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2-Hydroxychromene-2-carboxylate isomerase from bacteria that degrade naphthalenesulfonates

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Abstract

2-Hydroxychromene-2-carboxylate isomerase activity was found in cell-free systems from bacteria that degrade naphthalenesulfonates. The enzyme from *Pseudomonas testosteroni* A3 was activated by incubation with glutathione, dithiothreitol or mercaptoethanol. The highest enzyme activity was found after preincubation of the enzyme with glutathione at alkaline pH-values. A highly purified enzyme preparation converted besides 2-hydroxychromene-2-carboxylate also 2-hydroxybenzo[*g*]chromene-2-carboxylate (the 2-hydroxychromene-2-carboxylate formed from 1,2-dihydroxyanthracen). The addition of various metal ions or EDTA did not significantly change the catalytic activity of the enzyme. A possible reaction mechanism is proposed.

Abbreviations: 2,5-DHCCA – 2,5-dihydroxychromene-2-carboxylate, 2,6-DHCCA – 2,6-dihydroxychromene-2-carboxylate, 1,2-DHN – 1,2-dihydroxynaphthalene, GSH – glutathione, 2HBCCA – 2-hydroxybenzo[*g*]chromene-2-carboxylate, HBP – 2'-hydroxybenzalpyruvate, HBPA – 2'-hydroxybenzalpyruvate aldolase, 2HCCA – 2-hydroxychromene-2-carboxylate, 2HCCAI – 2-hydroxychromene-2-carboxylate isomerase, 2NS – naphthalene-2-sulfonate, R_t – retention time

Introduction

1,2-Dihydroxynapthalene is an intermediate in the bacterial degradation of naphthalene and naphthalenesulfonates (Fernley & Evans 1958; Davies & Evans 1964; Patel & Gibson 1974; Patel & Barnsley 1980; Brilon et al. 1981b; Nörtemann et al. 1986). In both degradative pathways, 1,2-dihydroxynaphthalene is oxidized by 1,2-dihydroxynaphthalene dioxygenase to 2-hydroxychromene-2-carboxylate (Barnsley 1976; Kuhm et al. 1991; Eaton & Chapman 1992). An isomerase-activity which converts 2-hydroxychromene-2-carboxylate (2HCCA) to 2'-hydroxybenzalpyruvate (HBP) was found by Barnsley (1976) in various naphthalene degrading pseudomonads. It was recently suggested, that the product of this reaction was *trans*-2'-hydroxybenzalpyruvate (Fig. 1; Eaton & Chapman 1992). 2-Hydroxychromene-2-carboxylates have also been described as ring-fission products of 1,2-dihydroxyanthracene, 3,4-dihydroxyphenanthrene, 1,2-dihydroxybenzothiophene, and 1,2- and 3,4-dihydroxypyrene (Evans et al. 1965; Kodama et al. 1973; Monticello et al. 1985; Walter et al. 1991). Isomerases which open the ring-structures of 2-hydroxychromene-2-carboxylates are therefore presumably necessary for the degradation of all polyaromatic hydrocarbons. Nevertheless almost no information

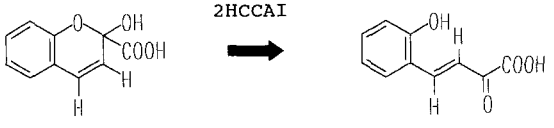

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Fig. 1. Conversion of 2HCCA to *trans*-HBP by 2HCCAI (according to Eaton & Chapman 1992). Chemical designations: (I) 2-hydroxychromene-2-carboxylate; (II) *trans*-2'-hydroxybenzalpyruvate.

is available about this group of enzymes. Therefore, 2-hydroxychromene-2-carboxylate isomerase has been studied from two bacteria that degrade naphthalenesulfonates.

Material and methods

Bacterial strains and media

Pseudomonas testosteroni A3 (DSM 676), *Pseudomonas vesicularis* BN6 (DSM 6383) and the culture conditions have been described previously (Brilon et al. 1981a; Nörtemann et al. 1986; Kuhm et al. 1991).

High pressure liquid chromatography (HPLC)

The HPLC apparatus and the column used have been described (Kuhm et al. 1991). The solvent system consisted of 25% [v/v] methanol, 75% [v/v] water, and 0.1% H_3PO_4 (flow rate 1 ml/min). Under these conditions 2-hydroxychromene-2-carboxylate (2HCCA) and 2'-hydroxybenzalpyruvate (HBP) were clearly separated (R_t 2HCCA = 14.6 min, R_t HBP = 17.4 min).

Preparation of cell extracts

Cell suspensions in Na/K-phosphate buffer (pH 7.3, 50 mM) were disrupted by using a French press (Aminco, Silver Spring, MD, U.S.A.) at 80 MPa. Cell debris was removed by centrifugation at 100,000 X *g* for 30 min at 4° C.

Protein was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

Enzyme assays

One unit of enzyme activity was defined as the amount of enzyme that converts 1 µmol of substrate per min.

For 2-hydroxychromene-2-carboxylate isomerase (2HCCAI) the reaction mixture contained (in 1 ml) 100 µmol of Na-glycine-buffer (pH 10) and 0.2 µmol of 2-hydroxychromene-2-carboxylate (2HCCA). The reaction was started by addition of cell-free extract. The increase of absorbance was recorded at 420 nm. The molar reaction coefficient was $\varepsilon = 10.5\ l \times mmol^{-1} \times cm^{-1}$ (Barnsley 1976). Cell-free extracts were incubated prior to the assay for 30 min with 5 mM-glutathione.

2'-Hydroxybenzalpyruvate aldolase (HBPA) was assayed spectrophotometrically as described previously (Kuhm et al. 1991).

Enzyme purification

Protein was purified at room temperature by use of a fast-protein liquid chromatography system (Pharmacia, Uppsala, Sweden), which has been described previously (Kuhm et al. 1991).

To determine enzyme activities during the purification procedure, it was necessary to reactivate the enzyme with glutathione. The maximal reactivation was achieved after about 30 min incubation of the enzyme with glutathione (2.5 mM).

For purification of 2HCCAI *Pseudomonas testosteroni* A3 was grown in 1.4 l of mineral medium containing naphthalene-2-sulfonate (5 mM) as growth substrate. Cells were harvested in the late exponential growth phase and an extract was prepared as described above. This crude extract was applied to a Q Sepharose-Fast Flow Column (HR 16/10; Pharmacia, Uppsala, Sweden) and eluted with 250 ml of a linear gradient of 50 mM-Tris/HCl buffer, pH 7.5 into 50 mM-Tris/HCl buffer, pH 7.5 plus 500 mM-NaCl at a flow rate of 3 ml/min. Fractions (5 ml each) were collected and fractions containing 2HCCAI activity were pooled (fractions 17–21). The combined fractions were desalted by ultrafiltration (Model 8050, filter PM 10, Amicon, Beverly, MA, U.S.A.) and applied to a Mono-Q column (HR5/5; Pharmacia). The sample was eluted with 30 ml of a linear gradient of 50 mM-Tris/HCl buffer, pH 7.5, into 50 mM-Tris/HCl buffer, pH 7.5 plus 200 mM-NaCl at a flow rate of 1 ml/min. Fractions (1 ml each) with the highest activity (fractions

14–20) were pooled and concentrated by ultrafiltration. The concentrate was applied to a Superose-6 column (HR 10/30; Pharmacia) and eluted with 30 ml of 50 mM-Tris/HCl, pH 7.5, plus 100 mM-NaCl at a flow rate of 0.3 ml/min. Fractions (0.5 ml each) with the highest activity were pooled (fractions 37–41). The sample was applied to a Chelating Superose column (HR 10/2, Pharmacia) loaded with Zn^{2+}-ions (9 ml of 0.2 M-$ZnSO_4$) and eluted with 50 mM-Tris/HCl, pH 7.5, plus 100 mM-NaCl and a linear gradient of EDTA (0–50 mM) at a flow rate of 0.5 ml/min. Fractions (1 ml each) were collected and those containing 2HCCAI (fraction 3 + 4) pooled.

2HCCAI and HBPA from *Pseudomonas vesicularis* BN6 were separated by anion-exchange chromatography (Mono-Q column, HR 5/5; Pharmacia) using a gradient of Tris/HCl (50 mM, pH 7.5) into Tris-HCl (50 mM, pH 7.5) plus 500 mM-NaCl and a flow rate of 0.5 ml/min; 0.7 ml fractions were collected. The maximum activities of 2HCCAI and HBPA were eluted at concentrations of 0.23 M-NaCl and 0.21 M-NaCl, respectively.

Reactivation of 2HCCA-isomerase from Pseudomonas vesicularis BN6

2HCCAI from strain BN6 was partially purified by anion-exchange chromatography. Aliquots of this enzyme preparation (10 µl each) were incubated for 1 h with 0, 2, 5 or 10 mM-glutathione and then 2HCCAI activity was determined with 0, 0.2, 0.4, 0.6, 0.8 or 1.0 mM-glutathione added to the enzyme assay (final volume 500 µl). No enzyme activity (< 0.001 U/ml) was found when the enzyme preparations had been preincubated without or with 10 mM-glutathione. After preincubation with 2 mM-glutathione the highest activity was found with 0.2 mM–1mM-glutathione added supplementary to the assay (0.052–0.057 U/ml, without GSH in the assay = 0.01 U/ml).After preincubation with 5 mM-glutathione highest activities were obtained with 0.2–0.6 mM-GSH added to the assay (0.070–0.075 U/ml).

Optimum pH

In the enzyme assay for 2HCCAI the formation of 2'-hydroxybenzalpyruvate (HBP) is determined spectrophotometrically (Barnsley 1976). The UV/Vis-spectrum of HBP changes intensively dependent on the pH of the solution. Therefore the optimum pH of 2HCCAI was determined by HPLC. The reaction mixtures contained in 100 µl 0.024 µmol 2HCCA and 8.0 µmol of the respective buffer. The reaction was started by the addition of 4 µl of a purified fraction of 2HCCAI. Samples were taken at short time intervalls (1.0–2.0 min) and immediately analyzed by HPLC. Spontaneous reactions of 2HCCA did not occur to a significant extent at any of the pH-values tested.

Determination of molecular weight

SDS-PAGE and gel filtration were performed as described earlier (Kuhm et al. 1990).

Chemicals

2HCCA was prepared enzymatically with whole cells of strain BN6. The cells were induced with naphthalene-2-sulfonate (2NS) (Kuhm et al. 1991), harvested at the end of the exponential growth phase, washed in Na/K-phosphate buffer (50 mM, pH 7.3) and resuspended in 100 ml of Na-acetate-buffer (100 mM, pH 5.5) to an optical density A_{546nm} of 5. The cell-suspension was incubated at 30° C, vigorously stirred and additionally aerated with compressed air. Over a period of 15 min a total of 32 mg of 1,2-dihydroxynaphthalene (1,2-DHN) was added. As soon as the medium turned yellow (= formation of 1,2-naphthoquinone) the addition of 1,2-DHN was slowed down to prevent the formation of the autoxidation product. After complete turnover of 1,2-DHN the suspension was centrifuged and 2HCCA isolated from the supernatant as described by Davies & Evans (1964). 2,5- and 2,6-Dihydroxychromene-2-carboxylate (2,5- and 2,6-DHCCA) were synthesized in the same way from 1,2,5- and 1,2,6-trihydroxynaphthalene. These 2-hydroxychromene-2-carboxylates were more unstable than 2HCCA. Therefore 1,2,5- and 1,2,6-trihydroxynaphthalene were completely converted to the corresponding 2-hydroxychromene-2-carboxylates, cells removed by centrifugation and the supernatants directly used for the assay of 2HCCAI. The spectral characteristics of 2,5- and 2,6-DHCCA have been described previously (Kuhm et al. 1991).

2-Hydroxybenzo[g]chromene-2-carboxylate (2HBCCA) was produced enzymatically from 1,2-dihydroxyanthracene by purified 1,2-dihydroxynaphthalene dioxygenase from Pseudomonas vesicularis BN6 as described previously for the synthesis of 2-hydroxychromene-2-carboxylates (Kuhm et al. 1991). During the reaction the solution became orange and slightly turbid. After complete conversion of 1,2-dihydroxyanthracene the reaction mixture was filtered (Millex-GV, 0.2 µm, Millipore, Molsheim, France). The resulting clear, colourless solution was used directly for enzyme assays.

1,2-Dihydroxyanthracene was kindly provided by Dr. S. Schacht (Essen, Germany). All other chemicals used for mineral salts media and buffer solutions were obtained from Merck, Darmstadt, Germany.

Results

2-Hydroxychromene-2-carboxylate isomerase in cell extracts

Two bacterial strains were studied which degrade naphthalenesulfonates. *Pseudomonas testosteroni* A3 was enriched with naphthalene-2-sulfonate (2NS) as sole source of carbon and energy (Brilon et al. 1981a). *Pseudomonas vesicularis* BN6 was isolated from a 6-aminonaphthalene-2-sulfonate degrading mixed culture (Nörtemann et al. 1986). In cell extracts from *Pseudomonas testosteroni* A3 a 2-hydroxychromene-2-carboxylate isomerase (2HCCAI) activity was demonstrated spectrophotometrically (0.005 U/mg of protein). The enzyme activity increased to 0.2 U/mg of protein when the cell extract was incubated for 15 min with glutathione (5 mM) prior to the assay. The addition of glutathione (0.2 mM) directly to the assay did not increase enzyme activity.

Reactivation of 2-hydroxychromene-2-carboxylate isomerase from Pseudomonas testosteroni A3 by glutathione or other thiols

In the spectrophotometric assay for 2HCCAI the formation of 2'-hydroxybenzalpyruvate (HBP) was measured at 420 nm. Thus all determinations of 2HCCAI activity in cell extracts were hindered by the action of 2'-hydroxybenzalpyruvate aldolase (HBPA). Therefore both enzymes were separated by anion exchange chromatography (Mono-Q column; Pharmacia) prior to further analysis.

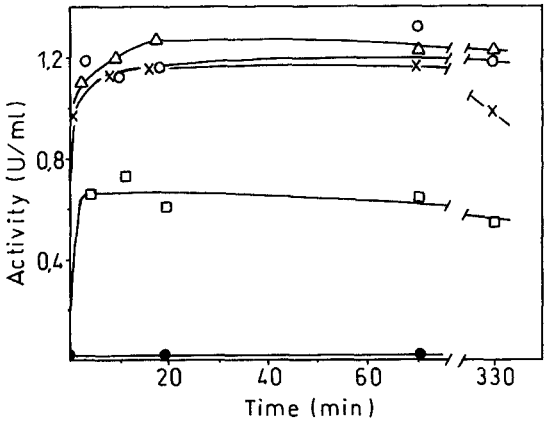

Fig. 2. Reactivation of 2HCCAI from *Pseudomonas testosteroni* A3 by glutathione. 2-Hydroxychromene-2-carboxylate isomerase from *Pseudomonas testosteroni* A3 was partially purified by anion exchange chromatography (see Material and methods). The protein fractions containing 2HCCAI activity were incubated with 1 mM-glutathione (×), 2.5 mM-glutathione (△), 5 mM-glutathione (○), 10 mM-glutathione (□), or without glutathione (●). After different time intervals enzyme activities were determined spectrophotometrically.

The enzyme activities increased significantly when the protein fractions containing 2HCCAI were preincubated with glutathione. The optimal glutathione concentration was 2.5 mM. Thus an almost immediate activation was observed and the enzyme activity increased more than 30-fold (Fig. 2). The effect of glutathione was optimal between pH 8 and pH 9.3. In contrast, no activation was observed when the enzyme was incubated with glutathione at pH 5.0. The same enzyme preparation was incubated for 2 h with glutathione (GSH), oxidized glutathione (GSSG), dithiothreitol or mercaptoethanol. 2HCCAI activity was determined with or without glutathione in the assay. Thus it was shown that glutathione could be replaced by other thiols for the reactivation of 2HCCAI (Table 1).

Purification of 2-hydroxychromene-2-carboxylate isomerase from Pseudomonas testosteroni *A3*

A typical purification scheme for 2HCCAI is shown

Table 1. Influence of various thiols on the activity of 2HCCAI from *Pseudomonas testosteroni* A3.

Compound added	Relative activities [%]	
	– GSH in assay	+ GSH in assay
glutathione	100	100
GSSG	6	36
dithiothreitol	30	63
mercaptoethanol	17	64
None	2	4

GSSG glutathione, oxidized form
2HCCAI from *Pseudomonas testosteroni* A3 was enriched 300-fold. The purified enzyme was incubated for 2 h in 50 mM-Tris/HCl-buffer (pH 7.5 + 100 mM-NaCl) with the compounds indicated (2 mM each). 2HCCAI activity was determined spectrophotometrically without or with glutathione (0.2 mM) added to the cuvette. Specific activities were 30 U/mg of protein (= 100%) without glutathione added to the assay and 28.6 U/mg of protein (= 100%) with glutathione added to the assay.

in Table 2. After the final step of the purification procedure the specific activity of the enzyme in the purest fractions ranged from 55 to 160 units/mg of protein, indicating that at least part of them did not contain homogeneous protein. The activity of the enzyme in the purest fractions represented an approx. 900-fold purification with a recovery of about 8%. The purifed 2HCCAI was relatively stable, after one month storage in Tris/HCl-buffer (pH 7.5, 50 mM, + 100 mM-NaCl) at 4° C 48% of the initial activity were recovered.

Estimation of molecular weight

Independent of the purification strategy the analysis of all purified enzyme preparation by SDS-PAGE revealed one clearly dominant protein band of M_r 24700 ± 2500. In all preparations obtained small amounts of impurities were found. By gel-filtration of the 2HCCAI activity a molecular weight lower than 30.000 was determined. Thus it appears that 2HCCAI consists of one single subunit.

k_M-Value, optimum pH and effect of metal-ions

The apparent k_M-value for 2HCCA, calculated from Lineweaver-Burk plots, was 0.23 mM. A pH optimum of 9 for enzyme activity was observed (in glycine-NaOH-buffer). Activities higher than 70% of this optimal activity were found at pH-values in the range 8.0–9.5 in glycine-NaOH- or Tris/HCl-buffer). At pH 7 about 50% and at pH 5.5 10% of the optimal activity was observed.

No increase of enzyme activity was found after (pre)incubation of the enzyme with $ZnSO_4$, $CoCl_2$, $FeCl_2$ or $MnCl_2$ (each 2 mM) or a trace element solution according to Pfennig & Lippert (1966). No significant inhibition was observed after one day incubation with 50 mM-EDTA.

Substrate specificity of 2-hydroxychromene-2-carboxylate isomerase from Pseudomonas testosteroni A3

A 900-fold enriched preparation of 2HCCAI from *Pseudomonas testosteroni* A3 converted 2HCCA (λ_{max} = 254 nm and 292 nm) into a compound with the spectral characteristics of HBP (λ_{max} = 300 nm and 414 nm). These changes of the absorption spectrum were characterized by two isosbestic points at 248 nm and 267 nm (Fig. 3A). The identity of the reaction product with HBP was confirmed by HPLC. From the other substrates tested only 2-hydroxybenzo[*g*]chromene-2-carboxylate

Table 2. Purification of 2-hydroxychromene-2-carboxylate isomerase from *Pseudomonas testosteroni* A3.

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (unitsXmg^{-1})	Recovery of activity (%)	purification factor
Crude extract	15.0	29.9	164.3	0.18	100	1.0
Ion-exchange chromatography, Q-Sepharose FF	25.0	26.0	32.3	0.81	87	4.4
Ion-exchange chromatography, Mono-Q	7.0	12.2	8.3	1.46	41	8.1
Gelfiltration	2.5	6.9	0.17	40.4	23	220
Chelating Superose	2.0	3.9	0.07	55.4	13	300

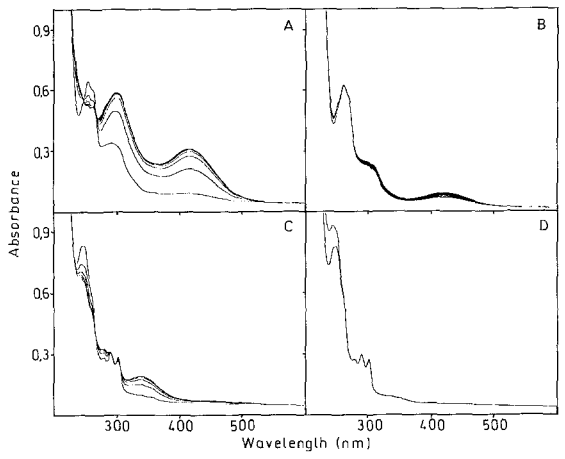

Fig. 3. Conversion of (substituted) 2-hydroxychromene-2-carboxylates by the purified 2HCCAI from *Pseudomonas testosteroni* A3. A 900-fold enriched preparation of 2HCCAI (0.009 U) was preincubated with glutathione (2.5 mM, 15 min). The reaction mixture contained (in 1 ml) 100 µmol of Na-glycine-buffer, pH 10.0, and 0.08 µmol of the respective substrate. For the determination of the respective spontaneous reaction glutathione was added to the same final concentration as in the enzyme assays. Every 3 min a spectrum was recorded. The reaction with 2HCCA (A, enzymatic; B, spontaneous) and 2HBCCA (C, enzymatic; D, spontaneous) were recorded spectrophotometrically.

(2HBCCA) was a substrate for the enzyme. The product of this reaction showed a λ_{max} = 334 nm (Fig. 3C). In contrast to 2HCCA and 2HBCCA, 2,5- and 2,6-dihydroxychromene-2-carboxylate (2,5- and 2,6-DHCCA) reacted already spontaneously. No significant turnover of 2,5-DHCCA or 2,6-DHCCA by the purified enzyme was observed.

Conversion of (substituted) 2-hydroxychromene-2-carboxylate(s) by resting cells of Pseudomonas testosteroni A3 and Pseudomonas vesicularis BN6

Resting cells of *Pseudomonas testosteroni* A3 and *Pseudomonas vesicularis* BN6 converted 2HCCA, 2,6-DHCCA and 2HBCCA (chromene formed from 1,2-dihydroxyanthracene), but not 2,5-DHCCA (Table 3). Cells of strain BN6 converted 2HCCA to salicylate and 2,6-DHCCA to 5-hydroxysalicylate. (The products were identified by their retention time and in-situ-spectra using HPLC.) In contrast, no metabolites were excreted by cells of *Pseudomonas testosteroni* A3. Both strains excreted a metabolite during turnover of 2-HBCCA. The product was identified by its HPLC retention time, in-situ-spectroscopy and comparison with an authentic standard as 3-hydroxynaphthalene-2-carboxylate (R_t = 7.6 min, λ_{max} = 235 nm, 280 nm and > 350 nm, λ_{min} = 225 nm, 265 nm and 310 nm).

2-Hydroxychromene-2-carboxylate isomerase from Pseudomonas vesicularis BN6

No 2HCCAI activity was found in cell extracts from strain BN6 (± glutathione). Surprisingly, after anion-exchange chromatography of such a cell extract this enzyme activity was detected. The protein fractions had to be preincubated with glutathione (c = 2.5 mM–5 mM, t = 15 min–30 min) and furthermore glutathione had to be added to the assay (0.2 mM–0.6 mM). Again, reactivation by glutathione showed a maximal effect at slightly alkaline pH-values. The enzyme eluted as a single activity peak from the column. Only 0.018 U 2HCCAI activity were found per mg of protein layered on the column. Under the same conditions with *Pseudomonas testosteroni* A3 0.12 U per mg of protein

Table 3. Turnover of (substituted) 2-hydroxychromene-2-carboxylates by resting cells of *Pseudomonas testosteroni* A3 and *Pseudomonas vesicularis* BN6.

Compound	relative activity, strain	
	Ps. testosteroni A3	*Ps. vesicularis* BN6
2-Hydroxychromene-2-carboxylate	100	100
2,5-Dihydroxychromene-2-carboxylate	< 3	< 3
2,6-Dihydroxychromene-2-carboxylate	68	230
2-Hydroxybenzo[*g*]-chromene-2-carboxylate	19	133

Pseudomonas testosteroni A3 was grown with 2NS and *Pseudomonas vesicularis* BN6 with glucose and induced with 2NS. The cells were harvested at the end of the exponential growth phase, washed and resuspended to an absorbance (A_{546nm}) of 6.3 or 8.2 in Na/K-phosphate buffer (50 mM, pH 7.3). The substrates were added to the cell suspensions (0.27 mM each) and the turnover of substrate was determined by HPLC. The values given were corrected for the spontaneous reactions of the respective substrates. The reaction rates are expressed as percentages of that for 2HCCA taken as 100%. Specific activities for 2HCCA were 0.27 U/mg of protein for *Ps. testosteroni* A3 and 0.14 U/mg of protein for *Ps. vesicularis* BN6.

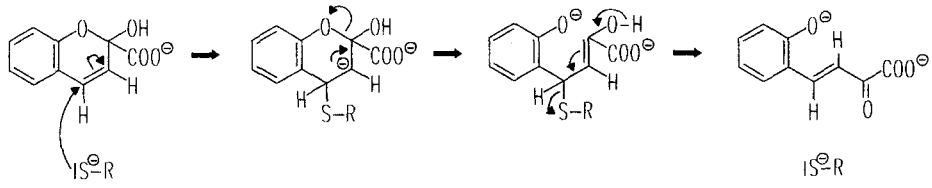

Fig. 4. Possible mechanism for the conversion of 2HCCA to *trans*-HBP catalyzed by 2HCCAI.

were recovered. In contrast to the enzyme from *Ps. testosteroni* A3 glutathione was essential for the reactivation of the enzyme and could not be replaced by dithiothreitol or mercaptoethanol. The apparent k_M-value for 2HCCA, calculated from Lineweaver-Burk plots was 0.27 mM. The optimal pH for activity was pH 9.5. At pH 9.0 50% and at pH 8.0 only 5% of this maximal activity was found.

Discussion

2-Hydroxychromene-2-carboxylates have been described as metabolites formed during the bacterial degradation of naphthalene, naphthalenesulfonates, anthracene, phenanthrene, benzothiophene, and pyrene (Davies & Evans 1964; Evans et al. 1965; Kodama et al. 1973; Barnsley 1976; Monticello et al. 1985; Kuhm et al. 1991; Walter et al. 1991). Nevertheless, in most cases it was suggested that they were only artefacts which were formed during isolation from the true acyclic extradiol ring-fission products. In contrast, Barnsley (1976) showed that 2-hydroxychromene-2-carboxylate (2HCCA) was the real product of the enzymatic oxidation of 1,2-dihydroxynaphthalene (1,2-DHN) by *Pseudomonas putida* NCIB 9816. It was suggested that the formation of 2-hydroxychromene-2-carboxylates was caused by the chemical stabilization of an enzyme bound *meta*-cleavage product by an electron shift (Patel & Barnsley 1980).

Barnsley (1976) and Eaton & Chapman (1992) demonstrated that under neutral conditions 2HCCA only slowly isomerizes to 2'-hydroxybenzalpyruvate (HBP). Therefore a 2HCCAI activity seems to be necessary to allow bacteria to grow with naphthalene. The genetic analysis of the *nah*-operon from plasmid NAH7 suggested that the gene coding for 2HCCAI was located within the 'upper' *nah*-operon (Yen & Gunsalus 1982; Yen & Serdar 1988; Eaton & Chapman 1992).

The 1,2-dihydroxynaphthalene dioxygenases from *Pseudomonas vesicularis* BN6 and *Pseudomonas testosteroni* A3 also oxidize 1,2-DHN to 2HCCA as sole product (Kuhm et al. 1991; Kuhm unpublished). Therefore 2HCCA converting enzymes are also necessary for the microbial metabolism of naphthalenesulfonates.

It was originally suggested by Barnsley (1976) that 2HCCAI converts 2HCCA to *cis*-HBP. Recently Eaton & Chapman (1992) suggested that not *cis*-HBP but its *trans*-isomer is formed by 2HCCAI. The formation of *trans*-HBP from 1,2-DHN by cell-free extracts of *Corynebacterium renale* in the presence of glutathione had been described previously (Dua & Rao 1986).

2HCCAI from *Pseudomonas putida* NCIB 9816 showed maximal activity at alkaline pH-values (pH 10) and was dependent on the presence of glutathione or other thiols (Barnsley 1976). The same was found in the present study for the enzyme from *Pseudomonas testosteroni* A3. The enzyme from strain A3 was activated by preincubation with glutathione or other thiols, but not by the direct addition of glutathione to the enzyme assay. This suggests that thiols are required for the activation of enzyme bound cysteine residues, but that glutathione does not act as a cofactor. The requirement for SH-groups and the alkaline pH-optimum of the enzyme suggests that an anionic cysteine residue is involved in catalysis (pK_a of the cysteine sulphydryl = 8.4; Palmer 1985) and a nucleophilic attack of the sulphydryl group on the carbon-carbon double bond of 2HCCA. This reaction mechanism would also allow the formation of *trans*-HBP from 2HCCA in a single reaction (Fig. 4). A similar nu-

cleophilic attack of a sulphydryl group on a carbon-carbon bond has also been proposed for the *cis-trans*-isomerization of maleylpyruvate (or maleylacetone) to fumarylpyruvate (or fumarylacetone) (Lack 1961; Seltzer & Lin 1979).

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