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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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4-Carboxymethyl-4-methylbut-2-en-4-olide (4-methyl-2-enelactone) isomerase, transforming 4-methyl-2-enelactone to 3-methyl-2-enelactone, was purified from a derivative strain of *Pseudomonas* sp. B13, named B13 FR1, carrying the plasmid pFRC2OP. This plasmid contained the isomerase gene cloned from *Alcaligenes eutrophus* JMP 134, which uses 4-methyl-2-enelactone as a carbon source. The enzyme consists of a single peptide chain of M_r 40000 as judged by SDS/PAGE. In addition to 4-methyl-2-enelactone, the putative reaction intermediate, 1-methyl-3,7-dioxo-2,6-dioxy-bicyclo[3.3.0]octane (1-methylbislactone), was a substrate for the enzyme, but kinetic data presented did not favour its role as a reaction intermediate. Isomeric methyl-substituted 4-carboxymethylbut-2-en-4-olides were neither substrates nor inhibitors. Possible reaction mechanisms are discussed.

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-carboxymethylbut-2-en-4-olides (trivially methyl-2-enelactones) as dead-end metabolites (Catelani *et al.*, 1971; Knackmuss *et al.*, 1976). Assimilation of methylcatechols via an *ortho*-cleavage pathway was postulated by Miller (1981) and subsequently demonstrated by Pieper *et al.* (1985), Powlowski & 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 circumvented by means of an enzyme-catalysed isomerization to 3-methyl-2-enelactone. 3-Methyl-2-enelactone was further degraded analogously to the classical 3-oxoadipate pathway.

Whereas in bacteria such as *Alcaligenes eutrophus* JMP 134 and *Pseudomonas desmolyticum* (Catelani *et al.*, 1971) 3-methyl-*cis*,*cis*-muconate, produced from 4-methylcatechol, is cycloisomerized to 4-methyl-2-enelactone, the fungus *Trichosporon cutaneum*, in contrast, cycloisomerizes 3-methyl-*cis*,*cis*-muconate directly to 3-methyl-2-enelactone (Powlowski & Dagley, 1985). Miller (1981) believed that this latter mode of cycloisomerization occurred also in the actinomycete *Rhodococcus ruber* N75, but recently Bruce & Cain (1988) showed that this organism, like *A. eutrophus* JMP 134 (Pieper *et al.*, 1985), degraded 4-methylcatechol via 4-methyl-2-enelactone as an intermediate, which in turn was metabolized by a 4-methyl-2-enelactone isomerase. The *Rhodococcus* enzyme has recently been purified and characterized and 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 the 3-methyl-2-enelactone-degrading *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 (pFRC2OP) 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 (pFRC2OP) 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-en-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 mineral 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 (pFRC2OP). 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 100000 *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 μmol 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 $\epsilon_{4\text{-methyl-2-enelactone}}$ 5200 litre·mol^{-1}·cm^{-1} and $\epsilon_{3\text{-methyl-2-enelactone}}$ 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-methylbislactone) were also measured using both h.p.l.c. and the photometric test. Because of poor absorption of this compound ($\epsilon_{210} \approx$ 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* B13 FR1 (pFRC2OP) 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 $(NH_4)_2SO_4$. Solid $(NH_4)_2SO_4$ 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 redissolved 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-$(NH_4)_2SO_4$ 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 ultracentrifugation through a membrane with an M_r 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 50–300 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_m) and $V_{max.}$ values, substrate concentrations of 30–1000 μM for 4-methyl-2-enelactone and of 12.5–500 μM for 1-methylbislactone were used. For determination of the inhibitor constant (K_i) for 4-carboxymethylbut-2-en-4-olide, inhibitor concentrations of 100–1000 μM were used. K_m as well as K_i and $V_{max.}$ values ± 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_r 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_r 670000), bovine γ-globulin (M_r 158000), ovalbumin (M_r 44000), horse myoglobin (M_r 17000) and vitamin B-12 (M_r 1350) as references (Bio-Rad). The subunit size was determined by SDS/PAGE with rabbit myosin (M_r 205000), β-galactosidase (M_r 116000), rabbit phosphorylase *b* (M_r 97000), BSA (M_r 66000), chicken ovalbumin (M_r 45000), rabbit glyceraldehyde-3-phosphate dehydrogenase (M_r 36000), carbonic anhydrase (M_r 29000), soybean trypsinogen (M_r 24000), soybean trypsin inhibitor (M_r 20100) and bovine γ-lactalbumin (M_r 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 (pFRC2OP) 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 (pFRC2OP) when measured by h.p.l.c. using a substrate concentration of 2 mM. Activities from both organisms showed pH optima at pH 6.5–7 in phosphate buffer. Variation of buffer concentrations in the range between 10 mM and 1 M had little influence upon activity.

Configuration of 3-methyl-2-enelactone

To characterize the product of the isomerase reaction, (+)-4-methyl-2-enelactone (10 mM in 300 ml of phosphate buffer, pH 6.5) was treated (3 h, 30 °C) with an extract (20 ml, 100 mg of protein) from cells of *A. eutrophus* JMP 134 induced with this lactone. After total conversion the culture fluid was acidified with H_3PO_4 to pH 2.5 and centrifuged at 5000 *g* for 10 min. 3-Methyl-2-enelactone was extracted from the supernatant and purified as previously described for chemically synthesized 3-methyl-2-enelactone (Pieper *et al.*, 1985). The optical activity of this biologically prepared 3-methyl-2-enelactone gave $[\alpha]_D^{22} = -22.4°$ in water (10.5 mg/ml). Cells of *A. eutrophus* JMP 134 were incubated with this biologically prepared material or with chemically synthesized 3-methyl-2-enelactone (5 mM). Whereas biologically synthesized 3-methyl-2-enelactone was degraded totally, only 70% of chemically synthesized 3-methyl-2-enelactone was transformed. This indicated that the chemical preparation consisted of a mixture of both optical isomers. To produce larger amounts of the non-degradable enantiomer of 3-methyl-2-enelactone, whole cells of JMP 134, induced with (+)-4-methyl-2-enelactone (2 mM), were incubated in 200 ml of phosphate buffer, pH 7.4, with chemically synthesized racemic 3-methyl-2-enelactone (2 mM). When turnover was complete cells were removed by centrifugation and the undegraded 3-methyl-2-enelactone was purified as previously described for the chemically synthesized 3-methyl-2-enelactone (Pieper *et al.*, 1985). The optical activity of this preparation of 3-methyl-2-enelactone was found to be $[\alpha]_D^{22} = +18.1°$ (9.8 mg/ml). When the optical activity of chemically synthesized (racemic) 3-methyl-2-enelactone was determined, it showed $[\alpha]_D^{22} = -11.0°$ 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 (pFRC2OP), 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 1. Purification of 4-methyl-2-enelactone isomerase from *Pseudomonas* sp. B13 FR1 (pFRC2OP)

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).

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

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-Superose 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^{-3} units/ml.

Addition to the assay mixture	(*a*) Conc. (mM)	(*a*) Relative enzyme activity	(*b*) Concn. (mM)	(*b*) Relative enzyme activity (%)
None	–	100	–	100
$CuSO_4$	0.1	10	0.5	0*
p-Chloromercuribenzoate	0.01	50	0.02	0†
N-Ethylmaleimide	0.05	60	0.5	0‡
Dithiothreitol	1	100	1	100
2-Mercaptoethanol	1	100	5	105
EDTA	0.5	100	5	100
1,10-Phenanthroline	0.02	100	0.1	100
Tiron	0.05	100	0.5	105
2,2'-Bipyridyl	0.05	100	0.2	110
8-Hydroxyquinoline	0.05	100	0.2	105
Dithionite	0.1	100	2	95

* 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.

purity by SDS/PAGE. Whereas the peak fraction B (see Table 1) revealed a single protein band of M_r 40000 (±2000), small amounts of impurities were found in the flanking fractions A and C. Generally, whenever a high isomerase activity was found, the protein band of M_r 40000 was clearly dominant. Gel filtration of the peak fraction B exhibited a homogeneous protein band, the M_r of which was calculated to be 25000 (±2000). This M_r was reproducibly determined throughout repeated gel-filtration purification procedures. Several chelating agents (Table 2) had no effect on the enzyme activity. In contrast, $CuSO_4$ significantly inhibited the enzyme. Inhibition was also achieved by addition of *N*-ethylmaleimide or *p*-chloromercuribenzoate. Inactivation with *p*-chloromercuribenzoate could be reversed by addition of excess dithiothreitol. Inactivation with *N*-ethylmaleimide, however, was irreversible. Whereas the crude or partially purified enzyme (step 3 in Table 1) could be stored for several weeks at 4 °C or −20 °C with slight loss of activity, the highly purified enzyme was rather unstable. About 10% of activity was lost within 24 h of storage at 4 °C. Addition of thiol-group-modifying reagents like dithiothreitol or chelating agents like EDTA significantly stabilized the enzyme. Under these conditions loss of activity was only 5%/day.

Binding and turnover of substituted lactones and muconates

The mechanism of 4-methyl-2-enelactone isomerase could 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 muconates and 1-methyl-3,7-dioxo-2,6-dioxabicyclo[3.3.0]octane (1-methylbislactone) as possible intermediates. For 4-methyl-2-enelactone, a K_m value of 176 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 h.p.l.c. tests, only 1-methylbislactone served as a substrate of the enzyme. Analysis by h.p.l.c. revealed that initially both 4-methyl- and 3-methyl-2-enelactone were enzymically produced in equal amounts from 1-methylbislactone at rates substantially higher than by chemical hydrolysis. Prolonged incubation of 1-methylbislactone resulted in quantitative formation of 3-methyl-2-enelactone as the end product of the enzymic reaction. When the kinetics of 1-methylbislactone metabolism by the isomerase were determined by h.p.l.c. 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-methylbislactone 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-methylbislactone (see above) the maximum rate of production of the 3-methyl-2-enelactone can thus only be half of the overall 1-methylbislactone 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-olide; *trans*-4-carboxymethylbut-2-en-4-olide; *cis,cis*-muconate and 3-methyl-*cis,cis*-muconate. Of these compounds, only 4-carboxymethylbut-2-en-4-olide exhibited a significant inhibitory effect upon the enzymic 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

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

Enzyme activity was assayed by h.p.l.c. 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.

Substrate or inhibitor	$k_{cat.}$ (s^{-1})	K_m (μM)	K_i (μM)
4-Carboxymethyl-4-methylbut-2-en-4-olide	98.5 ± 5.5 (150)	176.1 ± 24.1 (230)	–
4-Carboxymethylbut-2-en-4-olide	–	–	744 ± 76
1-Methyl-3,7-dioxo-2,6-dioxabicyclo[3.3.0]octane	37.5 ± 1.0 (170)	28.0 ± 2.7 (170)	–

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 methyl-substituted 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 *Pseudomonas* sp. B13, named *Pseudomonas* sp. B13 FR1 (pFRC20P), which harbours the gene coding for this isomerase from *A. eutrophus* JMP 134. In SDS/PAGE the enzyme showed an M_r of about 40000. It is not clear at the moment 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. The 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-methylbislactone 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_m and $k_{cat.}$ for 4-methyl-2-enelactone. With 1-methylbislactone, however, the *Alcaligenes* enzyme showed lower K_m and $k_{cat.}$ values compared with the *Rhodococcus* enzyme (see Table 3).

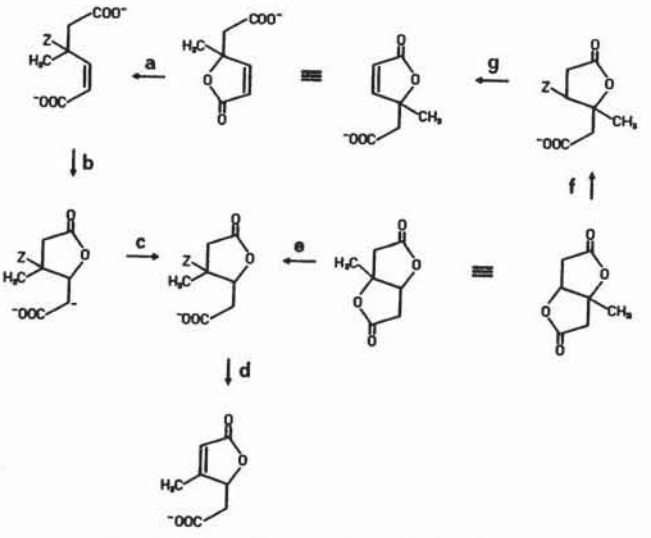
From kinetic data obtained with the *Rhodococcus* isomerase, Bruce *et al.* (1989) postulated 1-methylbislactone to be an intermediate of the isomerization reaction of 4-methyl-2-enelactone to 3-methyl-2-enelactone.

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 bislactone in which opening of the opposite ring yields 3-methyl-2-enelactone. Such an involvement of bislactonic structures in aromatic catabolism was postulated by Elsden & Peel (1958), but was considered later to be an artefact of isolation. 1-Methylbislactone, 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-methylbislactone, 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 methylbislactone as *free* intermediates, because (i) 3-methyl-*cis,cis*-muconate is not a substrate for the enzyme and (ii) 1-methylbislactone 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-methylbislactone is converted more quickly than 4-methyl-2-enelactone.

(2) 3-Methyl-*cis,cis*-muconate or 1-methylbislactone could, however, be non-covalently bound intermediates. Analysis of the specificity constants ($k_{cat.}/K_m$) of the isomerase for 4-methyl-2-enelactone ($5.6 \times 10^5 \pm 0.8 \times 10^5$ $s^{-1} \cdot M^{-1}$) and 1-methylbislactone ($1.34 \times 10^6 \pm 0.13 \times 10^6$ $s^{-1} \cdot M^{-1}$) indicates that the K_m for these substrates approximates the dissociation constant of the enzyme–substrate complex, K_s (Fersht, 1985). With the data from Table 3 this interpretation would indicate that 1-methylbislactone was bound better, but converted more slowly, by the isomerase than is 4-methyl-2-enelactone and that non-covalently bound 1-methylbislactone 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 carbanion, a carbonium ion or a covalently bound intermediate. By analogy, the enzymic conversion of 4-methyl-into 3-methyl-2-enelactone may involve the four steps, a–d (Scheme 1). The exclusion of 1-methylbislactone and 3-methyl-*cis,cis*-muconate as free intermediates, however, implicates 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



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-Methylbislactone 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-methylbislactone into both 3- and 4-methyl-2-enelactones may be explained if the 1-methylbislactone is considered as a structure partially analogous to 4-methyl-2-enelactone, but on which enzymic attack could occur on either lactonic ring. Transient accumulation of 4-methyl-2-enelactone from 1-methylbislactone 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-enelactone product (reactions f and g, Scheme 1); the former compound would subsequently undergo normal isomerization to the natural product which eventually accumulated.

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