate | 530       | 840            | 4.8            | 6.7            | 21  | 28  |
| Ammonium sulfate | 160          | 220            | 33             | 33             | 30  | 24  | 12  | 14  |
| DE-52           | 110           | 110            | 3.2            | 4.5            | 50  | 24  | 7.6 | 7.1 |
| DEAE-Toyopearl 650S    | 59            | 55             | 1.0            | 1.3            | 84  | 68  |
| Toyopearl HW55 SF   | 9.2           | -              | 0.11           | -              | 1.2 | -   |

* MC II was purified electrophoretically to homogeneity without the ether-Toyopearl 650S column chromatography.
Enzymatic activities were measured with various inhibitors present. EDTA (1.0 mM), CuSO₄ (1.0 mM), and HgCl₂ (0.5 mM) completely inhibited both enzymatic activities. MC I and MC II treated with p-chloromercuribenzoic acid retained 78% and 63% of the activities of untreated enzymes.

MC I and MC II showed maximal activities at pH 6.0 and more than 80% activities between pH 5.5 to 7.5 and pH 6.0 to 7.0, respectively. These results show that MC I is more active in a wide range of pHs than MC II.

Enzymatic activities of MC I and MC II were assayed with various bivalent metal salts in place of MnCl₂. MC I and MC II showed relative activities of 28% and 17% in reaction mixtures containing MgSO₄ in place of MnCl₂. MC II also showed 6.0% and 0.4% activities with CoCl₂ and FeSO₄.

NH₂-terminal amino acid sequences

The NH₂-terminal amino acid sequences of the purified MC I and MC II were identified as SSVTIEQIETXLVD- and MIATPVIESVE-, respectively (Fig. 5).

Induction of catechol 1,2-dioxygenase and muconate cycloisomerase

Figure 6 shows induction of both enzymes by catechol and cis,cis-muconate. cis,cis-Muconate was found to induce production of both enzymes rapidly. In particular, the catechol 1,2-dioxygenase activity reached the maximum after incubation for 3 h and decreased gradually. Induction of muconate cycloisomerase showed the maximum activity after incubation for 5 h and it was kept at a high level after one decrease of specific activity. On the other hand induction of both enzymes by catechol was later, more gradual, and lower than that by cis,cis-muconate. Aniline was found to lead to no production of both enzymes even after incubation for 35 h (data not shown).

Analysis of induced isozymes

Table II shows the total activity estimated from adding each isozyme fraction after DE52 column chromatography. Four enzymes, CD I, CD II, MC I, and MC II were induced simultaneously in all cell extracts examined. The enzymatic activities of CD II and MC II reached the maximum after incubation for 3 h (10.7 and 5.40 units for CD II and MC II, respectively) and decreased gradually. On the other hand, the maximum activities of CD I and MC I were found after incubation for 7 and 5 h (3.40 units for CD I and 27.0 units for MC I, respectively). These results show that production of CD II and MC II is linked and more sensitive than that of CD I and MC I to the inducer of cis,cis-muconate.

Discussion

We purified two muconate cycloisomerase isozymes, MC I and MC II, from aniline-assimilating Fraterura sp. ANA-18 and characterized them. MC I and MC II showed narrow substrate specificity and belonged to the catechol branch. Fraterura sp. ANA-18 also produces catechol 1,2-dioxygenase isozymes, CD I and CD II, with narrow substrate specificity. These results suggest that this bacterium has two catechol branches.

We compared NH₂-terminal amino acid sequences of MC I and MC II with the NH₂-terminal amino acid sequences of CatB (muconate cycloisomerase) reported previously (Fig. 5). MC I and two Pseudomonas CatB25,26 shared 5 identical amino acids in comparable sequences (hatched in Fig. 5). MC I was also similar to CatB from Acinetobacter calcoaceticus ADP1.27 On the other hand, the NH₂-terminal amino acid sequence of MC II showed no sequence similarity to that of MC I. This result shows that the enzymes are encoded by differ-
ent genes. In addition, it is interesting that the sequences of MC II and other muconate cycloisomerases had no identical amino acids.

cis,cis-Muconate induced production of catechol 1,2-dioxygenase and cis,cis-muconate cycloisomerase more rapidly than catechol (Fig. 6). These results show that cis,cis-muconate is a direct inducer for the synthesis of both enzymes as in the case of P. putida described by Ornston. Pseudomonas enzymes are also induced rapidly by benzoate, the first compound in a benzoate metabolic pathway. But it is interesting that induction with aniline in Fratureia sp. ANA-18 led to no production of enzymes in 35 h of the incubation. It seems that this observation is attributed to the toxicity of aniline and difference of an upper pathways leading to catechol from initial compounds. Though the muconate cycloisomerase activity induced by cis,cis-muconate was kept at a high level, the catechol 1,2-dioxygenase activity was decreased to a half of the maximum. As one of the reasons for this decrease, it is thought that cofactors are lacking or native catechol 1,2-dioxygenase is modified imperfectly in resting cells.

As shown in Table II, MC I, MC II, CD I, and CD II were induced simultaneously. But types of induction differed between CD I and CD II, MC I and MC II, and were similar between CD II and MC II, CD I and MC I, respectively. These observations suggest that Fratureia sp. ANA-18 has at least two catechol branches consisting of CD I and MC I, CD II and MC II, respectively, which are independent in gene expression. The occurrence of two catechol branches, particularly the branch consisting of CD II and MC II, is advantageous to aniline assimilation of this Fratureia because induction of CD II and MC II is more sensitive than that of CD I and MC I.

In previous papers, muconate cycloisomerase isozymes have been reported in some bacteria.5-8) But these enzymes are chloromuconate cycloisomerases and muconate cycloisomerases, which seem to be responsible for distinguishing the pathways, the chlorocatechol and catechol branches, respectively. Furthermore, it has been reported that the former enzymes are produced in cells grown on chlorinated aromatic compounds and the latter enzymes have been produced in cells grown on non-chlorinated aromatic compounds. However, Fratureia sp. ANA-18 produced not only two catechol 1,2-dioxygenase isozymes but also two muconate cycloisomerase isozymes simultaneously when the bacterium metabolized aniline. Therefore, this is the first report about two catechol branches induced simultaneously and shown by enzymes purification. Only the yeast Trichosporon cutaneum has been reported to produce two muconate cycloisomerase isozymes, which differ in their pi and content of free thiol groups.6) But this yeast was found to show only one peak of enzymatic activity for catechol 1,2-dioxygenase on a DEAE-Sephadex column chromatogram.7)

Recently, two kinds of gene clusters containing catA, catB, and catC, encoding catechol 1,2-dioxygenase, cis,cis-muconate cycloisomerase, and muconolactone isomerase, respectively, have been cloned from Acinetobacter lwofii K24.20) The Acinetobacter catechol 1,2-dioxygenase isozymes belong to the catechol branch because they have shown high enzymatic activities for catechol only.20) We are interested in the relationship between the catechol branches from Fratureia sp. ANA-18 and Acinetobacter lwofii K24 and now proceeding to clone the genes related to the catechol branch of Fratureia sp. ANA18.

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References