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A New Type of Muconate Cycloisomerase from *Rhodococcus rhodochrous* Strain 89

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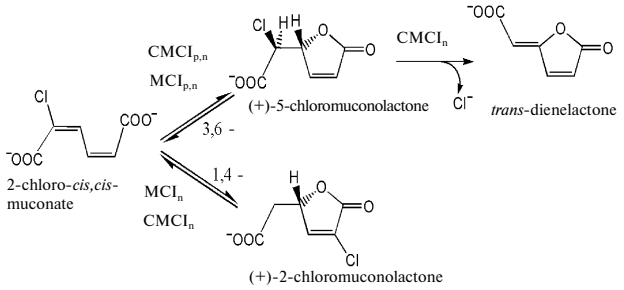
Abstract—Muconate cycloisomerase (MCI) was purified from *Rhodococcus rhodochrous* 89 grown on phenol. The enzyme appears to contain two different type subunits with molecular masses 35.5 and 37 kD. The N-terminal amino acid sequence of both subunits showed more similarity to corresponding enzymes from gram-negative bacteria than to one from *Rhodococcus opacus* 1CP. MCI from *R. rhodochrous* 89, like analogous enzymes from gram-negative bacteria, can convert 2-chloromuconate (2-CM) with the formation of both, 2- and 5-chloromuconolactones (CML) as intermediates. Nevertheless, its unique ability to convert 5-CML to *cis*- but not to *trans*-dienelactone sets it apart from all known chloromuconate cycloisomerases from gram-negative and gram-positive bacteria.

Key words: (chloro)muconate cycloisomerase, transformation, 2-chloromuconate, chloromuconolactones, dienelactones, *Rhodococcus rhodochrous* 89

Muconate cycloisomerases (MCIs) are involved in the conversion of catechol (1,2-dihydroxybenzene) via a so-called ordinary *ortho*-cleavage pathway for the degradation of aromatic compounds [1, 2] and are responsible for the cycloisomerization of *cis,cis*-muconate, formed as a result of the cleavage of the aromatic ring of catechol by catechol 1,2-dioxygenase, to muconolactone. Two other enzymes of this pathway, muconolactone isomerase and enol-lactone hydrolase, convert muconolactone to β-ketoadipate. Many MCIs from gram-negative strains have been purified and characterized [3-7]. All these enzymes were shown to be thermostable homooctamers with subunit molecular mass ~40 kD. MCIs from gram-negative *Pseudomonas putida* and *Acinetobacter calcoaceticus* have homologous amino acid sequences (52.4% of residues are identical) [8]. The similarity of these two enzymes with two MCIs from *Acinetobacter lwoffii* K-24 was even higher [9]. MCIs are highly effective in conversion of unsubstituted muconate and are unable to convert chlorinated substrates [10, 11].

It is known that chlorocatechols are key intermediates in the conversion of chlorinated aromatic compounds under aerobic conditions. Furthermore, the conversion of chlorocatechols proceeds via a modified *ortho*-cleavage pathway with enzymes chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase (CMCI), dienelactone hydrolase, and maleylacetate reductase participating in this pathway. The main difference between the sets of enzymes of the ordinary and modified *ortho*-cleavage pathways is that the during conversion of chlorinated substrates by enzymes of the modified *ortho*-cleavage pathway the elimination of a chloride ion takes place at the step of the cycloisomerization of the chloromuconate, while MCI of the ordinary *ortho*-cleavage pathway are unable to perform this reaction.

Cycloisomerization of 2-chloromuconate (2-CM) by different (chloro)muconate cycloisomerases has been studied in more detail on (C)MCIs from gram-negative bacteria (n) and the enzymes from *R. opacus* 1CP (p) (numbers adjacent to the arrows indicate whether the reaction is a 1,4- or 3,6-cycloisomerization):



Abbreviations: CMCI) chloromuconate cycloisomerase; MCI) muconate cycloisomerase; 2-CM) 2-chloromuconate; 2-CML) 2-chloromuconolactone; 5-CML) 5-chloromuconolactone.

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The CMCI from strains *Ralstonia eutropha* JMP134 and *Pseudomonas putida* pAC27 convert 2-CM to *trans*-dienelactone in two-steps reaction. First, they cycloisomerize 2-CM with the formation of 2- and 5-chloromuconolactones (CML). In the second step, they eliminate a chloride ion from 5-CML resulting in the formation of the final product of the reaction, *trans*-dienelactone [12]. In contrast, MCI of the ordinary *ortho*-cleavage pathway from the strains *Pseudomonas* sp. B13 as well as MCIs from strains *Pseudomonas putida* PRS2000 and *Ac. calcoaceticus* ADP1 were shown to convert 2-CM to 2- and 5-CML, but they fail to perform the second step of this reaction, the dechlorination of 5-CML [10]. This is the main explanation why strains with the ordinary *ortho*-cleavage pathway enzymes are unable to utilize chlorinated aromatic compounds.

MCIs and CMCIs from the gram-positive strain *Rhodococcus opacus* 1CP differ significantly from analogous enzymes from gram-negative bacteria by their ability to discriminate between two possible directions of the cycloisomerization of 2-CM so as only one product, 5-CML, is formed [13]. Both enzymes can catalyze the second part of the reaction, so no *trans*-dienelactone appears. This significant difference in kinetic properties between cycloisomerases from a number of gram-negative bacteria and gram-positive *R. opacus* 1CP is consistent with the difference in amino acid sequences of these enzymes: MCI and CMCI from *R. opacus* 1CP are more similar to each other than to analogous enzymes from the gram-negative bacteria [14].

Here we report on a new type of muconate cycloisomerase from gram-positive *R. rhodochrous* 89 that significantly differs in its properties from all known types of cycloisomerases and show the unique properties of this enzyme in catalyzing the conversion of 2-CM to *cis*-dienelactone.

MATERIALS AND METHODS

Microorganism, growth conditions, and preparation of crude extract. Strain *R. rhodochrous* 89 was grown in mineral medium with phenol as the sole source of carbon and energy as described earlier [15]. Crude extract was obtained according to standard procedure [15] except that $MnSO_4$ (final concentration 2 mM) was added to all solutions.

Enzyme assays. The activity of MCI was measured spectrophotometrically at 260 nm as described earlier [13]. For the routine procedure, muconate was used as the substrate.

Purification of MCI. Crude extract was fractionated by precipitation by ammonium sulfate (20-70% saturation). The pellet was collected by centrifugation, dissolved in 50 mM Tris-HCl buffer, pH 7.2, containing 2 mM $MnSO_4$ (buffer A), and dialyzed overnight against the same buffer. Undissolved particles were removed by centrifugation (40,000*g*, 20 min), and the supernatant was applied onto a DEAE-Toyopearl 650M column (26 × 40, bed volume 136 ml) (Toyo Soda, Japan) pre-equilibrated with buffer A. The enzyme was eluted in a linear 0-0.5 M gradient of NaCl in 2 liters of starting buffer (flow rate, 4 ml/min; fraction volume, 10 ml). The most active fractions were combined and heated at 60°C for 20 min. Precipitated proteins were removed by centrifugation (20,000*g*, 20 min), and the supernatant was applied onto a phenyl-Sepharose column (16 × 10, bed volume 8 ml) (Pharmacia, Sweden) pre-equilibrated with buffer A containing 1.6 M $(NH_4)_2SO_4$ (starting buffer). The column was washed with 16 ml of the starting buffer, and proteins were eluted by a decreasing linear gradient 1.6-0 M of ammonium sulfate in 160 ml of the starting buffer (flow rate, 1.5 ml/min; fraction volume, 2 ml). The fractions containing the peak of MCI activity were combined, desalted by concentration in an Amicon unit with PM30 membrane (Amicon, USA), and applied after centrifugation onto a Resource Q (V = 1 ml) (Pharmacia) column. The enzyme was eluted by a linear gradient of 0-1 M NaCl in 25 ml of buffer A (flow rate, 1.5 ml/min; fraction volume, 0.3 ml). Fractions containing the peak of MCI activity were concentrated in a Centricon 30 unit (Amicon) and applied in two runs on a Superdex 200 column (16 × 70, bed volume 120 ml) (Pharmacia) equilibrated with buffer A with 0.1 M NaCl (flow rate, 1 ml/min; fraction volume, 0.5 ml). To the most active fractions of MCI, ammonium sulfate was added to the final concentration of 1.6 M and, after centrifugation, the preparation was twice applied on the Resource ISO (V = 1 ml) column. Proteins were eluted by a decreasing linear gradient 1.6-0 M of $(NH_4)_2SO_4$ in 30 ml of starting buffer (flow rate, 1.5 ml/min; fraction volume, 0.3 ml). Fractions with the peak of MCI activity were combined, desalted in a Centricon 30 unit, and used as the main protein preparation for all experiments.

HPLC and UV analysis. Transformation of 2-CM and 2- and 5-CML by MCI was checked by HPLC and UV-spectral analysis. For HPLC, a Grom SIL 100 C_8 reversed-phase column (Grom, Germany) with 4.6-mm inner diameter and 125-mm length was used. The flow rate was 0.9 ml/min. The compounds were monitored at 210 and 280 nm. The mobile phase was an aqueous solution of 5% (v/v) methanol and 0.1% (w/v) H_3PO_4. The retention times for the standards were as follows: 2-CM, 7.26 min; 2-CML, 4.11 min; 5-CML, 2.54 min; *cis*-dienelactone, 5.41 min; *trans*-dienelactone, 3.66 min.

Estimation of subunit molecular mass, protein determination, and N-terminal amino acid sequencing. The purity of MCI was evaluated and subunit molecular masses were determined by SDS-PAGE by a modified Laemmli [16] procedure on slab gels with 15% acrylamide in the separating gel. The Sigma (USA) Low Molecular Weight Electrophoresis calibration kit con-

taining phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD) was used for the determination of molecular masses. The gel was stained with Coomassie G-250 [17].

The N-terminal amino acid sequence of MCI was determined after SDS-PAGE and electroblotting the gel onto an Immobilon P membrane (Millipore, USA) as described earlier [13].

The protein content was determined by a modification of the Bradford method [18] with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Purification of MCI. MCI was purified from the biomass of the strain *R. rhodochrous* 89 grown on phenol in seven steps (table). The specific activity of MCI increased during the first steps from 0.056 to 27.26 U/mg; further purification led to the elimination of contaminating proteins but was accompanied by decreasing of specific activity of MCI to 15.6 U/mg. The enzyme was purified 185.8-fold with 8.7% yield. The final enzyme preparation gave two bands on SDS-PAGE (Fig. 1). All attempts to separate these bands by changing pH, varying salt gradients, introducing additional purification steps (e.g., isochromatofocusing) failed to produce an enzyme preparation with a single band on SDS-PAGE. One the other hand, 4-20% polyacrylamide gel electrophoresis under native conditions gave only one band of protein (data not shown), suggesting that the MCI from *R. rhodochrous* 89 consists of two different types of subunits. The question of the stoichiometry of the subunits in the whole protein remains open because the determination of the molecular mass of native cycloisomerases by the gel filtration gives lower value than the one calculated on the basis of subunit molecular mass and number of subunits [13, 19].

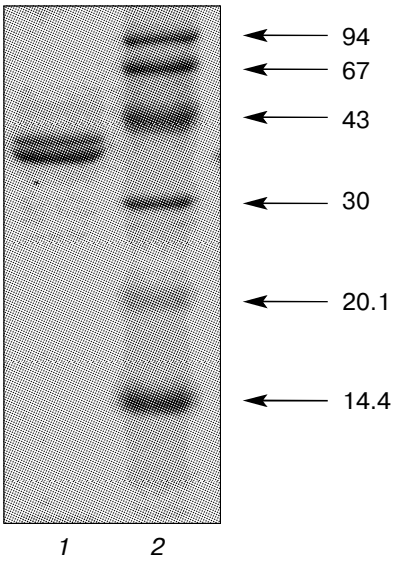

Fig. 1. SDS-polyacrylamide gel electrophoresis (in 12% gel) of purified MCI (5 μg protein) from *R. rhodochrous* 89 (lane *1*). Standards (lane *2*) are showed by arrows, the molecular weights of the protein markers being given on the right in kD: phosphorylase b (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), soybean trypsin inhibitor (20.1), α-lactalbumin (14.4).

Subunit molecular mass and N-terminal amino acid sequence. The molecular masses of the subunits of the MCI determined on the basis of SDS-PAGE in 15% gel were 35.5 and 37 kD.

The N-terminal amino acid sequence of the larger subunit of the MCI from *R. rhodochrous* 89 was determined to be MYKTI(K)ETLLMEIPQIRPxIIHMHQQxS ("x" designates amino acids which were not determined

Purification of MCI from *R. rhodochrous* 89

Purification step	Volume, ml	Total protein, mg	Total activity, U	Specific activity, U/mg protein	Purification, fold	Yield, %
Crude extract	550	4180.42	234.10	0.056	—	—
$(NH_4)_2SO_4$ fractionation	110	3215.71	270.12	0.084	1	100
DEAE-Toyopearl	158	498.46	265.68	0.533	6.45	98.4
Heating (60°C, 20 min)	150	216.72	218.89	1.01	12.0	81.0
Phenyl-Sepharose	320	17.06	197.90	11.60	138.1	73.3
Resource Q (6 ml)	3.6	6.84	186.46	27.26	324.5	69.0
Superdex 200	16.0	2.20	51.33	23.33	277.7	19.0
Resource ISO (1 ml)	5.6	1.51	23.57	15.61	185.8	8.7

a

MCI *R. rhodochrous* 89	1	**MYK**T**I**(K)	**ET***L***L***M***EIP**Q**IRP**X**I**----I**H***M***HQQ**X**S**	27
CatB *Ac. calcoaceticus*	1	**MYK***SV*	**ET**I**L***VD***IP**T**IRP**HKLSVTTMQT**Q**TL	30
CatB *R. opacus* 1CP	4	SIV*SV*	**ET**T*ILDV***P**L*V***RP**HKFATTSMTA**Q**PL	33
CatB2 *Frateuria* sp. ANA-18	7	KI*ESV*	**ET***I***L***V***E***L***P**T**IRP**HRLSVATMNC**Q**TL	36
CatBI-2 *A.lwoffii* K-24	7	KI*ESV*	**ET***I***L***VDV***P**T**IRP**HRLSVATMNC**Q**TL	36
TfdD *B.cepacia* CSV90	2	KIDA**I**	**E***AVIVDV***P**TK**RP-I**QMSITT*V***HQQ-S**	30
TfdD *R.eutropha* JMP134	2	KIDA**I**	**E***AVIVDV***P**TK**RP-I**QMSITT*V***HQQ-S**	30

b

MCI *R. rhodochrous* 89	1	**AVATMQTQTLV**M**VKI**(K)	S**TDDGF***I***G**X**X**E**ATTIGG**	32
CatB *Ac. calcoaceticus*	21	*S***V***I***TMQTQTLV***L***I**K**I**	I**T***E***DG***IV***G**W**G**E**ATTIGG**	52
CatBI *P.putida*	26	**A***M***H**T**MQTQTLV***LIRV*	RC*S***DG**VE**G**IG**E**A**TTIG**L	58
CatB2 *Frateuria* sp. ANA-18	27	*S***VATM***N***C**Q**TLV***L***V***R***I**	RCA**DG**V*V***G**VG**E***G***TTIGG**	58
CatBI-2 *A.lwoffii* K-24	27	*S***VATM***N***C**Q**TLV***L***V***R***I**	RCA**DG**V*V***G**VG**E***G***TTIGG**	58
CatB *P.putida*	25	**A***M***H**T**MQ**Q**QTLV***VLRV*	RC*S***DG**VE**G**IG**EATTIGG**	58
CatBI-1 *A.lwoffii* K-24	24	*S***VATM**YG**QTL***ML***VK***V*	YCT**DG**A*V***G**IG**E***G***TTI***A***G**	58
CatB1 *Frateuria* sp. ANA-18	25	*S***VATM**YG**QTL***ML***VK***V*	YCT**DG**A*V***G**IG**E***G***TTI***A***G**	56
CatB *R. opacus* 1CP	25	**A**NH*SIDA***QT**Y***LL***V***EV*	V**TDAGF***V***G**LG**E***GVS***PGG**	56

Fig. 2. Comparison of N-terminal amino acid sequences of the large (a) and small (b) subunits of the MCI from *R. rhodochrous* 89 with other MCIs (CatB) and CMCI (TfdD). Accession numbers and references for the published sequences: CATB *A. calcoaceticus*, AF009224 [8], CatB *R. opacus* 1CP, AF003948 [14], CatB1 and CatB2 *Frateuria* sp. ANA-18, AB009343 and AB009373, respectively [21], CatBI-1 and CatBI-2 *A. lwoffii* K-24, U77658 and U77659, respectively [9], TfdD *B. cepacia* CSV90, D16356 [22], TfdD *Ralstonia eutropha* JMP134, M35097 [23], CatBI *P. putida*, M19460 [24], CatB *P. putida*, U12557 [25]. Amino acid residues similar in MCI 89 and in any other sequences are indicated in bold, and residues with conserved groups are indicated in italic. Numbers before and after the sequences refer to positions in individual sequences.

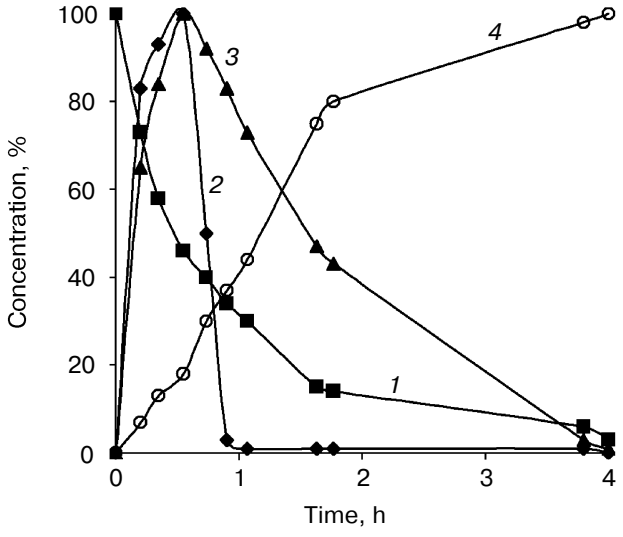

Fig. 3. HPLC analysis of the conversion of 2-chloro-*cis,cis*-muconate by MCI from *R. rhodochrous* 89. The reaction mixture contained 50 mM Tris-HCl, pH 7.2, 2 mM $MnSO_4$, 0.1 mM muconate, and 50 μg protein in a total volume of 1 ml. *1*) 2-CM; *2*) 5-CML; *3*) 2-CML; *4*) *cis*-dienelactone. Concentrations of 0.1 mM for 2-CM, 0.09 mM for *cis*-dienelactone, and the largest peak area for 2- and 5-CML were taken as 100%.

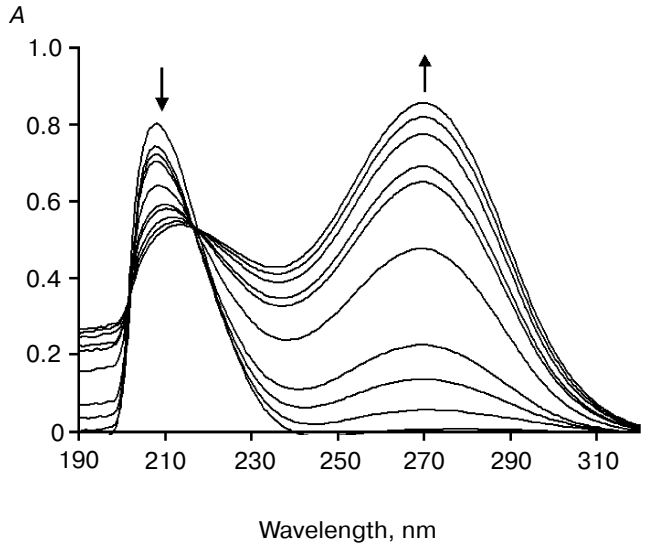

Fig. 4. Spectral changes during conversion of 5-CML by MCI from *R. rhodochrous* 89. The reaction mixture (1 ml) contained 50 mM Tris-HCl (pH 7.2), 2 mM $MnSO_4$, ~0.1 mM 5-CML, and 50 μg of purified MCI. The UV spectrum between 190 and 320 nm was measured every 1.5 min.

during sequencing). The N-terminal amino acid sequence of the smaller subunit of the MCI was determined to be AVATMQTQTLVMVKI(K)STDDGFIGxxEATTIGG. Comparison of the N-terminal sequences of both subunits of the MCI from strain 89 with all known MCIs from gram-negative and gram-positive bacteria by FASTA search [20] showed that both subunits are more similar to analogous enzymes from gram-negative bacteria than to ones from gram-positive bacteria (Fig. 2). It is necessary to stress that because of the difference in molecular mass of the small subunit of MCI from *R. rhodochrous* 89 (35.5 kD) and all known MCIs (40 kD), the N-terminal sequence of the former is similar to the part of other MCIs starting at position 24-28 from the beginning.

Conversion of 2-chloromuconate by MCI. The MCI from *R. rhodochrous* 89 was shown to be able to convert 2-CM. The UV spectra of this conversion showed decreasing absorption of a peak at 275 nm corresponding to 2-CM and some shift of the absorption maximum to 280 nm (data not shown). Results of HPLC analysis of metabolites formed during conversion of 2-CM by this MCI are demonstrated in Fig. 3. Most of the 2-CM is converted during the first half an hour after addition of the enzyme. After 4 h, less than 8% of the added 2-CM was detected by HPLC. Two intermediates with retention time 2.54 and 4.05 min and absorbing at 210 nm corresponding to 5- and 2-CML, respectively, were detected during conversion of 2-CM. Both intermediates appeared within the first 10 min after the MCI was added. The maximum of the amount of both muconolactones was determined 30 min from the beginning of the experiment, and then the amount of both intermediates began to decrease. During this experiment only one product of the reaction was formed. It had retention time 5.29 min and absorption at 280 nm, this corresponding to *cis*-dienelactone.

Both 2- and 5-CML were substrates for the MCI from *R. rhodochrous* 89. The UV spectra of the conversion of 5-CML are shown in Fig. 4. After the addition of the MCI, the maximum of absorption at 207 nm corresponding to 5-CML decreased and shifted to 215-220 nm because of the formation of 2-CML, and another peak at 270 nm increased. HPLC analysis of the metabolites formed showed that the conversion of 5-CML proceeded via 2-CML and 2-CM with the formation of *cis*-dienelactone as the final product.

All MCIs and CMCIs that have been described so far can irreversibly transform 2-CM to chloromuconolactones [10, 12]. Only CMCIs of gram-negative bacteria are able to convert 5-CML to *trans*-dienelactone as the final product [12]. Recently, it was reported that muconolactone isomerase, the third enzyme of the ordinary *ortho*-cleavage pathway from the strain *R. eutropha* JMP134, can convert 5-CML to *cis*-dienelactone [26]. For this enzyme, 5-CML was a better substrate than muconolactone. It is known that muconate is a substrate for MCI but not for muconolactone isomerase of the ordinary *ortho*-cleavage pathway, and MCI is not able to convert muconolactone, which is the substrate for muconolactone isomerase, to enol-lactone, but catalyzes interconversion of *cis,cis*-muconate and (+)-muconolactone [4, 27]. In the case of MCI from strain 89, this enzyme is able to conduct not only the reversible conversion of 2-CM and 2- and 5-CML, but also to catalyze irreversible conversion of 5-CML into *cis*-dienelactone. Thus, the described enzyme combines the properties of different enzymes.

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