Stereo- and regiospecific cis,cis-muconate cycloisomerization by 
Rhodococcus rhodochrous N75

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Abstract

cis,cis-Muconate cycloisomerase was purified to homogeneity from cells of Rhodococcus rhodochrous N75 grown at the expense of benzoate and p-toluic acid as the sole sources of carbon. A single cycloisomerase was found to be induced in this organism with no isoforms being detected when R. rhodochrous N75 was grown on either benzoate or p-toluic acid as the sole source of carbon. The enzyme is hexameric with a single subunit Mr of 40,000. cis,cis-Muconate cycloisomerase from R. rhodochrous N75 displayed strict regio- and stereospecificity whereby cis,cis-muconate is cycloisomerized to (4S)-muconolactone and 2-methyl- and 3-methyl-substituted muconates are cycloisomerized to 2-methyl- and 4-methyl-substituted muconolactones by 1,4- and 3,6-cycloisomerization, respectively.

1. Introduction

cis,cis-Muconate cycloisomerase (also referred to as cis,cis-muconate lactonizing enzyme) is a member of enolase superfamily [1] and the second enzyme mediating the catechol branch of the β-ketoadipate pathway for the metabolism of aromatic compounds by aerobic microorganisms [2]. It catalyzes reversible γ-lactonization, which is an intramolecular conjugate addition–elimination reaction. cis,cis-Muconate is cycloisomerized to (+)-muconolactone with the insertion of a proton.

The cycloisomerization of cis,cis-muconate by lactonizing enzymes from Pseudomonas putida and the yeast Tri-
chosporon cutaneum occurs by a syn (4S) absolute stereochemical course [3,4]. Carboxymuconate cycloisomerase from the ascomycete fungus Neurospora crassa has also been established as a syn cycloisomerase, with the same absolute stereochemical course [5], whereas carboxymuconate cycloisomerase from P. putida proceeds by an anti addition [6]. Cain et al. [7] also reported that the cycloisomerization of 3-methyl-cis,cis-muconate also proceeds by syn addition of carboxyl groups to double bonds to form (4S)-3-methylmuconolactone in the fungus Aspergillus niger and (4S)-4-methylmuconolactone in P. putida.

Many studies on these enzymes have been focused on those from proteobacteria with regard to the stereospecificity of cycloisomerization and dechlorination [8,9]. Studies on the cycloisomerases from Gram-positive bacteria are limited to those reported in Rhodococcus opacus 1CP (formerly R. erythropolis), which suggested a functionally convergent evolution among bacterial cycloisomerases [10,11]. Recently, a new type of muconate cycloisomerase with two different type subunits was reported from Rhodococcus rhodochrous 89 [12]. However, there is little information on the cycloisomerization of alkyl-substituted substrates. This paper reports the first description of the regio- and stereospecific cycloisomerization of cis,cis-muconate from a Gram-positive bacterium R. rhodochrous N75.
2. Materials and methods

2.1. Microorganism and culture conditions

*Rhodococcus* N75 (NCTC1068) was originally provided by Professor R.B. Cain, University of Newcastle, UK. Culture conditions were those described previously unless otherwise stated [13].

2.2. Chemicals

* cis,cis*-Muconate, * cis,trans*-muconate and (+)-muconolactone were kindly provided by Dr. Alan Sutherland, University of North London, UK. Other muconates were prepared enzymically using purified catechol 1,2-dioxygenase [14] and catechols as starting substrates; 3-alkyl-*cis,cis*-muconates from 4-alkylcatechols, 2,3-dimethyl-*cis,cis*-muconate from 3,4-dimethylcatechol, and 2,4-methyl-*cis,cis*-muconate from 3,5-dimethylcatechol. Catechol or its derivatives (1 mM) in 1 ml of 50 mM Tris–HCl buffer (pH 7.3) were incubated at room temperature with purified catechol 1,2-dioxygenase (0.14–0.37 U) which was free of cycloisomerase activity. The reaction was monitored spectrophotometrically at OD260 or by high-performance liquid chromatography (HPLC). The when the substrate was depleted and no further increase at OD260 was observed, the protein was removed by ultrafiltration using a YM3 Diaflo membrane (3000 M₆ cut-off, Amicon) and the filtrate was collected. This solution was immediately used for muconate cycloisomerase assays at the time of preparation.

2.3. Enzyme assay

The activity of muconate cycloisomerase was assayed spectrophotometrically by measuring the rate of decrease in OD260 as described by Ornston and Stanier [15]. The reaction mixture contained: 50 mM MOPS buffer (pH 7.3), and approximately 1 mM substrate and 2–3 U of purified cycloisomerase. The reaction was initiated by adding 10 U of purified cycloisomerase after the pH was readjusted to 7.2. Samples were taken at intervals and the reaction was monitored by HPLC. The reaction was stopped by acidifying the reaction mixture to pH 2 with concentrated phosphoric acid. A clarified supernatant solution was obtained by centrifugation at 19000 rpm for 20 min. The enzymically produced muconolactone was then characterized by A.G. Sutherland, University of North London, UK.

2.4. Enzyme purification

Purification of *cis,cis*-muconate cycloisomerase was attempted from both benzoate-grown and p-toluate-grown cells of *Rhodococcus* N75 to determine if any isofunctional enzymes were induced when the cells were grown at the expense of p-toluate as a sole carbon source. Each batch of cells was subjected to the same purification procedure. All the procedures were performed at 4°C. Frozen cells were used to prepare cell-free crude extracts as described previously [13]. The crude extract was loaded onto a column of DEAE-Toyopearl 650C (2.5 x 10 cm) which had been previously equilibrated with buffer A (50 mM MOPS, pH 7.4, 1 mM dithiothreitol). The column was then washed with the same buffer until no protein was evident in the eluate. The enzyme was eluted with a linear gradient of 0–0.5 M KCl in 500 ml of buffer A. Fractions (5 ml) were collected at a flow rate of 1 ml min⁻¹. Active fractions were pooled and then concentrated by ultrafiltration with a YM100 Diaflo membrane (100000 M₆ cut-off, Amicon). The concentrate was applied to a Sephacryl S-300 HR gel filtration column (1.6 x 83 cm) pre-equilibrated with buffer A. Subsequent elution of the enzyme activities was carried out at 15 ml h⁻¹ and 1-ml fractions were collected. Fractions containing the highest enzyme activity were combined and loaded onto a Mono Q HR 5/5 column (Pharmacia) pre-equilibrated with buffer A. The column was then washed with 30% buffer B (buffer A containing 1 M NaCl) and the enzyme was eluted with a linear gradient of 30–60% buffer B. Fractions (1 ml) were collected at a flow rate of 1 ml min⁻¹ and active fractions were combined and stored at −20°C until required.

The enzyme preparation from benzoate-grown cells was used for characterization studies unless otherwise stated.

2.5. Enzymatic cycloisomerization of muconates to muconolactones

The enzymatic cycloisomerization of *cis,cis*-muconate to (+)-muconolactone was performed in 50 ml reaction mixture containing 50 mM MOPS buffer (pH 7.2), 2 mM MnCl₂, and 0.284 g (40 mM) of *cis,cis*-muconate. The reaction was initiated by adding 10 U of purified cycloisomerase after the pH was readjusted to 7.2. Samples were taken at intervals and the reaction was monitored by HPLC. The reaction was stopped by acidifying the reaction mixture to pH 2 with concentrated phosphoric acid. A clarified supernatant solution was obtained by centrifugation at 19000 rpm for 20 min. The enzymically produced muconolactone was then characterized by A.G. Sutherland, University of North London, UK.

Enzymatic cycloisomerization of substituted muconates was performed using substrates that had been prepared with purified catechol 1,2-dioxygenase. The reaction mixture contained: 50 mM MOPS buffer (pH 7.3), 2 mM MnCl₂, approximately 1 mM substrate and 2–3 U of purified cycloisomerase in a final volume of 1 ml. After 30–60 min, the reaction was stopped by the addition of concentrated phosphoric acid and the products were analyzed by HPLC.

2.6. HPLC

HPLC was performed using a Hewlett-Packard 1050 series component system (Blacknell, UK). Samples (10 μl) were injected onto a Spherisorb-ODS (C₁₈) reverse-phase column (0.46 x 25 cm; particle size, 5 μm; HPLC Technology Ltd., Macclesfield, UK). The mobile phase was 40% (v/v) aqueous methanol with 0.1% (v/v) concentrated phosphoric acid. The flow rate was 1.5 ml min⁻¹ and the UV detector was set at 215 nm.
3. Results

3.1. Purification of muconate cycloisomerase from *R. rhodochrous* N75

Muconate cycloisomerase from *R. rhodochrous* N75 was purified to electrophoretic homogeneity from *p*-toluate-grown cells and benzoate-grown cells. The elution profiles from all the chromatographic steps for muconate cycloisomerase were similar from cells grown with either benzoate or *p*-toluate as the sole source of carbon. All the chromatography steps resulted in a single peak of muconate cycloisomerase activity. Data from a typical purification are shown in Table 1. These results imply that a single cycloisomerase is induced regardless of whether the cells were grown at the expense of benzoate or *p*-toluate as the sole carbon source. This was confirmed by both native and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the purified enzymes, as both enzyme preparations produced single bands with identical *R*~f~ values (Fig. 1).

3.2. Determination of *M*, and subunit composition

Both native and SDS–PAGE analysis of the cycloisomerases purified from benzoate- and *p*-toluate-grown cells resulted in a single band of protein. The subunit *M*~s~ of the enzyme was estimated to be 40 000 by SDS–PAGE analysis (Fig. 1). The bands in native PAGE displayed the cycloisomerase activity and both protein samples migrated as a single band at *M*~*s~ 40 000 when excised from the native gel and subjected to SDS–PAGE.

The native *M*~s~ of the cycloisomerase from *R. rhodochrous* N75 was estimated to be 236 000 by Superose 6 HR gel filtration chromatography, suggesting that the enzyme is homohexameric.

3.3. pH optimum

The pH optimum of the muconate cycloisomerase reaction was estimated by measuring its activity in 50 mM Bis-Tris-propane at various pH values between 6.5 and 8.0. At pH > 8.0, the enzyme activity could not be determined due to a rapid increase of absorbance in the absence of enzyme caused by the presence of Mn~2+~ ions in the reaction mixture. The pH optimum of the cycloisomerase activity was determined to be pH 7.5.

3.4. Substrate specificity and kinetic properties

The estimation of substrate specificity of the cycloisomerase against a range of alkyl-substituted *cis*,*cis*-*muconates* (3-methylmuconate, 3-ethylmuconate, 2,3-dimethylmuconate and 2,4-dimethylmuconate) was attempted using enzymically prepared substrate solutions on the basis of decrease in the peak area of HPLC chromatogram of the substituted muconates. After an incubation period of 24 h in the absence of the purified cycloisomerase, almost 90% of all the muconates formed from 4-alkyl-substituted catechols were found to be transformed to compounds which exhibited a very high absorbance near 210 nm. Although the enzyme activities against alkyl-substituted *cis*,*cis*-muconates could not be quantified due to the instability of those substrates, enzymic transformation of these muconates by the purified cycloisomerase was observed within 30–60 min by HPLC analysis. Lactonization of *cis*,trans-*muconate* was also examined using the purified cycloiso-

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<th>Total activity (U)</th>
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merase. The reaction mixture contained: 50 mM Tris–HCl buffer (pH 8.0), 1 mM MnCl₂, 0.1 mM cis,trans-muconate, and 15 U of the purified enzyme in a final volume of 3 ml. The reaction was monitored spectrophotometrically at OD₃₂₅. A very slow decrease in OD₃₂₅ was observed after an initial lag. In control experiments, where the enzyme was omitted, this reaction did not occur. Sistrom and Stainier [18] reported that the lactonizing enzyme from a strain of Pseudomonas catalyzed a reversible reaction between cis,trans-muconate and (−)-muconolactone and the conversion rate of cis,trans-muconate to the lactone was approximately 0.02% of that of the cis,cis-isomer. The enzyme from R. rhodochrous N75 also exhibited this reaction at 1.5% of the rate for the cis,cis-isomer. However, attempts to characterize the product were not successful since the activity with the cis,trans-isomer was extremely low and hence sufficient amounts of the product could not be obtained.

Michaelis–Menten kinetic parameters were determined using a range of substrate concentration (0.01–0.1 mM). Apparent $K_m$ and $V_{max}$ values for cis,cis-muconate were 0.16 ± 0.011 mM and 103.6 ± 4.1 U mg⁻¹, respectively.

3.5. N-terminal sequence of muconate cycloisomerase

The N-terminal amino acid sequence of muconate cycloisomerase from R. rhodochrous N75 was determined and is shown in Fig. 2. The sequences of the pure enzyme from both benzoate-grown cells and $p$-toluate-grown cells were identical, providing further evidence that only a single muconate cycloisomerase is produced by R. rhodochrous N75 when metabolizing aromatic compounds by the ortho-cleavage pathway. When the N-terminal amino acid sequence of muconate cycloisomerase from R. rhodochrous N75 was compared with those from other cycloisomerases quoted by Solyanikova et al. [10], it showed a high degree of similarity with other cycloisomerases and also contained the amino acids which were highly conserved in other organisms.

![Fig. 3. Different regiochemistry of cycloisomerization. $R=CH_3$, $CH_2CH_3$, $C(H_2)CH_3$, or $C(H_3)CH_3$; $R_1=CH_3$ and $R_2=H$, or $R_1=H$ and $R_2=CH_3$.](image)

3.6. Regiospecificity of enzymatic cycloisomerization

The course of enzymatic cycloisomerization of muconates to muconolactones was monitored by HPLC and the resulting products were identified by comparison of their retention times with those of authentic standards. Enzymically prepared muconates were shown to be cycloisomerized to their corresponding muconolactones by the purified cycloisomerase. Fig. 3 illustrates the types of cycloisomerization which are mediated by the cycloisomerase purified from R. rhodochrous N75. HPLC analysis showed that 3-methyl-cis,cis-muconate was lactonized to 4-methyl-muconolactone by 3,6-intramolecular cycloisomerization, as previously shown with partially purified enzyme [16]. Since 3-ethyl-cis,cis-muconate was converted to a lactonic compound which did not co-elute with authentic 3-ethyl-muconolactone, it was assumed to be 4-ethylmuconolactone. Therefore, a general reaction formula can be deduced that 3-alkyl-substituted cis,cis-muconates are cycloisomerized to 4-alkyl-substituted muconolactones (Fig. 3). In contrast, 2-methyl-cis,cis-muconate was lactonized to 2-methylmuconolactone by 1,4-intramolecular cycloisomerization. 2,3-Dimethyl-cis,cis-muconate and 2,4-dimethyl-cis,cis-muconate were also found to undergo 1,4-intramolecular cycloisomerization to yield 2,3-dimethylmuconolactone and 2,4-dimethylmuconolactone, respectively. Schmidt et al. [18] showed that these dimethyl-lactones were accumulated as dead-end metabolites in the metabolism of 3,4-dimethylbenzoate and 3,5-dimethylbenzoate, respectively, by $p$-toluate-grown cells of R. rhodochrous N75. Likewise, the Gram-negative strainRalstoniaeutrophajMP134wasreportedtoproducethesedimethyl-lactonesvia3,4-dimethylcatecholand3,5-dimethylcatechol during the metabolism of dimethylphenols, suggesting the 1,4-type cycloisomerization [19]. These results, therefore, suggest that the methyl substitution at the C-2 position of muconate directs the cycloisomerization by...
1,4-addition rather than 3,6-addition (Fig. 3). The steric influence caused by the methyl substitution may encourage the 1,4-cycloisomerization.

4. Discussion

*R. rhodochrous* N75 was known to metabolize 4-methylcatechol via a modified β-ketoadipate pathway [13, 16,20]. Like catechol 1,2-dioxygenase [14], the cycloisomerase from *R. rhodochrous* N75 displayed a broad substrate specificity against a range of alkyl-substituted cis,cis-muconates though the relative activities could not be quantified due to the instability of those substrates. Schmidt et al. [21] reported that 3-methyl-cis,cis-muconate was non-enzymically cycloisomerized to racemic 4-methylmuconolactone at pH 6.5, whereas Cain et al. [7] discovered that the compound was rapidly transformed to 1,4-addition rather than 3,6-addition (Fig. 3). The steric cis substrate specificity against a range of alkyl-substituted isomerase from *R. rhodochrous* 16,20]. Like catechol 1,2-dioxygenase [14], the cycloisomerization was shown to be 4-methylmuconolactone. 3-Ethyl-cis,muconolactone at pH 6.5, whereas Cain et al. [7] discovered that 3-methyl-cis,cis-muconate was non-enzymically cycloisomerized to racemic 4-methylmuconolactone at pH 6.5, whereas Cain et al. [7] discovered that the compound was rapidly transformed to 3-methyl-cis,trans-muconate and 3-methylmuconolactone, followed by a slower conversion of the former into the latter. When examined in this study, non-enzymic conversion of 3-methyl-cis,cis-muconate to 3-methylmuconolactone was observed. HPLC analysis showed that the product from non-enzymic conversion co-eluted with authentic 3-methylmuconolactone, whereas the product from the enzyme-mediated conversion was shown to be 4-methylmuconolactone. 3-Ethyl-cis,cis-muconate also appeared to undergo non-enzymic conversion to 3-ethylmuconolactone.

Bacterial cycloisomerasers have been known to catalyze the cycloisomerization of 3-methyl-cis,cis-muconate to 4-methylmuconolactone, which resulted in a metabolic block in pseudomonads when metabolizing methyl-substituted aromatics by the ortho-cleavage pathway [22,23]. In contrast, eukaryotic cycloisomerasers produce 3-methylmuconolactone from 3-methyl-cis,cis-muconate [4,7,24,25]. The cycloisomerase from *R. rhodochrous* N75 was found to display a strict regiospecificity; 3-alkyl-substituted cis,cis-muconates were lactonized to form 4-alkyl-substituted muconolactones by 3,6-cycloisomerization, whereas 1,4-cycloisomerization was observed when a methyl group was substituted at the C-2 of muconate.

Muconolactone produced by the purified cycloisomerase from *R. rhodochrous* N75 was characterized by 1H nuclear magnetic resonance studies using chiral reagents (A.G. Sutherland, personal communication). The product was (4S)-muconolactone with an enantiomeric excess of 98%. Although the absolute stereochemistry of cycloisomerization in *R. rhodochrous* N75 was not presented in these studies, it can be predicted in an indirect manner based on the known stereochemistry that muconolactone produced from cis,cis-muconate has 4S absolute configuration [4]; muconolactone isomerase from *P. putida* has been known to act only on the (4S)-lactone [3,17]. The product resulting from cycloisomerization of cis,cis-muconate by the cycloisomerase from *R. rhodochrous* N75 was observed to be degraded when incubated with muconolactone isomerase and enol lactone hydrolase prepared from recombinant strains of *Escherichia coli* which contained genes encoding the pseudomonad enzymes (data not shown).

The regio- and stereochemistry, the native $M_c$ of enzyme, the subunit composition and the metal (Mn$^{2+}$) dependence of the cycloisomerase from *R. rhodochrous* N75 were found to be identical to those of other bacterial cycloisomerasers. Although the kinetic properties for halogenated muconates and the ability of dehalogenation were not determined with the cycloisomerase from *R. rhodochrous* N75, it is likely to be most closely related to *R. opacus* ICP muconate cycloisomerase according to the N-terminal sequence comparison. A new type of muconate cycloisomerase, which has been recently discovered from *R. rhodochrous* strain 89 grown on phenol [14], appeared to be distinct from that from *R. rhodochrous* N75 in two ways: (1) it consists of two different subunits with molecular masses of 33.5 and 37 kDa. (2) The N-terminal amino acid sequence of muconate cycloisomerase from *R. rhodochrous* N75 was more homologous to chloromuconate cycloisomerase from *R. opacus* ICP than the large subunit of cycloisomerase from *R. rhodochrous* 89 (Fig. 2). A single cycloisomerase found in *R. rhodochrous* N75 also implies that this strain may have evolved in a haloaromatic-deficient environment. The complete nucleotide sequence and structural studies would reveal its mechanical, structural and evolutionary relatedness to other cycloisomerasers.

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References