Purification and characterization of 4-methylmuconolactone methylisomerase, a novel enzyme of the modified 3-oxoadipate pathway in the Gram-negative bacterium Alcaligenes eutrophus JMP 134

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INTRODUCTION

Methyl-substituted catechols are commonly degraded via meta-cleavage pathways (Hegeman & Rosenberg, 1970; Murray et al., 1972; Sala-Trepat et al., 1972), whereas ortho-cleavage leads to methyl-substituted 4-carboxyhydroxybut-2-en-4-olides (trivially methyl-2-enelactones) as dead-end metabolites (Catelani et al., 1971; Knackmuss et al., 1976). Assimilation of methylketones via an ortho-cleavage pathway was postulated by Miller (1981) and subsequently demonstrated by Pieper et al. (1985), Powolowski & Dagley (1985) and Bruce & Cain (1988) in widely different genera of micro-organisms.

The degradation of 4-methyl-2-enelactone is of special interest because 2-enelactones carrying an alkyl substituent at C-4 cannot be degraded by the classical 3-oxoadipate pathway (Pieper et al., 1985). In the just-cited publication the accumulation of the dead-end metabolite 4-methyl-2-enelactone was shown to be catalyzed by the enzyme methyl-substituted 4-carboxyhydroxybut-2-en-4-olide isomerase, transforming 4-methyl-2-enelactone to 3-methyl-2-enelactone. The enzyme consists of a single peptide chain of M, 40000 (Pieper et al., 1987) and has an enzyme mechanism proposed (Bruce et al., 1989). Rojo et al. (1987) successfully cloned the A. eutrophus gene coding for the 4-methyl-2-enelactone isomerase into a derivative strain of Pseudomonas sp. B13 and thus obtained a genetically engineered strain which was able to utilize 4-methylbenzoate exclusively via the ortho-cleavage pathway. The present paper describes the purification and characterization of the enzyme from Pseudomonas sp. B13 FR1 (pFRC20P) which converts 4-methyl-2-enelactone into 3-methyl-2-enelactone. The significant differences between this enzyme and that from R. ruber N75 may implicate different mechanisms for the isomerization reactions in these two organisms.

EXPERIMENTAL

Materials

Organisms. The gene encoding 4-methyl-2-enelactone isomerase (Pieper et al., 1985) was transferred into Pseudomonas sp. B13 FR1 on a hybrid cosmid pLAFR3, which contained a 26 kb DNA fragment from the A. eutrophus JMP 134 chromosome (Rojo et al., 1987). High levels of 4-methyl-2-enelactone isomerase were measured in acetate-grown cells of the constructed organism. Deletion and subcloning analysis of the inserted Alcaligenes DNA localized the region that encoded the isomerase to a segment 3 kb in length (Rojo et al., 1987). As small pieces of DNA could be removed from both ends of the insert without any loss of enzyme activity, the 3 kb fragment enclosed obviously represents the full isomerase gene.

A. eutrophus JMP 134 was originally isolated by its ability to grow with 2,4-dichlorophenoxyacetic acid (2,4-D) as sole source of carbon and energy (Don & Pemberton, 1981). Pseudomonas sp. B13 FR1 (pFRC20P) is a derivative of Pseudomonas sp. B13

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(Dorn et al., 1974). A Tn5 hybrid transposon carrying the xylX, xylY, xylZ, xylL and xylS genes plus the Pm promoter of the TOL plasmid pWWO (Harayama et al., 1986) has been transposed into the chromosome, thereby enabling the strain to transform 4-methyl- and 4-chloro-benzoates. The gene encoding 4-methyl-2-enelactone isomerase (Pieper et al., 1985) was transferred into Pseudomonas sp. B13 FR1 on a hybrid cosmid pLAFR3 as described by Rojo et al. (1987). High levels of 4-methyl-2-enelactone isomerase were measured in acetate-grown cells of the constructed organism.

Chemicals. 4-Methyl-2-enelactone and 2-methyl-2-enelactone were prepared as described by Knackmuss et al. (1976). 3-Methyl-2-enelactone was prepared chemically as previously described (Pieper et al., 1985). 1-Methylbislactone was prepared by incubation of 4-methyl-2-enelactone under acidic conditions. Full details of the preparation procedure for, and characterization of, the 1-methylbislactone are obtainable from K. H. E. on request, as are details of the preparation of 4-carboxymethyl-2,4-dimethylbut-2-ene-4-olide from 2,4-dimethylphenol. cis,cis-Muconate was prepared as described by Schmidt et al. (1980); 4-carboxymethylbut-2-ene-4-olide as described by Elvidge et al. (1950) and trans-4-carboxymethylenebut-2-en-4-olide as described by Reineke & Knackmuss (1984).

Methods

Culture conditions and preparation of cell extracts. Cells were grown in Luria broth medium (Dorn et al., 1974) containing 4-methyl-2-enelactone (5 mM) for growth of JMP 134 or acetate (10 mM) for growth of Pseudomonas sp. B13 FR1 (pFRC20P). Tetracycline (20 μg/ml) was added to cultures of the latter strain to avoid loss of the cosmid encoding the 4-methyl-2-enelactone isomerase. For induction experiments, cells of JMP 134 were grown with fructose (5 mM) as sole carbon source. During the late-exponential growth phase 4-methyl-2-enelactone (2 mM) was added as inducer. Cells were harvested after an induction period of 2 h. For preparation of cell extracts, cells were harvested during late-exponential growth phase and suspended in Tris/HCl buffer (20 mM, pH 7.5). The cell suspensions were disrupted with a French press (Aminco, Silver Spring, MD, U.S.A.) at an internal pressure of 80 MPa and the cell debris was removed by centrifugation at 10000 g for 1 h at 4 °C.

Assay procedures for 4-methyl-2-enelactone isomerase. The activity of 4-methyl-2-enelactone isomerase was measured by reversed-phase h.p.l.c. using the solvent system described by Pieper et al. (1985). Disappearance of 4-methyl-2-enelactone, as well as formation of 3-methyl-2-enelactone, were monitored at 210 nm throughout the incubation period of 20 min. Samples were taken at intervals of 5 min and were directly analysed. During the whole incubation period, less than 10% of the substrate had been converted into product. This method was used at high concentrations of 4-methyl-2-enelactone (> 500 μM) and for measurement of enzyme activity in inhibition experiments using inhibitor concentrations of greater than 500 μM. A photometric test procedure was based on the increase of absorbance in the range of 210-230 nm during conversion of 4-methyl-2-enelactone into 3-methyl-2-enelactone (Pieper et al., 1985). Routinely, enzyme activity was measured in phosphate buffer, pH 6.5, at 220 nm. The assay mixtures contained 0.2 μl of 4-methyl-2-enelactone in 100 mM phosphate buffer. At 220 nm the difference in molar absorption was calculated to be 5400 litre·mol⁻¹·cm⁻¹, based on 4-methyl-2-enelactone 5200 litre·mol⁻¹·cm⁻¹ and 3-methyl-2-enelactone 10600 litre·mol⁻¹·cm⁻¹. Enzyme activities at substrate concentrations in excess of 0.2 mM were measured at 230 nm, to avoid absorption values in excess of 2. The molar absorption difference at 230 nm was calculated to be 2200 litre·mol⁻¹·cm⁻¹. Enzyme activities against 1-methyl-3,7-dioxo-2,6-dioxabicyclo[3.3.0]octane (1-methylbishlactone) were also measured using both h.p.l.c. and the photometric test. Because of poor absorption of this compound (ε₂₃₀ ≈ 500 litre·mol⁻¹·cm⁻¹) activity was calculated from the rate of formation of 4-methyl- and 3-methyl-2-enelactone in both analytical systems. For h.p.l.c. analysis, samples were taken at intervals of 5 min and were directly injected. During the incubation period of 20 min, less than 10% of the substrate has been converted into product. For determination of end products of conversion the reaction was monitored throughout a reaction period of 60 min with enzyme concentrations converting 90% of substrate into product within 30 min. At the beginning of the reaction 4-methyl- and 3-methyl-2-enelactone were generated at comparable rates. During this phase the photometric test was based on a molar absorption coefficient of 7900 litre·mol⁻¹·cm⁻¹ (the arithmetic mean of ε = 5200 litre·mol⁻¹·cm⁻¹ and 10600 litre·mol⁻¹·cm⁻¹ for the respective two methyl-2-enelactones). A unit of activity is the amount of protein necessary to convert 1 μmol of substrate into product/min at 25 °C.

Purification of 4-methyl-2-enelactone isomerase. Pseudomonas sp. B13 FR1 (pFRC20P) was grown in 3 litres of mineral medium containing acetate (10 mM) as growth substrate. Cells were harvested during late-exponential growth and a cell extract was prepared as described above. This extract (64.4 mg of protein in 11.2 ml) was fractionated with (NH₄)₂SO₄. Solid (NH₄)₂SO₄ was added to the extract with constant stirring to give 40% saturation. After 30 min the resulting precipitate was removed by centrifugation at 5000 g for 10 min and discarded. The supernatant was adjusted to 60% saturation. The precipitate was collected by centrifugation and redissovled in about 3 ml of Tris/HCl (20 mM, pH 7.5). Further protein purification was performed by use of an h.p.l.c. system consisting of an LCL 500 controller, pump 500, UV-1 monitor, REL-482 recorder and FRAC autosampler from Pharmacia (Uppsala, Sweden).

Hydrophobic-interaction column. The dissolved precipitate was applied to a phenyl-Superose gel column (HR 10/10; Pharmacia, Uppsala, Sweden) and eluted with 60 ml of a linear gradient of 1-0 M-(NH₄)₂SO₄ in Tris/HCl (20 mM, pH 7.5) at a flow rate of 1 ml/min. Fractions (1 ml each) were collected, the activity of the isomerase was determined and those fractions with the highest activity were pooled and concentrated by ultra-centrifugation through a membrane with an Mₘ cut-off of 10000 (Amicon, Danvers, MA, U.S.A.).

Gel-filtration column. The concentrate was applied to a Superose-6 column (HR 10/30; Pharmacia, Uppsala, Sweden) and eluted with 25 ml of Tris/HCl (20 mM, pH 7.5) containing 100 mM-NaCl at a flow rate of 0.3 ml/min. Fractions (0.5 ml each) with the highest activity were pooled. Ion-exchange column. The pooled fractions were applied to a Mono-Q column (HR 55; Pharmacia, Uppsala, Sweden). The applied sample was eluted with 30 ml of a linear gradient of 0-300 mM-NaCl in Tris/HCl buffer (20 mM, pH 7.5) at a flow rate of 0.5 ml/min. Fractions (0.5 ml each) with the highest activity were retained.

Page. Aliquots of the individual fractions from the final purification step were subjected to SDS/PAGE by the method of Laemmli (1970). The resulting gels were silver-stained by the method of Merril et al. (1981) using the Bio-Rad (Richmond, CA, U.S.A.) silver-stain kit.

Analysis of kinetic data. For determination of Michaelis
constants \((K_i)\) and \(V_{\text{max}}\) values, substrate concentrations of 30–1000 \(\mu\text{M}\) for 4-methyl-2-enelactone and of 12.5–500 \(\mu\text{M}\) for 1-methylbutyrate were used. For determination of the inhibitor constant \((K_i)\) for 4-carboxymethylbut-2-en-4-olide, inhibitor concentrations of 100–1000 \(\mu\text{M}\) were used. \(K_i\) as well as \(K_i\) and \(V_{\text{max}}\) values \(\pm\) s.d. were calculated by non-linear-regression analysis using the least-squares method (STSC Inc., 1987).

Analytical methods. Protein was determined by the Bradford (1976) procedure. The method of Scopes (1974) was used for precise quantification of homogeneous protein. The \(M\), of native enzyme was determined by gel filtration using a Superose-6 column (see under ‘Purification of 4-methyl-2-enelactone isomerase’ above) calibrated with bovine thyroglobulin \((M, 670000)\), bovine \(\gamma\)-globulin \((M, 158000)\), ovalbumin \((M, 44000)\), horse myoglobin \((M, 17000)\) and vitamin B-12 \((M, 1350)\) as references (Bio-Rad). The subunit size was determined by SDS/PAGE with rabbit myosin \((M, 205000)\), \(\beta\)-galactosidase \((M, 116000)\), rabbit phosphorylase \(b\) \((M, 97000)\), BSA \((M, 66000)\), chicken ovalbumin \((M, 45000)\), rabbit glyceraldehyde-3-phosphate dehydrogenase \((M, 36000)\), carbonic anhydrase \((M, 29000)\), soybean trypsinogen \((M, 24000)\), soybean trypsin inhibitor \((M, 20100)\) and bovine \(\gamma\)-lactalbumin \((M, 14200)\) as reference proteins (Sigma).

RESULTS

Activity of 4-methyl-2-enelactone isomerase in cell extracts

The activity of 4-methyl-2-enelactone isomerase was found in crude cell extracts of both \(A.\ eutrophus\) JMP 134 grown on 4-methyl-2-enelactone and \(Pseudomonas\) sp. B13 FR1 (pFRC20P) grown on acetate. The activities were 605 units/g of protein for \(A.\ eutrophus\) JMP 134 and 360 units/g of protein for \(Pseudomonas\) sp. B13 FR1 (pFRC20P) when measured by h.p.l.c. using a \(\mu\)-column of chemically synthesized 3-methyl-2-enelactone was determined, it showed \([\alpha]_{22}^{22} = +18.1^\circ(9.8\text{ mg/ml})\). When the optical activity of chemically synthesized (racemic) 3-methyl-2-enelactone was determined, it showed \([\alpha]_{22}^{22} = -11.0^\circ\) in water (10.3 mg/ml). These results showed that only the \((-\)-)-isomer is biologically active.

Purification of 4-methyl-2-enelactone isomerase

Purification of the enzyme was carried out using acetate-grown cells of \(Pseudomonas\) sp. B13 FR1 (pFRC20P), because large amounts of enzyme could be easily prepared by growth on this commercially available compound. A typical balance sheet of the purification is shown in Table 1. 4-Methyl-2-enelactone isomerase activity was generally eluted from the final ion-exchange chromatographic step at approx. 0.2 m-NaCl with two or three fractions containing more than 90\% of the activity. Specific activity in those fractions ranged from 30 to 80 units/mg of protein approximately, indicating that at least part of them did not contain homogeneous enzyme. The activity of the enzyme in the purest fractions (e.g. fraction B, Table 1) represents an approx. 700-fold purification with a recovery of about 8\%.

Physical properties of the enzyme

The different fractions eluted from the Mono-Q column containing 4-methyl-2-enelactone isomerase were examined for

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery of activity (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>11.2</td>
<td>7.6</td>
<td>69.4</td>
<td>0.11</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. 40–60% satn. (NH_4)_2SO_4 precipitate</td>
<td>3</td>
<td>7.0</td>
<td>39.0</td>
<td>0.18</td>
<td>92</td>
<td>1.7</td>
</tr>
<tr>
<td>3. Hydrophobic-interaction-chromatography eluate</td>
<td>2</td>
<td>4.7</td>
<td>1.46</td>
<td>3.2</td>
<td>61</td>
<td>29</td>
</tr>
<tr>
<td>4. Superose 6 gel-filtration eluate</td>
<td>1.5</td>
<td>2.9</td>
<td>0.135</td>
<td>21.5</td>
<td>38</td>
<td>195</td>
</tr>
<tr>
<td>A</td>
<td>0.5</td>
<td>0.286</td>
<td>0.0076</td>
<td>37.6</td>
<td>3.8</td>
<td>342</td>
</tr>
<tr>
<td>B</td>
<td>0.5</td>
<td>0.631</td>
<td>0.0080</td>
<td>78.9</td>
<td>8.3</td>
<td>718</td>
</tr>
<tr>
<td>C</td>
<td>0.3</td>
<td>0.140</td>
<td>0.0031</td>
<td>45.2</td>
<td>1.8</td>
<td>411</td>
</tr>
</tbody>
</table>

Table 1. Purification of 4-methyl-2-enelactone isomerase from \(Pseudomonas\) sp. B13 FR1 (pFRC20P)

Experimental details are given in the Experimental section. Individual fractions eluted from the Mono-Q column were collected in three pools: A, B and C (see the text).
Table 2. Effect of chelating agents, heavy metals and reducing agents on the activity of 4-methyl-2-enelactone isomerase

The eluate from the phenyl-Sepharose column was used as the enzyme source. The enzyme was incubated with the indicated reagents (a) for 10 min at room temperature and (b) for 18 h at 4°C before enzyme activity was determined by addition of the substrate. A relative activity of 100% corresponds to an absolute activity of 4.7 × 10⁻⁹ units/ml.

<table>
<thead>
<tr>
<th>Addition to the assay mixture</th>
<th>(a) Conc. (mm)</th>
<th>Relative enzyme activity</th>
<th>(b) Conc. (mm)</th>
<th>Relative enzyme activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>0.1</td>
<td>10</td>
<td>0.5</td>
<td>0*</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>0.01</td>
<td>50</td>
<td>0.02</td>
<td>0†</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>0.05</td>
<td>60</td>
<td>0.5</td>
<td>0†</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>1</td>
<td>100</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>1</td>
<td>100</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.02</td>
<td>100</td>
<td>0.1</td>
<td>100</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>0.05</td>
<td>100</td>
<td>0.5</td>
<td>105</td>
</tr>
<tr>
<td>Tiron</td>
<td>0.05</td>
<td>100</td>
<td>0.2</td>
<td>105</td>
</tr>
<tr>
<td>2,2'-Bipyridyl</td>
<td>0.05</td>
<td>100</td>
<td>0.2</td>
<td>105</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>0.05</td>
<td>100</td>
<td>0.2</td>
<td>105</td>
</tr>
<tr>
<td>Dithionite</td>
<td>0.1</td>
<td>100</td>
<td>2</td>
<td>95</td>
</tr>
</tbody>
</table>

* 35% of the activity was recovered after further incubation with 3 mM-dithiothreitol for 24 h at 4°C.
† Whole activity was recovered after further incubation with 3 mM-dithiothreitol for 24 h at 4°C.
‡ Irreversible inactivation.

Table 3. $K_{m}$ or $K_{cat}$ and $k_{cat}$ values of substituted lactones for 4-methyl-2-enelactone isomerase

Enzyme activity was assayed by HPLC as well as by photometric test using 0.01–0.04 μg of enzyme/ml (substrate concentration were between 5 μM and 2 mM). The kinetic constants were calculated by non-linear regression analysis. $k_{cat}$ values were calculated on the basis of $M_r$ 40000 and correspond to concentrations extrapolated to infinity. The values in parentheses were calculated from the data of Bruce et al. (1989) and are given for comparative purposes.

<table>
<thead>
<tr>
<th>Substrate or inhibitor</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_{m}$ (μM)</th>
<th>$K_{cat}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Carboxy methyl-4- methy1but-2-en-4-0lide</td>
<td>98.5 ± 5.5</td>
<td>176.1 ± 24.1</td>
<td>–</td>
</tr>
<tr>
<td>4-Carboxymethylbut-2-en-4-0lide</td>
<td>–</td>
<td>–</td>
<td>28.0 ± 2.7</td>
</tr>
<tr>
<td>1-Methyl-3,7-dioxo-2,6- dioxabicyclo(3.3.0)octane</td>
<td>37.5 ± 1.0</td>
<td>28.8 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>cis,cis-muconate and 3- methyl-cis,cis-muconate</td>
<td>37.5 ± 1.0</td>
<td>(170)</td>
<td>(170)</td>
</tr>
<tr>
<td>4-carboxy methylbut-2-en-4-0lide</td>
<td>–</td>
<td>–</td>
<td>744 ± 76</td>
</tr>
</tbody>
</table>

The mechanism of 4-methyl-2-enelactone isomerase can involve reactions of opening and closing of lactone rings. The examination of 4-methyl-2-enelactone and its analogues as substrates was extended to include 4-carboxy methylbut-2-en-4-0lide as possible intermediates. For 4-methyl-2-enelactone, a $K_{m}$ value of 170 μM (Table 3) was determined. A specific activity of 140.0 ± 7.8 μmol·min⁻¹·mg of protein⁻¹ when measured in the $V_{max}$ range was calculated. On the basis of $M_r$ 40000, the catalytic constant, $k_{cat}$, is 98.9 ± 5.5 s⁻¹. Of all other compounds tested using both the photometric and HPLC tests, only 1-methylbis lactone served as a substrate of the enzyme. Analysis by HPLC revealed that initially both 4-methyl- and 3-methyl-2-enelactone were enzymatically produced in equal amounts from 1-methylbis lactone at rates substantially higher than by chemical hydrolysis. Prolonged incubation of 1-methylbis lactone resulted in quantitative formation of 3-methyl-2-enelactone as the end product of the enzymic reaction. When the kinetics of 1-methylbis lactone metabolism by the isomerase were determined by HPLC and photometric analysis, they revealed $K_{m}$ and $k_{cat}$ values to be substantially lower than those found for 4-methyl-2-enelactone as a substrate. The maximum velocity of 1-methylbis lactone conversion by the purified enzyme was only about 40% of that found for 4-methyl-2-enelactone. As equivalent amounts of both isomeric methyl-2-enelactones are produced enzymically from 1-methylbis lactone (see above) the maximum rate of production of the 3-methyl-2-enelactone can thus only be half of the overall 1-methylbis lactone conversion rate (i.e. 20%).

The transformation rate of the following compounds was less than 1% of that found for 4-methyl-2-enelactone: 2-methyl- and 2,4-dimethyl-2-enelactone and 4-carboxymethylbut-2-en-4-0lide; trans-4-carboxymethylbut-2-en-4-0lide; cis,cis-muconate and 3-methyl-cis,cis-muconate. Of these compounds, only 4-carboxymethylbut-2-en-4-0lide exhibited a significant inhibitory effect upon the enzymatic conversion of 4-methyl-2-enelactone (Table 3). Non-linear-regression analysis showed the inhibition to be of the competitive type. A slight inhibition of $V_{max}$ of about 25% was
achieved when the enzyme was incubated with 4-methyl-2-enelactone (100 μM) and a 20-fold excess of 2,4-dimethyl-, 3-methyl-, 2-methyl-2-enelactone or trans-dienelactone. cis,cis-Muconate as well as 3-methyl-cis,cis-muconate exhibited no such effect.

DISCUSSION

A new metabolic pathway for the degradation of methylsubstituted aromatic compounds via a modified ortho-cleavage pathway has recently been described in *A. eutrophus* JMP 134 (Pieper et al., 1985) and *Rhodococcus* species (Bruce & Cain, 1988). (+)-4-Methyl-2-enelactone, described as a dead-end metabolite in some *Pseudomonas* species (Catelani et al., 1971; Knackmuss et al., 1976), can be converted by the former organisms into (−)-3-methyl-2-enelactone by a novel isomerase. This 4-methyl muconolactone methylisomerase was purified over 700-fold to electrophoretic and gel-chromatographic homogeneity from a constructed derivative of *Rhodococcus* species. The enzyme, in contrast with the tetrameric *Pseudomonas* enzyme, is a single polypeptide. In SDS/PAGE the enzyme showed an *M*<sub>s</sub> of about 4000. It is not clear at present why gel-filtration experiments yielded a considerably lower value for the native enzyme. Nevertheless, it is obvious that the native enzyme consists of a single polypeptide. This enzyme, in contrast with the tetrameric *Rhodococcus* enzyme (Bruce et al., 1989), is therefore a monomer. With respect to other properties, however, there are strong similarities between both enzymes, as they were both inhibited by thiol-modifying agents, whereas chelating agents had no effect. Superficially, there are also common kinetic properties. Other than the natural substrate 4-methyl-2-enelactone, only 1-methylbis-lactone from a wide range of putative substrates is transformed by the two isomerases.

There are also only minor differences between the two enzymes concerning the kinetic constants *K*<sub>m</sub> and *k*<sub>cat</sub> for 4-methyl-2-enelactone. With 1-methylbis-lactone, however, the *Alcaligenes* enzyme showed lower *K*<sub>m</sub> and *k*<sub>cat</sub> values compared with the *Rhodococcus* enzyme (see Table 3).

From kinetic data obtained with the *Rhodococcus* isomerase, Bruce et al. (1989) postulated 1-methylbis-lactone to be an intermediate of the isomerization reaction of 4-methyl-2-enelactone to 3-methyl-2-enelactone.

A methyl-group shift on the intact lactonic ring, however, seemed rather improbable, because the purified enzyme obviously is able to cleave lactonic ring structures. This is true also for the isomerase described here. Two principal pathway alternatives were left for consideration: 4-methyl-2-enelactone may be ring-opened to 3-methyl-cis,cis-muconate, with subsequent lactonization to the isomeric 3-methyl-2-enelactone. It could be first lactonized to a bis-lactone in which opening of the opposite ring yields 3-methyl-2-enelactone. Such an involvement of bis-lactonic structures in aromatic catabolism was postulated by Eldsden & Peol (1988), but was considered later to be an artefact of isolation. 1-Methylbis-lactone, however, was found to be a substrate for 4-methyl-2-enelactone isomerase, yielding both 3-methyl- and 4-methyl-2-enelactone as products, which is in contrast with the *Rhodococcus* enzyme, where only 3-methyl-2-enelactone was produced. Both enzymes are obviously able to cleave lactonic ring structures. Whereas cleavage only of a lactone ring is necessary for conversion of 1-methylbis-lactone, the overall enzymic conversion of 4-methyl-2-enelactone isomerization includes both cleavage and ring formation. Therefore a more refined consideration has to take into account three reaction paths.

(1) The simplest model would assume ring cleavage and ring formation to occur in sequence, producing free intermediates. Kinetic data presented exclude such a mechanism that involves either 3-methyl-cis,cis-muconate or methylbis-lactone as free intermediates, because (i) 3-methyl-cis,cis-muconate is not a substrate for the enzyme and (ii) 1-methylbis-lactone is converted more slowly into 3-methyl-2-enelactone than is 4-methyl-2-enelactone. This is in contrast with the *Rhodococcus* enzyme, where 1-methylbis-lactone is converted more quickly than 4-methyl-2-enelactone.

(2) 3-Methyl-cis,cis-muconate or 1-methylbis-lactone could, however, be non-covalently bound intermediates. Analysis of the specificity constants (*K*<sub>cat</sub>/*K*<sub>m</sub>) of the isomerase for 4-methyl-2-enelactone (5.6 x 10<sup>4</sup> ± 0.8 x 10<sup>3</sup> s<sup>-1</sup> M<sup>-1</sup>) and 1-methylbis-lactone (1.34 x 10<sup>4</sup> ± 0.13 x 10<sup>3</sup> s<sup>-1</sup> M<sup>-1</sup>) indicates that the *K*<sub>m</sub> for these substrates approximates the dissociation constant of the enzyme-substrate complex, *K*<sub>s</sub> (Fersht, 1985). With the data from Table 3 this interpretation would indicate that 1-methylbis-lactone was bound better, but converted more slowly, by the isomerase than is 4-methyl-2-enelactone and that non-covalently bound 1-methylbis-lactone is unlikely to be an intermediate in the overall reaction. Because 3-methyl-cis,cis-muconate failed to serve as a substrate and also to exhibit any inhibitory effect, there is no indication for its involvement in the enzymic reaction.

(3) The analysis by Ngai et al. (1983) of the cycloisomerization of cis,cis-muconate to 4-carboxymethylbut-2-en-4-olide considered mechanisms involving a carbamion, a carbonium ion or a covalently bound intermediate. By analogy, the enzymic conversion of 4-methyl-2-enelactone to 3-methyl-2-enelactone may involve the four steps, a-d (Scheme 1). The exclusion of 1-methylbis-lactone and 3-methyl-cis,cis-muconate as free intermediates, however, implies either very improbable double-charged transition states or, more likely, a covalently bound transition state during the course of the reaction. A possible mechanism is shown in Scheme 1, in which attack of the lactone ring of 4-methyl-2-enelactone by an enzyme nucleophile results in a covalently bound intermediate. Ring closure in the opposite

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Scheme 1. Hypothetical mechanism of isomerization of 4-methyl-lactone to 3-methyl-lactone

As described in the text, isomerization of 4-methyl-lactone should take place through reaction steps a-d, similar to those described by Ngai et al. (1983). 1-Methylbis-lactone could be directly isomerized to 3-methyl-lactone (step e and d) or transformed into 4-methyl-lactone (step f and g). Z represents an OH group or an enzyme nucleophile.
direction (reaction b, Scheme 1) results in a formal ‘shift’ of the methyl group in the newly formed lactone, although it would remain attached to the same carbon atom. Further studies using deuterated and tritiated forms of the substrates should permit to determine action of syn or anti modes of lactone ring formation and opening and exclude finally a methyl-migration mechanism.

The enzymic conversion of 1-methylbis lactone into both 3- and 4-methyl-2-ename lactones may be explained if the 1-methylbis lactone is considered as a structure partially analogous to 4- methyl-2-ename lactone, but on which enzymic attack could occur on either lactonic ring. Transient accumulation of 4-methyl-2-ename lactone from 1-methylbis lactone could be explained by the latter binding in an ‘incorrect’ orientation so that the resultant ring-opening will produce 4-methyl- rather than the natural 3- methyl-2-ename lactone product (reactions f and g, Scheme 1); the former compound would subsequently undergo normal isomerization to the natural product which eventually accumulated.

REFERENCES


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