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cis,cis-Muconate cyclase from *Trichosporon cutaneum*

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The inducible enzyme catalysing the conversion of *cis,cis*-muconate to (+)-muconolactone was purified 300-fold from the yeast *Trichosporon cutaneum*, grown on phenol. The enzyme has a sharp pH optimum at pH 6.6. It reacts also with several monohalogen derivatives and with one monomethyl derivative of *cis,cis*-muconate, but not with *cis,trans*- or *trans,trans*-muconate or 3-carboxy-*cis,cis*-muconate. In contrast with the corresponding enzymes in bacteria, the yeast enzyme does not require added divalent metal ions for activity and is not inhibited by EDTA. The purified enzyme can be resolved into two peaks by isoelectric focusing. The two forms have pI 4.58 (*cis,cis*-muconate cyclase I) and pI 4.74 (*cis,cis*-muconate cyclase II), respectively. Each of these is homogenous on polyacrylamide-gel electrophoresis in the absence or presence of sodium dodecyl sulphate. The two enzyme forms have the same molecular weight (50000) as determined by gel filtration and by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. They have the same K_m value (25 μM) for *cis,cis*-muconate. They differ with respect to their content of free thiol groups. *cis,cis*-Muconate cyclase I contains one thiol group, essential for activity, but relatively stable upon storage. *cis,cis*-Muconate cyclase II contains two thiol groups that are readily oxidized during storage with concomitant loss of activity.

Degradation of aromatic compounds has been studied mainly in procaryotes. The β-ketoadipate pathway has been reviewed by Stanier & Ornston (1973). However, Cain and coworkers have studied aromatic metabolism in certain yeasts and mycelial fungi (Cain *et al.*, 1968; Cook & Cain, 1974; Huber *et al.*, 1975). The enzyme catalysing the conversion of the ring fission product of protocatechuate to 3-carboxymuconolactone in *Aspergillus niger* was purified and characterized by Thatcher & Cain (1974*a*,*b*). Our group has earlier studied the enzymes involved in the metabolism of simple phenols in yeast (Neujahr & Varga, 1970; Neujahr *et al.*, 1974). We have demonstrated that, in *Trichosporon cutaneum*, the metabolism of phenol follows the known β-ketoadipate pathway, whereas that of resorcinol involves different metabolites and, partly, also different enzymes (Neujahr, 1978; Gaal & Neujahr, 1979, 1980). The first two enzymes leading to the cleavage of the aromatic ring, phenol hydroxylase (EC 1.14.13.7) and catechol 1,2-oxygenase (EC 1.13.11.1), are common to both pathways. They have been purified to homogeneity and studied in detail (Varga & Neujahr, 1970; Neujahr & Gaal, 1973, 1975; Neujahr & Kjellén, 1978). The present paper describes the purification and characterization of the third enzyme of phenol metabolism in *T. cutaneum*, catalysing the formation of (+)-4-carboxymethylbut-2-en-4-olide from *cis,cis*-muconate. Such an enzyme has not been isolated before from a eucaryotic organism. The yeast enzyme differs in several respects from the enzyme catalysing a similar reaction in *Pseudomonas putida* (Sistrom & Stanier, 1954; Ornston, 1966*b*; Meagher & Ornston, 1973).

Experimental

Materials

Equipment and commercially available chemicals were as described previously (Gaal & Neujahr, 1979). *cis,cis*-Muconic acid was prepared by oxidizing phenol with peracetic acid; *trans,trans*-muconic acid was prepared by inversion of *cis,cis*-muconic acid (Elvidge *et al.*, 1950*a*). *cis,trans*-Muconic acid was prepared from *cis,cis*-muconic acid by inversion in boiling water (Elvidge *et al.*, 1950*b*). Trisodium β-carboxy-*cis,cis*-muconate was prepared from vanillin (Ainsworth & Kirby, 1968). Halogen and methyl derivatives of *cis,cis*-muconate were prepared enzymically (Gaal & Neujahr, 1979).

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Analytical procedures

Protein was measured by the biuret method (Gornall *et al.*, 1949), or it was calculated from the u.v. absorbance at 280 and 260nm (Layne, 1957). Carbohydrate was analysed by the phenol method (Dubois *et al.*, 1956). Thiol groups were determined with 5,5'-dithiobis-(2-nitrobenzoic acid) as described by Habeeb (1972). A molar absorption coefficient of $13600\,M^{-1}\cdot cm^{-1}$ at 412nm was used for each thiol group.

Polyacrylamide-gel electrophoresis and molecular weight determination

The standard disc electrophoresis method (Davis, 1964) and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber *et al.*, 1972) were used with Bromophenol Blue as front indicator. After completion of runs, the gel rods were cut off at the front, stained with Amido Black and destained electrophoretically. Molecular weight was also determined by gel filtration (Andrews, 1965) on a Sephadex G-150 column (2.5cm × 85cm) equilibrated with 50mM-Tris/H_2SO_4, pH7.6.

Electrofocusing

Commercial Ampholine solution (3.5ml of 40% w/v, pH4–6) was fractionated by electrofocusing for 15h at 1200V. The fractions between pH4.0 and 5.0 were pooled. The pooled solution was supplemented with 0.3ml of Ampholine solution (40% w/v, pH5–8). This mixture was used for the isoelectric focusing of the enzyme, which was applied as a narrow zone at pH5.0 (3ml of solution, approx. 25mg of protein), after the pH gradient had been formed.

Enzyme assays

cis,cis-Muconate cyclase was determined spectrophotometrically (Sistrom & Stanier, 1954) in 1.0cm cells at room temperature by monitoring the disappearance of its substrate (ΔA_{260}/min). The final volume of 1.0ml contained 33mM-Mes (4-morpholine-ethanesulphonic acid) buffer adjusted to pH6.6 with NaOH, 0.1μmol of *cis,cis*-muconate and 0.5–200 mg of enzyme protein. In this system, one enzyme unit is equivalent to a decrease of 17.2 absorbance units/min.

Induction and purification of the enzyme

Cultures (50 litres) of *T. cutaneum* were grown in an aerated fermentor with phenol as carbon source. Essentially the same procedure was used as described by Varga & Neujahr (1970). Phenol concentration in the culture medium was measured with a bioprobe as described by Neujahr & Kjellén (1979). The cell paste was stored at −25°C until disrupted. All buffers and purification steps (listed in Table 1) were as described previously (Gaal & Neujahr, 1980).

Identification of the reaction product

Muconate cyclase I (Table 1) was used as enzyme source. The reaction mixture contained 100 units of enzyme, 100ml of 0.05M-potassium phosphate buffer, pH6.6, and 1mM-2-mercaptoethanol. The incubation and isolation were carried out by a modification of the procedure of Ornston & Stanier (1966). Portions of about 50mg of *cis,cis*-muconic acid (0.85g total) were added to the reaction mixture during 2h of stirring at 30°C. The pH was maintained between 6.4 and 6.7 by adding 0.1M-NaOH and *cis,cis*-muconic acid. The enzymic conversion was followed spectrophotometrically at 260nm with small aliquots. When the reaction approached completion, the pH was adjusted to 5.5 and the incubation was continued for another 20min. The solution was then acidified to pH2.0 with conc. H_2SO_4 and was extracted continuously with diethyl ether for 48h. The diethyl ether was evaporated *in vacuo* and the residual water was

Table 1. *Purification of cis,cis-muconate cyclase from Trichosporon cutaneum*

Step	Volume (ml)	Protein concn. (mg/ml)	Specific activity (units/mg)	Purification factor (-fold)	Recovery (%)
1. Crude extract	975	11.7	0.39	—	—
2. Protamine-treated supernatant	1020	6.9	0.50	0.91	79
3. DEAE-Sephadex A-50 eluate crude separation	550	4.8	1.45	2.6	86
4. DEAE-Sephadex A-50 eluate	125	1.8	12.1	22.0	61
5. Hydroxyapatite eluate	50	1.0	27.9	50.7	31
6. Sephadex G-150 eluate	106	0.28	42.4	77.1	28
7. Isoelectric focusing					25*
Peak fraction 1†	2.0	0.56	180	327	4.4
Peak fraction 2†	2.0	0.60	210	381	5.7

* Total recovery in peak 1 + 2.

† As marked in Fig. 1; 1 corresponds to enzyme I, 2 corresponds to enzyme II.

removed in a freeze-dryer. The remaining yellowish oil was dissolved in a minimum amount of ethyl acetate. Crystallization was effected by cooling to −25°C. The crude crystals were recrystallized from ethyl acetate/benzene (1:1,v/v) at 4°C, yielding colourless crystals, m.p. 75–76°C (uncorrected). The yield was 0.62g (73%). The optical rotation was $[\alpha]_D^{24} + 83.4°$ (*c* 2 in ethyl acetate). Literature values: m.p. 76–77.5°C; $[\alpha]_D^{24} + 53.2$ (Sistrom & Stanier, 1954); m.p. 75°C (Ornston & Stanier, 1966).

Results

Yield and homogeneity of cis,cis-muconate cyclase purified from phenol-grown cells

The results of the final purification procedure, repeated three times with different batches of cells, are summarized in Table 1. The enzyme in the most active fractions was purified more than 300-fold, with a total recovery of about 10%. The enzyme was eluted as a single activity peak from DEAE-Sephadex A-50, from Sephadex G-150 and from hydroxyapatite. However, on isoelectric focusing, it separated into two peaks (Fig. 1). Peak 1 had pI4.58, peak 2 had pI4.74. They will be referred to as *cis,cis*-muconate cyclase I (enzyme I) and *cis,cis*-muconate cyclase II (enzyme II), respectively. The two forms were detectable on disc electrophoresis gels even before the electrofocusing step (results not shown).

Purified enzyme was homogenous on disc electrophoresis, even when the enzyme had been stored frozen for several weeks (Fig. 2, gels 1 and 2). In contrast, stored enzyme II gave two bands on disc electrophoresis (Fig. 2, gels 4 and 5). The slower moving band was absent from enzyme II freshly incubated with a thiol compound (Fig. 2, gel 6). This correlates with the higher content and higher reactivity of thiol groups in enzyme II and in enzyme I, as discussed in a subsequent section.

Physical properties of the enzyme

The u.v.-absorption spectrum of either enzyme I or enzyme II exhibits a single peak at 278nm. There is no detectable peak in the visible region. Molecular weight determination by gel filtration (Fig. 3*a*) gave a value of 52000 ± 4000. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of enzyme I or enzyme II (Fig. 3*b*) gave a single protein band corresponding to a mol.wt. of 48000 ± 2000. These results indicate that enzymes I and II have a similar molecular weight (close to 50000) and that each form probably consists of a single polypeptide chain.

pH optimum, substrate saturation and substrate specificity

The enzyme has a relatively narrow activity plateau at pH6.6 with less than 50% activity below

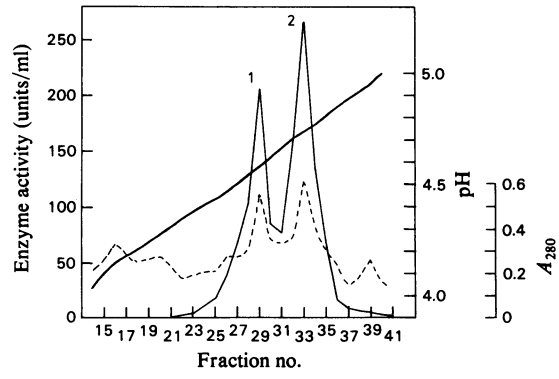

Fig. 1. *Resolution of cis,cis-muconate cyclase into two forms by isoelectric focusing*

——, Enzyme activity; ——, pH; – – –, absorbance; 1, enzyme, pI4.58; 2, enzyme II, pI4.74. Other details were as described in the Experimental section.

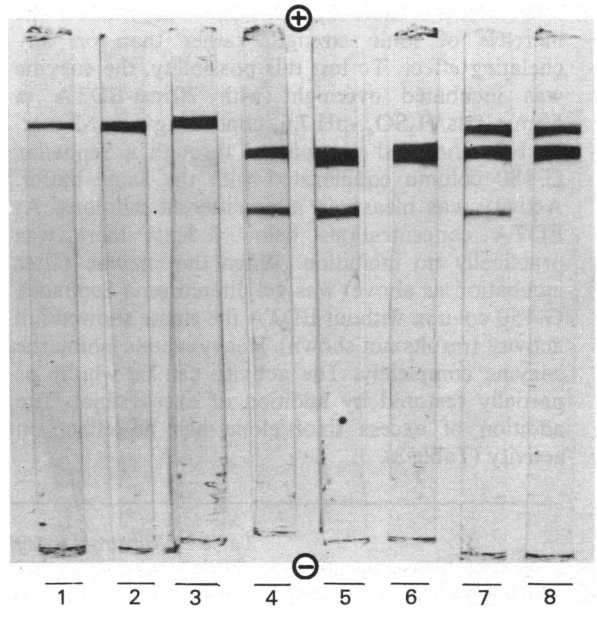

Fig. 2. *Polyacrylamide-disc-gel electrophoresis of purified cis,cis-muconate cyclase isoenzymes I and II (cf. Fig. 1)*

All samples were enzyme preparations stored for several weeks at −25°C after the last purification step. The approximate amounts of enzyme applied are shown in μg of protein. Other details were as described in the Experimental section. 1, Enzyme I, 10μg; 2, enzyme I, 25μg; 3, enzyme I, 25μg, freshly incubated with 0.1M-2-mercaptoethanol for 30min; 4, enzyme II, 20μg; 5, enzyme II, 35μg; 6, enzyme II, 35μg, freshly incubated with 0.1M-2-mercaptoethanol for 30min; 7, a mixture of enzymes I and II, 15μg each; 8, a mixture of enzymes I and II, 15μg each, freshly incubated with 0.1M-2-mercaptoethanol for 30min.

pH 6 or over pH 7 (Fig. 4). In sodium citrate buffer, the activity is less than 20% of that in 4-morpholine-ethane-sulphonate or phosphate buffers at the same pH. Enzymes I and II have the same K_m value for *cis,cis*-muconate. The K_m, as determined from double-reciprocal plots, is 25 μM. The enzyme is also active against several *cis,cis*-muconate derivatives, as shown in Table 2. However, there is no detectable activity against *cis,trans*- and *trans,trans*-muconate or against 3-carboxy-*cis,cis*-muconate.

Effect of divalent metal ions, chelating agents and heavy metals

The activity of the enzyme remains practically unchanged upon prolonged dialysis against 50 mM-Tris/H_2SO_4 buffer, pH 7.6, containing 1 mM-2-mercaptoethanol and 0.1 mM-EDTA. Divalent metal ions and chelating agents have no significant effect on enzyme activity (Table 3). However, high concentrations of EDTA (5–20 mM) do have inhibitory effects (Table 3). This may depend on the increase of ionic strength rather than on any chelating effect. To test this possibility, the enzyme was incubated overnight with 20 mM-EDTA in 50 mM-Tris/H_2SO_4, pH 7.6, containing 1 mM-2-mercaptoethanol and was passed through a Sephadex G-150 column equilibrated with the same buffer. Activity was measured after different dilutions. At EDTA concentrations below 1.5 mM there was practically no inhibition. When the enzyme (after incubation as above) was gel filtered on a Sephadex G-150 column without EDTA the eluate showed full activity (results not shown). Heavy metals inhibit the enzyme completely. The activity can be wholly or partially restored by addition of excess thiol. The addition of excess thiol alone has no effect on activity (Table 3).

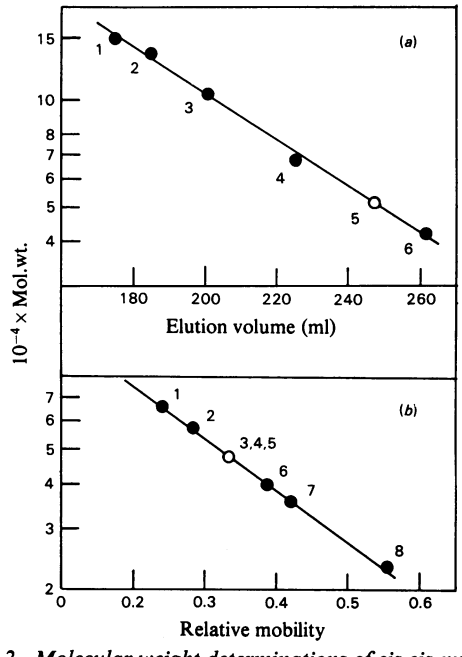

Fig. 3. *Molecular weight determinations of cis,cis-muconate cyclase*

●, Reference proteins; ○, *cis,cis*-muconate cyclase. Other details were as described in the Experimental section. (*a*) Gel filtration on Sephadex G-150. 1, Alcohol dehydrogenase (mol.wt. 150000); 2, lactate dehydrogenase (mol.wt. 136000); 3, hexokinase (mol.wt. 105000); 4, avidin (mol.wt. 68000); 5, *cis,cis*-muconate cyclase, eluate from Sephadex G-150 (step 6 of Table 1); 6, peroxidase (mol.wt. 42000). (*b*) sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. 1, Bovine serum albumin (mol.wt. 68000); 2, catalase (mol.wt. 58000); 3, enzyme I; 4, enzyme II; 5, enzyme I + enzyme II; 6, aldolase (mol.wt. 40000); 7, glyceraldehyde 3-phosphate dehydrogenase (mol.wt. 36000); 8, trypsin (mol.wt. 23300).

Table 2. *Substrate specificity of cis,cis-muconate cyclase*

Activity measurements were carried out as described in the Experimental section except that lower amounts (0.05 μmol) of *trans,trans*-muconic acid were used as substrate. Details of the enzyme assays with halogen and methyl derivatives of *cis,cis*-muconate were as described by Gaal & Neujahr (1979). An enzyme activity of 100% corresponds to a ΔA_{260} of −1.50/min. The detection limit was 0.03%.

Substrate	Source of enzyme ...	Relative enzyme activity (%) I	Step 6 of Table 1*	II
cis,cis-Muconate		100	100	100
trans,trans-Muconate		<0.03	<0.03	<0.03
cis,trans-Muconate		<0.03	<0.03	<0.03
3-Carboxy-*cis,cis*-muconate		<0.03	<0.03	<0.03
2-Fluoro-*cis,cis*-muconate			7	
3-Fluoro-*cis,cis*-muconate			20	
3-Chloro-*cis,cis*-muconate			26	
2-Methyl-*cis,cis*-muconate			<0.5	
3-Methyl-*cis,cis*-muconate			25	

* Immediately preceding the electrofocusing step.

Table 3. *Effect of divalent metal ions, chelating agents, heavy metals and reducing agents on the activity of cis,cis-muconate cyclase*

Eluate from the Sephadex G-150 column (step 6 of Table 1) was used as the enzyme source. If not otherwise stated, the enzyme was incubated with the indicated reagents for 3 min before the addition of the substrate. Other details of the assay are described in the Experimental section. 100% activity corresponds to an ΔA_{260} of −0.67/min.

Addition to the assay mixture	Concentration (mM)	Relative enzyme activity (%)
None	—	100
$MnSO_4$	1	88*†
	10	81
$MgSO_4$	1	102*†
	10	98
$FeSO_4$	1	72
$CaCl_2$	1	102
	10	70
$ZnCl_2$	0.1	40
	1	5
$CuSO_4$	0.1	<1
$CuSO_4$ + dithiothreitol	0.1 + 10	98
$AgNO_3$	0.1	<1
$AgNO_3$ + dithiothreitol	0.1 + 10	35
$HgCl_2$	0.01	<1
$HgCl_2$ + dithiothreitol	0.01 + 10	101
p-Mercuribenzoate	0.1	<1†
p-Mercuribenzoate + 2-mercaptoethanol	0.01 + 10	73†
Dithiothreitol	10	98†
EDTA	1	99†
	5	71†
	20	35
Tiron	0.1	94
	1	84
1,10-*o*-Phenanthroline	0.02	90
α-α-Dipyridyl	0.1	90
Sodium diethyldithiocarbamate	0.03	92

* Essentially the same results after incubation overnight at 5°C.
† Essentially the same results with enzymes I and II.

Thiol groups

Reduced thiol groups are essential for activity (Table 3), but it is not clear whether they are involved in actual catalysis or whether they influence catalysis although situated further away from the active site. Determination of the thiol content in the two enzyme forms under dissociating conditions gave the results shown in Fig. 5. Enzyme I contains nearly one reactive thiol group/molecule and enzyme II approx. 0.6 thiol group/molecule (mol.wt. 50000) when the enzymes are stored without added thiol.

In freshly reduced enzyme II (freed from excess thiol by gel filtration), the reaction rate with 5,5′-dithiobis-(2-nitrobenzoic acid) is rapid until one thiol group has reacted and then slows down. After 5 h the reaction seems to level off to give nearly two thiol groups/molecule of enzyme. The corresponding reaction rate in freshly reduced enzyme I is rapid until one thiol group/molecule has reacted with no increase thereafter. These results indicate that enzyme I contains only one reactive thiol group, whereas enzyme II contains two. The reactive thiol group in enzyme I is comparatively stable upon storage, whereas one of those in enzyme II is less stable. During storage, enzyme activity decreases much faster in enzyme II than in enzyme I (Table 4). The activity can be easily restored by incubation with 2-mercaptoethanol (Table 4). These observations can be correlated with the occurrence of two protein bands in aged enzyme II, but only one in aged enzyme I (Fig. 2, compare gels 4 and 5 with gels 1 and 2). The slower moving band in aged enzyme II, which disappears after treatment with a thiol (Fig. 2, compare gel 6 with gels 4 and 5), can thus be ascribed to the formation of intermolecular disulphide bond(s) upon storage.

Stability

In buffers of pH 5–8 (50 mM-sodium acetate or Tris/H_2SO_4) containing 0.1 mM-EDTA and 1 mM-2-mercaptoethanol, the enzyme(s) can be stored for

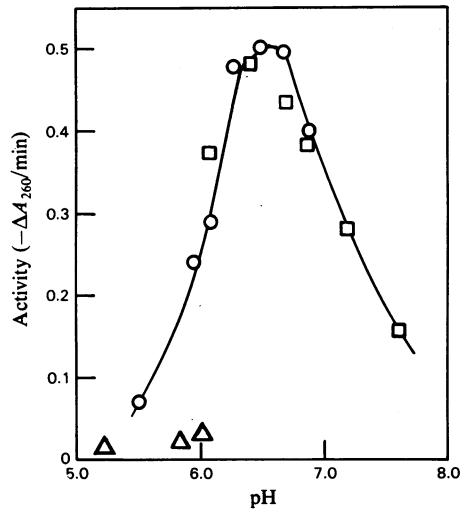

Fig. 4. *Effect of pH and buffer on the activity of cis,cis-muconate cyclase*

Buffers (30mM) are indicated as follows: O, 4-morpholine-ethane sulphonate; □, potassium phosphate; △, sodium citrate. The eluate from the Sephadex G-150 column (step 6 of Table 1) was used as the enzyme source. The enzyme activity curves had similar shapes when either enzyme I or enzyme II were tested. Other details were as described in the Experimental section.

several days at 5°C without significant loss of activity. When stored at −25°C in Tris/H_2SO_4 buffer, supplemented as above, the enzymes are stable for several weeks. About 40–50% of activity is lost upon freeze-drying or when the enzyme is kept at 45°C for 5 min.

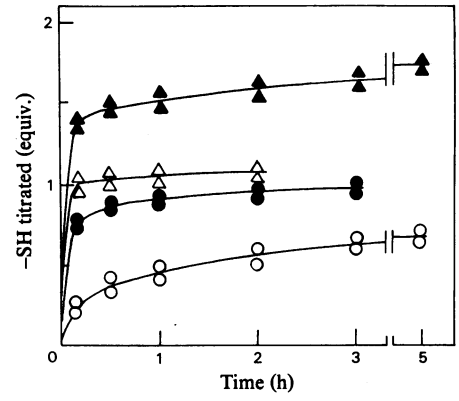

Fig. 5. *Thiol groups in the two enzyme forms of cis,cis-muconate cyclase*

The thiol groups were determined with 5,5′-dithiobis-(2-nitrobenzoic acid) as described in the Experimental section. The results of two different experiments are shown. The aged enzymes were stored for several weeks at −24°C. ●, Aged enzyme I; O, aged enzyme II. △, ▲, Enzymes I and II respectively after incubation with 5mM-dithiothreitol for 1.5h at room temperature followed by gel filtration on Sephadex G-25.

Discussion

The enzyme described here catalyses the conversion of *cis,cis*-muconate to (+)-4-carboxymethylbut-2-en-4-olide, as shown by the crystallization and identification of the reaction product. It is the first such enzyme to be isolated from a eucaryote, and the first that separates into two active forms that are not subunits. The two forms have the same affinity for the natural substrate, they have the same molecular size (Fig. 3), they differ signifi-

Table 4. *Effect of storage and 2-mercaptoethanol on enzymes I and II*

Freshly prepared enzyme was dissolved in 50mM-Tris/H_2SO_4, pH 7.6, containing 0.1mM-EDTA and assayed immediately. For storage, the enzyme was kept in the same buffer at varying temperature and time, as indicated. For reactivation, 2-mercaptoethanol was added to 1mM concentration and the enzyme solution was incubated for 1h before assay. Other details were as described in the Experimental section. 100% Activity for enzymes I and II corresponds to an ΔA_{260} of −0.82 and −0.95 respectively.

	Activity of enzyme (%)	
	I	II
Enzyme preparation		
Freshly prepared enzyme	100	100
Enzyme stored at −24°C for 3 weeks	95	64
As above but incubated with 1mM-2-mercaptoethanol before assay	96	97
Enzyme stored at 4°C for 5 days	96	66
Enzyme stored at 4°C for 8 days	94	55
Enzyme stored as above but incubated with 1mM-2-mercaptoethanol before assay	95	98

cantly in the content and reactivity of their thiol groups (Fig. 5) and they have different isoelectric points (Fig. 1). Preliminary analysis indicates that the two enzyme forms also have different carbohydrate contents.

Since the proteinase inhibitor phenylmethylsulphonyl fluoride was used during purification, it is unlikely that partial proteolytic degradation of the enzyme occurred. Thus it seems probable that enzyme I and enzyme II are isoenzymes of *cis,cis*-muconate cyclase. The amounts of enzymes I and II were too small for a detailed analysis of their composition.

The properties of various enzymes catalysing the lactonization of *cis,cis*-muconate or its 3-carboxy derivative are discussed by Thatcher & Cain (1974*a*). Common properties are K_m values in the range 18–98 μM, inhibition by heavy metals, one to two thiol groups/subunit and inhibition by phosphate and citrate.

The two procaryotic enzymes isolated from *P. putida* are characterized by good heat stability and a subunit size of about 40000 (Sistrom & Stanier, 1954; Meagher & Ornston, 1973; Patel *et al.*, 1973). The enzyme lactonizing *cis,cis*-muconate, but not that lactonizing 3-carboxy-*cis,cis*-muconate, is inhibited by EDTA (Sistrom & Stanier, 1954; Ornston, 1966*a*).

The three eucaryotic enzymes lactonizing 3-carboxy-*cis,cis*-muconate do not require divalent metal ions for activity, are not inhibited by EDTA and have poor heat stability. Widely different values have been reported for the molecular weight. The enzyme isolated from *Rhodotorula mucilaginosa* has a mol.wt. of 19000 (Cain *et al.*, 1968), whereas the *Aspergillus niger* enzyme has a mol.wt. of 190000 with eight subunits of mol.wt. 25000 (Thatcher & Cain, 1974*a*,*b*). The enzyme isolated from *Neurospora crassa* has a mol.wt. of 190000 (Cain *et al.*, 1968). No eucaryotic enzyme catalysing the lactonization of unsubstituted *cis,cis*-muconate has been described before. The muconate cyclase from *T. cutaneum* resembles more the enzymes from eucaryotes than those from procaryotes. This is because it has a poor heat stability, does not require added metal ions, and is not inhibited by EDTA.

The resemblance of the yeast enzyme to the three enzymes from mycelial fungi is interesting, because all those act on 3-carboxy-*cis,cis*-muconate as substrate, whereas the *T. cutaneum* enzyme acts on *cis,cis*-muconate. Properties distinguishing the *T. cutaneum* enzyme from related enzymes of either procaryotic or eucaryotic origin are a relatively sharp optimum at pH 6.6, no inhibition by phosphate (although citrate inhibits) and the occurrence of two enzyme forms.

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