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Subcloning of the Enterobactin Biosynthetic Gene *entB*: Expression, Purification, Characterization, and Substrate Specificity of Isochorismatase†

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Received July 19, 1989; Revised Manuscript Received October 3, 1989

ABSTRACT: The *Escherichia coli entB* gene, coding for the enterobactin biosynthetic enzyme isochorismatase, has been subcloned into the multicopy plasmid pKK223-3 under the control of the *tac* promoter. The resulting recombinant plasmid pFR1 expresses isochorismatase amounting to over 50% of the total cellular protein. The enzyme has been purified to homogeneity and a convenient assay developed. The enzyme has a K_m for isochorismate of 14.7 μM and a turnover number of 600 min^{-1}. By use of ^{1}H NMR spectroscopy, the progress of the reaction was followed with the expected formation of 2,3-dihydro-2,3-dihydroxybenzoate product. Several substrate analogues were also utilized by the enzyme including chorismic acid, the immediate precursor to isochorismic acid in the enterobactin biosynthetic pathway.

The biosynthesis of the bacterial siderophore enterobactin from chorismic acid and serine is accomplished by the enzymes encoded for by the *ent* genes in *E. coli*. In these biosynthetic reactions, chorismic acid is first converted into 2,3-dihydroxybenzoate by the *entC*, *entB*, and *entA* gene products (Young et al., 1971; Nahlik et al., 1987). The final assembly of 2,3-dihydroxybenzoate and L-serine is an ATP-requiring process carried out by the products of the *entD*, *entE*, *entF*, and *entG* genes (Luke & Gibson, 1971; Woodrow et al., 1975). Recently, the *entA* and *entE* genes have been subcloned into multicopy expression vectors and expressed in *E. coli* in

†Supported by NIH Grant GM20011 (C.T.W.), NIH Grant GM31958 (G.A.B), and NIH Postdoctoral Fellowship GM12806-01 (F.R.). J.L. acknowledges a Du Pont Graduate fellowship from the Massachusetts Institute of Technology.

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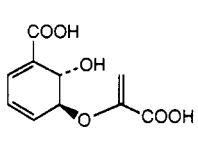
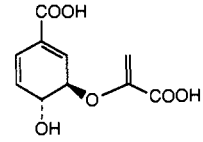
0006-2960/90/0429-1425$02.50/0

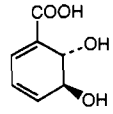
Chart I



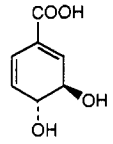
1 - Isochorismic Acid



2 - Chorismic Acid



3 - *trans*-2,3-Dihydro-2,3-dihydroxybenzoic Acid



4 - *trans*-3,4-Dihydro-3,4-dihydroxybenzoic Acid

quantities suitable for rigorous enzymological studies (Liu et al., 1989; Rusnak et al., 1989). In this and the preceding report (Liu et al., 1990), we report the successful subcloning, overexpression, and characterization of isochorismatase (product of the *entB* gene) and isochorismate synthase (product of the *entC* gene), respectively. Thus, all of the essential genes that comprise the *entCEBA* operon have now been subcloned and their respective protein products purified to homogeneity.

Enzymic transformations of chorismic acid (**2**; Chart I), a precursor common to (a) the aromatic amino acids via prephenic acid (Addadi et al., 1983; Guilford et al., 1987) and anthranilic acid (Policastro et al., 1984; Teng & Ganem, 1984), (b) the *p*-aminobenzoic acid moiety of folic acid (Walsh et al., 1987; Nichols et al., 1989), (c) the *p*-hydroxybenzoic acid precursor to ubiquinone (Lawrence et al., 1974), and (d) 2,3-dihydroxybenzoic acid (Earhart, 1987), have been the focus of many investigations. Recently, the more obscure metabolite isochorismic acid (**1**; Chart I) has been proposed to play equally important roles in aromatic biosynthetic conversions. Isochorismic acid was first discovered in growth cultures of *E. coli* mutants which accumulated this metabolite while grown under iron-deficient conditions (Young et al., 1971). These mutants, unable to synthesize 2,3-dihydroxybenzoic acid from chorismic acid, were blocked in the synthesis of isochorismatase, which converts isochorismic acid into 2,3-dihydro-2,3-dihydroxybenzoic acid (**3**) in the biosynthetic pathway to enterobactin. Isochorismic acid has also been proposed to be the precursor to *o*-succinylbenzoic acid in the biosynthesis of menaquinone (Bentley & Meganathan, 1987; Weische & Leistner, 1985; Weische et al., 1987) and may be involved in arene oxide formation in the biosynthesis of crotepoxide, senepoxide, and pipoxide (Ganem & Holbert, 1977). Isochorismate is also a likely intermediate in the formation of salicylic acid from chorismic acid in *Mycobacterium smegmatis* (Marshal & Ratledge, 1971) and is probably a precursor of *m*-carboxyphenylalanine in plants (Weiss & Edwards, 1980). Since the conversion of isochorismic acid to *o*-succinylbenzoic acid (Weishce & Leistner, 1985) and the conversion of chorismic acid to salicylic acid (Marshall & Ratledge, 1972) have been demonstrated only in crude preparations, this report and the preceding paper (Liu et al., 1990) detailing the reversible interconversion of **1** and **2** by isochorismate synthase represent the first reported instances of the purification and characterization of isochorismate-utilizing enzymes.

Materials and Methods

Materials. NADH disodium salt was from Boehringer-Mannheim Biochemicals. L-Lactate dehydrogenase was from Sigma Chemical Co., St Louis, MO. dGTP, dATP, and the Dale rapid-deletion subcloning primer (Dale et al., 1985), RD22-mer, were from IBI Inc., New Haven, CT. The 29-base oligonucleotide primer used in the deletion reaction was from Research Genetics, Huntsville, AL. D_2O (99.99%) was from Cambridge Isotope Laboratories, Woburn, MA.

Syntheses of Compounds Tested with Isochorismatase. (*a*) *Chemical Syntheses.* The following compounds used in this study (Tables III and IV) were synthesized according to the following procedures: (±)-isochorismic acid (**1**) (Busch & Berchtold, 1983); (±)-*trans*-2,3-dihydro-2,3-dihydroxybenzoic acid (**3**) (DeMarinis et al., 1974; Quinn and Berchtold, manuscript in preparation); (±)-*trans*-3,4-dihydro-3,4-dihydroxybenzoic acid (**4**) (Chiasson & Berchtold, 1974); (±)-disodium 3-[(1-carboxylatoethenyl)oxy]cyclohepta-1,6-diene-1-carboxylate (**6**) (Pawlak & Berchtold, 1988); dihydropyran (**12**) (Ganey, 1986); (±)-(1β,3β,4α)-dihydrochorismate (**9**) [prepared from (±)-**2**] and the dihydropyran compound **11** (Delaney, Padykula, and Berchtold, manuscript in preparation); (1α,3β,4α)-dihydrochorismate (**10**) and 3-[(carboxyethenyl)oxy]benzoic acid (**14**) [both prepared from (−)-**2**] (Ife et al., 1976); (±)-*trans*-3-[(1-carboxyethenyl)-oxy]-4-methoxy-1,5-cyclohexadiene-1-carboxylic acid (**16**) and (±)-*trans*-1-[(1-carboxyethenyl)oxy]-2-hydroxy-3,5-cyclohexadiene (**17**) (Pawlak et al., 1989); (±)-3-[(1-carboxyethenyl)oxy]-2-hydroxy-6-cyclohexene-1-carboxylic acid (**5**), (±)-*cis*-3-[(carboxyethenyl)oxy]-4-cyclohexene-1-carboxylic acid (**7**), (±)-3-[(carboxyethenyl)oxy]-1-cyclohexene-1-carboxylic acid (**8**), the TFA salt of (±)-3-[(carboxyethenyl)oxy]-2-amino-6-cyclohexene-1-carboxylic acid (**13**), and (±)-3-[(carboxyethenyl)oxy]-6-hydroxy-1-benzoic acid (**15**) (Quinn and Berchtold, manuscript in preparation).

(*b*) *Biosynthesis of* (−)-*Chorismic Acid and* (+)-*Isochorismic Acid.* (−)-Chorismic acid (**2**) was prepared from *Klebsiella pneumoniae* 62-1 (formerly *Aerobacter aerogenes* 62-1) according to the method of Gibson (1968). For the production of (+)-isochorismate (**1**), 39.2 mg of (−)-chorismic acid was dissolved in 100 mL of 100 mM pH 8.0 Tris-HCl buffer containing 10 mM $MgCl_2$. A preparation of isochorismate synthase (≈1.5 units), partially purified by chromatography on phenyl-Sepharose CL-4B resin (Pharmacia) from the overproducing *E. coli* strain K38/pJLT5053 harboring the *entC* gene (Liu et al., 1990), was added to initiate the reaction. The absorbance of the reaction mixture at 278 nm was monitored until no further increase was observed (about 1 h), at which point the solution was cooled to 0 °C and filtered through an Amicon PM-30 filter to remove isochorismate synthase. The filtrate was then acidified with concentrated HCl to pH 2 and extracted four times with 50 mL of ethyl acetate. The organic extracts were combined, dried with anhydrous magnesium sulfate at 0 °C, and filtered, and the ethyl acetate was removed in vacuo. The residue was dissolved in 2.0 mL of HPLC buffer (55% 0.01 M trifluoroacetic acid, 45% methanol), injected onto a Merck Lobar C_8 reverse-phase A column (240 × 10 mm, 40–63-μm particle size), and eluted with HPLC buffer. Fractions containing isochorismic acid were combined and concentrated by lyophilization. Yields were 52% based on the amount of isochorismic acid expected from equilibrium mixtures with chorismic acid (Liu et al., 1990). The optical rotation of this solution was measured at 22 °C. The final solution after lyophilization was divided into

0.5-mL quantities and frozen at −80 °C until further use.

Bacterial Strains and Plasmids. *E. coli* JM101 [*supE thi* Δ(*lac-proAB*)/F′(*traD36 proA*$^+$ *proB*$^+$ *laqI*^{q} *lacZ*ΔM15)] and JM105 [Δ(*lac-pro*)$_{XIII}$ *thi rpsL* (*str*^{r}) *endA sbcB supE hsdR*/F′(*traD36 proA*$^+$ *proB*$^+$ *laqI*^{q} *lacZ*ΔM15)] and the replicative form of M13mp19 were obtained from New England Biolabs, Beverly, MA. pKK223-3 was from Pharmacia, Piscataway, NJ. pMS111, a pBR322 derivative harboring the *entA*, *entB*, and *entE* genes on an *Eco*RI fragment, was provided by Dr. I. G. Young of the Australian National University.

DNA Manipulations. DNA manipulations were carried out as described (Maniatis et al., 1982). All restriction enzymes were obtained from New England Biolabs and were used in accordance with the manufacturers' instructions. T4 DNA ligase, T4 DNA polymerase, 10× ligase buffer, and 10× polymerase buffer were from International Biotechnologies Inc.; X-Gal, IPTG, and calf intestine alkaline phosphatase were from Boehringer-Mannheim Biochemicals. DNA sequencing was carried out by using the Sequenase kit, version 2, from United States Biochemical Corp., Cleveland, OH, with [α-^{35}S]thio-dATP (1000 Ci/mmol) from Amersham, Arlington Heights, IL.

Construction of an Isochorismatase-Overproducing Strain. (*a*) *Subcloning of the 2.0-kb entB-Containing Fragment into M13mp19.* The DNA sequence of a 3.25-kb portion of pMS111 containing the *entE*, *entB*, and *entA* genes indicated that the *entB* gene was located on a 2.0-kb *Hpa*I fragment (Liu et al., 1989). The 2.0-kb fragment containing the *entB* gene was isolated (after digestion of pMS111 with *Hpa*I) by electrophoresis in low melting point agarose and purified by ion-exchange chromatography on an Elutip (Schleicher & Schuell) column. The purified *entB*-containing fragment was subsequently ligated into the replicative form of M13mp19 which had been digested with *Sma*I and treated with calf intestine alkaline phosphatase. Ligation using T4 DNA ligase was carried out by incubation at 2 °C for 72 h. The ligation mixture was then transformed into *E. coli* JM101, and clear plaques were isolated from 2× TY (1.6% Difco bacto tryptone, 1% Difco yeast extract, 0.5% NaCl, 1.5% agar) plates. Preparation of single-stranded template following the Amersham protocol led to the isolation of single-stranded DNA. Sequencing of the template showed the presence of the insert in the proper orientation. The M13 phage containing the 2.0-kb *entB* insert is called mFR1 (Figure 1A).

(*b*) *Deletion of 801 bp Upstream of entB.* In order to reduce the distance between the *tac* promoter (in the multicopy plasmid pKK223-3, see below) and the start codon of the *entB* gene, 801 base pairs prior to the start of *entB* were deleted from mFR1 in a manner similar to the procedure described for constructing an overproducing system for the *entC* gene (Liu et al. 1990). Single-stranded mFR1 (≈1 μg) was primed with the oligonucleotide used in the Dale rapid-deletion subcloning method (RD22-mer, ≈40 ng; Dale et al., 1985) and cut with *Eco*RI for 2 h at 42 °C. After inactivation of *Eco*RI by heating at 65 °C for 10 min, 40 ng of a 29-base oligonucleotide primer corresponding to the first two amino acid residues of *entB*, 18 upstream residues, and five overhanging bases of an *Eco*RI restriction site was subsequently ligated to the resulting nicked mFR1 (Figure 1B). Deletion of single-stranded DNA in the 3′ to 5′ direction was carried out by using the exonuclease activity of T4 DNA polymerase at 37 °C for 30 min in the presence of 0.2 mM dGTP. Deletion of single-stranded DNA occurs up to the region of double-stranded DNA at which point the exonuclease activity is compensated

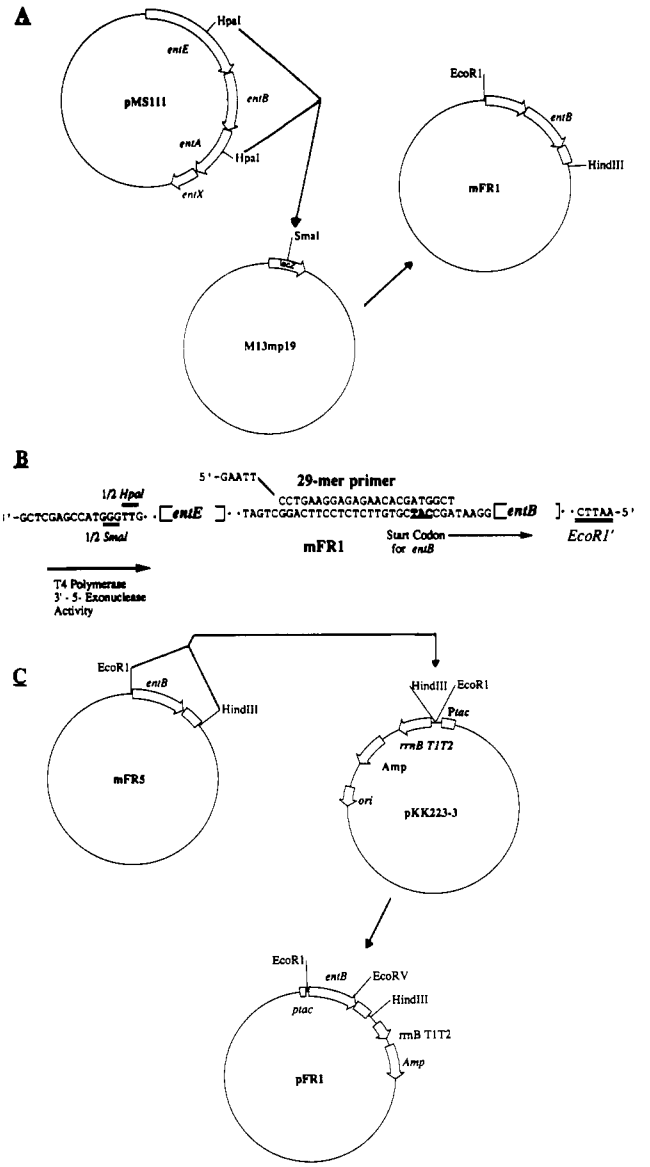


FIGURE 1: Subcloning strategy for the *entB* gene. (A) The 2.0-kb *entB*-containing fragment from pMS111 was ligated into the *Sma*I site of M13mp19. An M13mp19 clone selected with the 5′ end of the *entB* gene proximal to the *Eco*RI site in the polylinker cloning sites was called mFR1. The 2.0-kb insert of mFR1 codes for the carboxy terminus of *entE*, the complete *entB* gene, and the first ≈300 bases of the *entA* gene. (B) The single-stranded form of mFR1, corresponding to the antisense strand of the *entB* gene, was linearized by annealing RD22-mer primer and cutting with *Eco*RI as described under Materials and Methods. Subsequent annealing of the synthetic 29-base oligonucleotide was followed by deletion of 801 bases in the 3′–5′ direction by using the exonuclease activity of T4 polymerase. Reannealing produced a new M13 clone, mFR5, containing a truncated insert now 1.1 kb in length. The *Sma*I/*Hpa*I site produced by ligation of the 2.0-kb *Hpa*I fragment into the *Sma*I site of M13mp19 and the *Eco*RI site used to linearize single-stranded mFR1 are shown. (C) Digestion of double-stranded mFR5 with *Eco*RI/*Hin*dIII and subsequent ligation into *Eco*RI/*Hin*dIII-digested pKK223-3 produced pFR1.

by the polymerase activity in the presence of the dGTP, the nucleotide corresponding to the first base of the double-stranded region. T4 polymerase was then inactivated by heating for 10 min at 65 °C and the reaction mixture allowed to cool slowly to room temperature over 2 h. Ligation was accomplished by using T4 DNA ligase plus ATP to 1 mM at 24 °C for 2.5 h. The ligation mixture was subsequently transformed into competent JM101 cells. Several clear plaques that appeared were then isolated and used to prepare phage by inoculating 2 mL of freshly diluted JM101 cells in 2× TY

medium at 37 °C. After 6 h, the cells were collected by centrifugation and 5 μL of 10× SDS loading dye (250 mM EDTA, 1% SDS, 0.1% bromophenol blue, 50% glycerol, pH 8.0) was added to 25 μL of culture supernatant and the total loaded onto a 1% agarose gel without ethidium bromide. A substantial portion of DNA had been deleted from 10 out of a total of 54 clones screened. Sequencing of single-stranded template prepared from these phage confirmed that 801 base pairs had been deleted *and* the *Eco*RI site originally in the M13mp19 polylinker region was still intact in 8 of these clones. The resulting M13 phage containing the 1.1-kb *entB* fragment is called mFR5.

(*c*) *Subcloning into pKK223-3.* The replicative form of mFR5 was incubated with *Eco*RI/*Hin*dIII, and the 1.1-kb fragment containing the *entB* gene was isolated and purified by low melting point agarose gel electrophoresis and ion-exchange chromatography (Elutip). The plasmid pKK223-3 was linearized by *Eco*RI/*Hin*dIII, isolated, and purified in a similar fashion. Ligation of the 1.1-kb fragment containing the *entB* gene into pKK223-3 using T4 DNA ligase at 24 °C for 14 h was followed by transformation into *E. coli* JM105. After overnight incubation at 37 °C, individual colonies were picked from the plate and grown in 5 mL of LB broth containing 100 μg/mL ampicillin at 37 °C for 6 h and collected by centrifugation. A DNA miniprep and subsequent restriction mapping showed the presence of the recombinant pKK223-3 plasmid containing the 1.1-kb insert, herein described as pFR1.

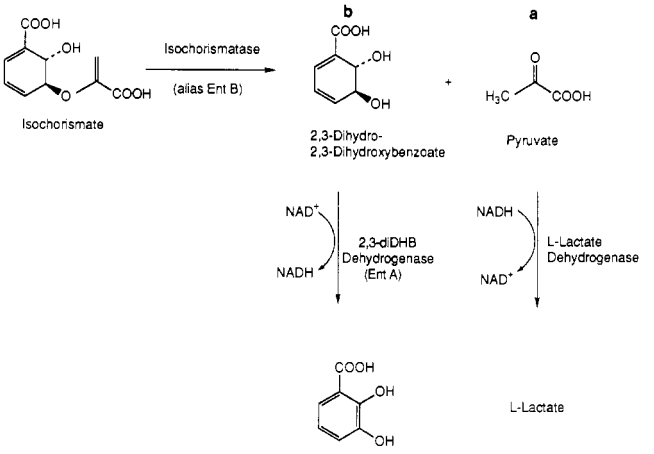
Purification of Isochorismatase. (*a*) *Crude Cell Extract.* A 1.1-L culture of *E. coli* JM105/pFR1 was grown in LB broth containing ampicillin (100 μg/mL) at 37 °C until the OD_{595} reached 0.6, at which point IPTG was added to a final concentration of 1 mM. Three hours later, the cells (2.8 g) were harvested by centrifugation, washed in 0.1 M Tris-HCl/5 mM DTT/pH 8.0, resuspended in 8.2 mL of the same buffer, and lysed by three passages through a French press cell operating at 12000 psi at the orifice. Cell debris was removed by centrifugation (15 min, 10000*g*), DNase added (≈1 mg), and the resulting crude extract incubated on ice for 1 h.

(*b*) *Ammonium Sulfate Fractionation.* Ammonium sulfate was added to the crude extract slowly while stirring on ice to give a final concentration of 30% saturation. After centrifugation (15 min, 10000*g*) to remove the precipitate, isochorismatase was precipitated by bringing the ammonium sulfate concentration of the supernatant to 50% saturation by slow addition while stirring on ice. The precipitate was collected by centrifugation (15 min, 10000*g*) and dissolved in 6.0 mL of 25 mM Tris-HCl buffer, pH 8.0, containing 5 mM dithiothreitol (buffer B).

(*c*) *Gel Filtration Chromatography.* The protein fraction after ammonium sulfate precipitation was applied to a 115 × 2.6 cm column of Sephadex G-150–120 (Sigma) and eluted with buffer B at a flow rate of 20 mL/h at 4 °C. Fractions (6.0 mL) were collected and assayed for protein by measuring the absorbance at 280 nm. Fractions of the major peaks that eluted were assayed for isochorismatase activity. A single peak which eluted after the void volume was found to catalyze the hydrolysis of isochorismate to pyruvate and 2,3-dihydro-2,3-dihydroxybenzoate. The fractions from this peak were combined for the next purification step.

(*d*) *Anion-Exchange Chromatography.* The fractions containing isochorismatase activity were applied to a prepacked Mono-Q 16/10 column (Pharmacia) at 4 °C and a flow rate of 5.0 mL/min. After washing with 250 mL of buffer B, isochorismatase was eluted with a linear 500-mL gradient of 0–0.6 M KCl; isochorismatase eluted at about 0.4 M KCl.

Scheme I: Coupled Assay for Isochorismatase



(*e*) *Analysis of Proteins.* Polyacrylamide gel electrophoresis under denaturing conditions was performed on 12–13% gels as previously described (Laemmli, 1970). High molecular weight standards were from Bethesda Research Laboratory, Gaithersburg, MD. Protein determinations used Bradford's method (Bradford, 1976) with bovine serum albumin as a standard.

(*f*) *Storage of Purified Protein.* After anion-exchange chromatography, the fractions containing isochorismatase were pooled and dialyzed quickly by repetitive concentration/dilution twice with buffer B in a 50-mL Amicon filtration cell with a PM-10 membrane and further concentrated to 15 mL. This solution was then dialyzed further in Spectra/Por 2 dialysis tubing (American Scientific Products Inc.) against a solution of 50% buffer B and 50% glycerol over 24 h with three changes of buffer. The resulting protein was stable for several months at −20 or +2 °C.

(*g*) *Native Molecular Weight Determination.* The native molecular weight for isochorismatase was determined by chromatography of the purified protein on a TSK-250 (Bio-Rad) gel filtration column using the following molecular weight standards (M_r): thyroglobulin (669000), ferritin (440000), catalase (232000), aldolase (149000), hemoglobin (64500), and carbonic anhydrase (29000). A flow rate of 1.0 mL/min with 25 mM Tris-HCl/25 mM NaCl/pH 7.0 at 24 °C as eluate was employed. A plot of $(V_e - V_0)/(V_t - V_0)$, where V_e = elution volume, V_0 = void volume, and V_t = total column volume, versus log (molecular weight) for the standards gave a linear curve which could be used to predict the molecular weight.

(*h*) *N-Terminal Sequence Determination.* Twelve residues of the N-terminal sequence of isochorismatase were determined by using the Edman degradation procedure by Dr. William Lane at Harvard Microchemistry Facility, Harvard University, Cambridge, MA.

Assays for Isochorismatase. (*a*) *Coupled Assay with* L-*Lactate Dehydrogenase.* Isochorismatase activity could be monitored by observing the *decrease* of absorbance at 340 nm due to NADH oxidation using L-lactate dehydrogenase (LDH) to convert the released pyruvate to L-lactate (Scheme Ia). The assay mixture contained isochorismic acid (**1**) (or a substrate analogue), NADH (0.10 mM), and LDH (approximately 3.5 units) in 50 mM potassium phosphate buffer, pH 7.01 at 37 °C. The order of addition was NADH and isochorismate followed by LDH; this allowed the consumption of extraneous pyruvate. Isochorismatase was added after the absorbance at 340 nm remained constant with time. In certain cases, it was necessary to add an additional aliquot of NADH if the

Table I: Purification of Isochorismatase Activity from a 1.1-L Shake Flask of JM105/pFR1

	volume (mL)	activity[a]	protein (mg/mL)	specific activity[b]	purification (x-fold)	yield (%)
crude extract	5.6	158.3	35.5	4.46	1	100
0–30% $(NH_4)_2SO_4$	5.4	155.5	35.2	4.42	0.99	95
30–50% $(NH_4)_2SO_4$	5.0	158.1	36.8	4.30	0.96	89
Sephadex G-150	54.0	14.5	2.7	5.37	1.20	88
Mono-Q anion exchange	73.0	10.4	1.2	8.67	1.94	86

[a] Activity is defined as the number of μmol of NADH oxidized min^{-1} mL^{-1} in the L-lactate dehydrogenase coupled assay after subtracting NADH oxidase activity in the absence of isochorismic acid. [b] Specific activity is defined as the number of μmol of NADH oxidized min^{-1} (mg of protein)$^{-1}$ in the L-lactate dehydrogenase coupled assay after subtracting NADH oxidase activity in the absence of isochorismic acid.

extraneous pyruvate consumed too much NADH.

(*b*) *Coupled Assay with 2,3-Dihydro-2,3-dihydroxybenzoate Dehydrogenase.* Alternatively, isochorismatase activity could be monitored by using a coupled assay with 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (2,3-diDHB dehydrogenase; Liu et al., 1989) and observing the *increase* of absorbance at 340 nm due to NAD reduction by the other product of the isochorismatase reaction, 2,3-dihydro-2,3-dihydroxybenzoate (Scheme Ib). The assay mixture contained isochorismate, NAD^+ (0.10 mM), and 2,3-diDHB dehydrogenase (≈10 μg) in 50 mM potassium phosphate buffer, pH 7.01, at 37 °C. The reaction was initiated by the addition of isochorismatase.

(*c*) *Determination of Substrate Concentrations.* (+)-Isochorismate (**1**) was determined by the absorbance at 278 nm by using ϵ_{278} = 8300 M^{-1} cm^{-1} as described below. (−)-Chorismate (**2**) was determined by the absorbance at 275 nm by using ϵ_{275} = 2630 M^{-1} cm^{-1} (Addadi et al., 1983). 3-[(Carboxyethenyl)oxy]benzoic acid (**14**) was determined by measuring the absorbance at 287 nm by using ϵ_{287} = 1530 M^{-1} cm^{-1} (Young et al., 1969b). The extinction coefficient used for isochorismic acid (**1**) (8830 M^{-1} cm^{-1}) differs from the value reported in previous reports (Young et al., 1969a; Busch & Berchtold, 1983). The extinction coefficient of 8300 M^{-1} cm^{-1} was determined by comparing the absorbance at 278 nm of a solution of isochorismic acid in pH 7.01, 50 mM potassium phosphate buffer, with the amount of NADH consumed or NAD^+ reduced in coupled assays with either LDH or 2,3-diDHB dehydrogenase, respectively [as described above in (a) and (b)]. For the determination of kinetic constants, the concentration of (±)-isochorismate (**1**) and compounds **5**–**8** was determined by the amount of pyruvate released after incubation with isochorismatase using the LDH coupled assay described above in (a). This method determines the concentration of only those isomers that are utilized by the enzyme. Concentrations of compounds **9**–**13** and **15**–**17** were determined gravimetrically.

(*d*) *Determination of pH Optimum for Isochorismatase.* The assay described above in (a) using LDH and natural isochorismate (900 μM) was performed in various buffers from pH 5.5 to 9.0 in increments of 0.5 pH unit. The following buffers were used instead of potassium phosphate: for pH 5.5–6.5, 50 mM 2-(*N*-morpholino)ethanesulfonic acid; for pH 7.0 and 7.5, 50 mM 3-(*N*-morpholino)propanesulfonic acid; for pH 8.0 and 8.5, 50 mM tris(hydroxymethyl)aminomethane; and for pH 9.0 and 9.5, 50 mM 2-(*N*-cyclohexylamino)ethanesulfonic acid. The amount of LDH in each assay was adjusted appropriately to keep the pyruvate to L-lactate reaction non rate limiting.

(*e*) *Kinetic Isotope Effect in D_2O.* Deuterated 50 mM potassium phosphate buffer was prepared by dissolving 340 mg of K_2HPO_4 and 415 mg of KH_2PO_4 in a 100 mL of D_2O after repeated lyophilization/rehydration D_2O. The pD of the resulting buffer was measured to be 6.81. Enolpyruvyl ether hydrolysis was measured at 37 °C by using the LDH coupled assay described above with (±)-isochorismate (**1**) at a final concentration of 370 μM. The control reaction was carried out identically except pH 7.01, 50 mM potassium phosphate buffer was used.

(*f*) *Determination of Kinetic Constants.* K_m and k_{cat} values for isochorismate and other substrates listed in Table III were determined by using the LDH coupled assay described above in (a) usually using eight different substrate concentrations. The values were determined by using the fitting routine HYPER (Cleland, 1979). Inhibitor constants for the compounds listed in Table IV were determined from reciprocal plots at four different inhibitor concentrations (Segel, 1975) according to the LDH coupled assay of enolpyruvyl ether hydrolysis of (±)-4,5-dihydroisochorismate (**5**).

NMR Experiments. NMR spectra were recorded at 500 MHz with a Varian VXR500 spectrometer using a water suppression pulse sequence. Samples were maintained at 30 °C during data acquisition. Standard NMR spectra of isochorismate (**1**), chorismate (**2**), 2,3-dihydro-2,3-dihydroxybenzoate (**3**), and 3,4-dihydro-3,4-dihydroxybenzoate (**4**) were obtained on 1–2 mg dissolved in 0.70 mL of the deuterated buffer described above for the kinetic isotope experiment. Enzyme was dialyzed three times by rapid Centricon filtration of a 50% glycerol enzyme solution against deuterated potassium phosphate buffer. Dialyzed isochorismatase (≈100 μg) was added to observe either the conversion of isochorismate (**1**) to 2,3-dihydro-2,3-dihydroxybenzoate (**3**) or that of (−)-chorismate (**2**) to 3,j-dihydro-3,4-dihydroxybenzoate (**4**). (±)-Isochorismate (**1**) purified by reverse-phase chromatography as described above was used for these experiments.

Results

Subcloning of entB and Overproduction of Isochorismatase. The strategy to subclone and express the *entB* gene eventually involved inserting the gene into the multicopy plasmid pKK223-3 containing the strong *tac* promoter. Subcloning of the fragment directly into this vector using the available restriction sites flanking the *entB* gene, however, would have resulted in a large stretch of untranslatable DNA (≥800 base pairs) between the start of the gene and the promoter element and would drastically reduce the efficiency of transcription. Therefore, the 2.0-kb *Hpa*I fragment containing the *entB* gene was first cloned into the *Sma*I site of M13mp19 (Figure 1A), and the method described in the preceding paper for overexpressing the *entC* gene product (Liu et al., 1990) was used to delete 801 bp from the *Eco*RI site in the polylinker region of M13mp19 to a region just upstream of the *entB* gene Shine–Delgarno sequence (Figure 1B). The proper clone resulting from the deletion reaction, mFR5, now contains an *Eco*RI site 23 base pairs upstream from the start of the *entB* gene. It is now straightforward to remove the *entB* fragment (now ≈1.2 kb) by digestion of double-stranded mFR5 with *Eco*RI/*Hin*dIII and subclone this fragment into *Eco*RI/*Hin*dIII-digested pKK223-3 (Figure 1C). The resulting plasmid pFR1 was

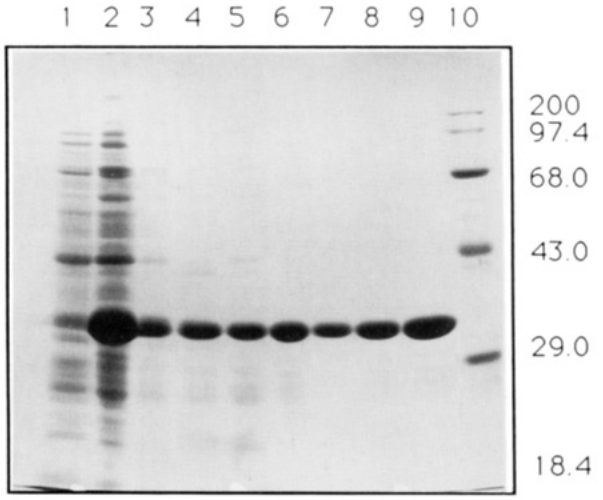


FIGURE 2: Purification of isochorismatase as followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Lane 1, whole cells of uninduced pFR1/JM105, 50 μg of protein. Lane 2, whole cells of pFR1/JM105 4 h after induction by IPTG, 50 μg of protein. Lane 3, crude extract from pFR1/JM105, 50 μg of protein. Lane 4, 0–30% ammonium sulfate precipitation of crude extract, 50 μg of protein. Lane 5, 30–50% ammonium sulfate precipitation, 40 μg of protein. Lane 6, combined isochorismatase fractions after Sephadex G-150 gel filtration chromatography, 15 μg of protein. Lanes 7–9, combined isochorismatase fractions after Mono-Q anion-exchnge chromatography. The amount of protein loaded was 5 μg in lane 7, 10 μg in lane 8, and 20 μg in lane 9. Lane 10, molecular weight standards.

transformed into JM105; after induction by IPTG, it was found that ≥50% of the total cellular protein expressed was isochorismatase on the basis of the yield of purified enzyme (Table I). This is qualitatively consistent with the amount of enzyme present in the crude extract as determined from sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (lane 2, Figure 2).

Purification and Characterization of Isochorismatase. The purification of isochorismatase was accomplished by a five-step procedure (Table I) yielding 88 mg of essentially homogeneous enzyme from 1.1 L of growth medium containing 2.8 g of cells. Crude extract was treated with DNase to remove nucleic acids since protamine sulfate precipitated isochorismatase. Gel filtration chromatography immediately following ammonium sulfate precipitation obviated the need for dialysis. SDS–PAGE of the protein fraction after anion-exchange chromatography indicated an essentially pure protein (Figure 2, lanes 7–9; overloading of the protein in lane 9 indicates <1% contamination remaining). The subunit molecular weight was calculated to be 35000 compared with the calculated molecular weight obtained from the DNA sequence of 32554 (Nahlik et al., 1989; Liu et al., 1989). Gel filtration of isochorismatase on a Superose 12 gel filtration column against molecular weight standards yielded a native molecular weight of 159000 (≈4.9 monomers/holoenzyme). Amino-terminal sequencing of the first 12 residues of isochorismatase gave the sequence: H_2N-A-I-P-K-L-Q-A-Y-A-L-P-E...-COOH. This sequence is identical with that predicted from the DNA sequence (Nahlik et al., 1989; Liu et al., 1989) after processing of the first methionine.

The strategy for assaying isochorismatase is outlined in Scheme I. The substrate isochorismate (**1**) is now readily available by either chemical or enzymic synthesis (see Materials and Methods). Progress of the isochorismatase reaction can be followed by using a coupled assay to monitor either of the products of the reaction, pyruvate or 2,3-dihydro-2,3-dihydroxybenzoate (2,3-diDHB; **3**) by their respective dehydrogenases, L-lactate dehydrogenase and 2,3-diDHB dehydrogenase. Table II validates the expected stoichiometry of the two products formed by using the coupled assays described in Scheme I. Table II also indicates that only half of (±)-isochorismate (**1**) is converted into pyruvate and 2,3-diDHB, suggesting that only one enantiomer is utilized by the enzyme. This is verified below by ^1H NMR spectroscopy. Although 2,3-diDHB dehydrogenase is available from an overproducing strain of *E. coli* harboring the *entA* gene (Liu et al., 1989), the coupled assay using L-lactate dehydrogenase is preferred when monitoring the time dependence of product formation due to the large amounts of auxiliary enzyme necessary.

Table II: Stoichiometry of the Isochorismatase-Catalyzed Reaction[a]

	substrate	
	(+)-isochorismic acid	(±)-isochorismic acid
nmol of isochorismate[b]	42.23	45.30
nmol of pyruvate formed[c]	44.21	22.35
nmol of 2,3-diDHB formed[d]	43.89	18.81
nmol of pyruvate/nmol of isochorismate	1.05	0.49
nmol of 2,3-diDHB/nmol of isochorismate	1.04	0.42

[a] Errors for each number are about 5%. [b] Nanomoles of isochorismate was determined by using $\epsilon_{278} = 8300\ M^{-1}\ cm^{-1}$. [c] Nanomoles of pyruvate was determined by the amount of NADH consumed in the LDH assay by using $\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$. [d] Nanomoles of 2,3-dihydroxy-2,3-dihydrobenzoate (2,3-diDHB) was determined by the amount of NADH produced in the 2,3-diDHB assay by using $\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$.

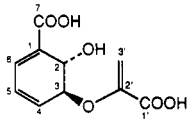
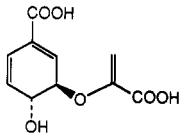
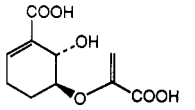
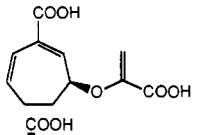
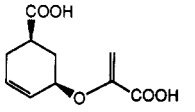
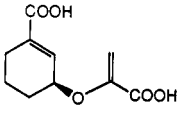
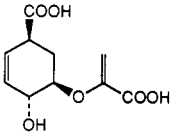
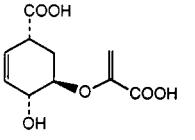
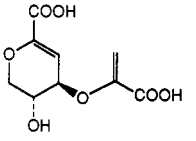
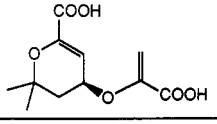
Calculated kinetic parameters at 37 °C for natural isochorismate (**1**) in the L-lactate dehydrogenase coupled assay are $K_m = 14.7\ \mu M$ and $V_{max} = 18.5\ \mu mol$ of NADH oxidized $min^{-1}\ mg^{-1}$. By use of the subunit molecular weight derived from the gene sequence (32554), the turnover number is $600\ min^{-1}$. Kinetic parameters have also been determined for synthetic (racemic) isochorismate. By use of concentrations of substrate based on the amount of pyruvate formed [i.e., concentration of substrate = $^1/_2$ (concentration of racemic compound)], $K_m = 10.6\ \mu M$ and $V_{max} = 16.0\ \mu mol$ of NADH oxidized $min^{-1}\ mg^{-1}$. These constants determined for both the natural and synthetic forms of isochorimate are in agreement if only one isomer of the racemic mixture is utilized by the enzyme.

Isochorismatase has a broad pH optimum with maximum activity extending from pH 6.5 to 7.5 (data not shown). The decline in activity with pH is sharper at higher pH with less than 3% of the maximum activity remaining at pH 9.0 while at pH 5.5, almost 50% of the maximum activity is retained.

A deuterium isotope effect is observed when the reaction is run in D_2O. Thus, when the reaction is buffered at pH 7.01 in H_2O, the velocity of the reaction is 19.00 $\mu mol\ min^{-1}\ mg^{-1}$. When the reaction is carried out in D_2O (pD = 6.81), the velocity of the reaction is 9.20 $\mu mol\ min^{-1}\ mg^{-1}$, a 2-fold decrease in the rate of the reaction. This result indicates that a step involving hydrogen transfer is partially rate limiting.

Substrate Analogues for and Inhibitors of Isochorismatase. A number of structural isomers of isochorismate have been tested as either alternate substrates or inhibitors of isochorismatase. Table III lists those compounds that were utilized by isochorismatase. This was verified by the production of pyruvate using the LDH coupled assay system (Scheme I). The compounds listed in Table III can be grouped into two classes on the basis of the representative values of K_m,k_{cat}, and the ratio of k_{cat}/K_m. Isochorismate (**1**) is the best substrate for enolpyruvyl hydrolysis in terms of the ratio k_{cat}/K_m.

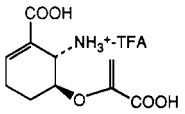
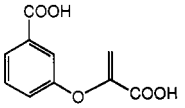
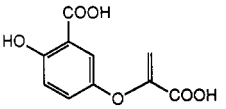
Table III[a]

compound	no.	K (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
	**1**	15	600	40
	**2**	≥37 000	≥1300	≥0.030
	**5**	23	310	13
	**6**	120	540	4.5
	**7**	280	980	3.5
	**8**	86	1300	15
	**9**	ND	≥2.1	ND
	**10**	ND	≥52.7	ND
	**11**	ND	≥2.3	ND
	**12**	ND	≥9.5	ND

[a] Abbreviation: ND, not determined.

However, 4,5-dihydroisochorismate (**5**), the cycloheptadienyl compound **6**, and compounds **7** and **8** are also very good substrates for the enzyme despite significant structural differences. Compounds **5–8** also have k_{cat} values comparable to the k_{cat} value of isochorismate. Compounds **7** and **8** are interesting since the k_{cat} values for these compounds are actually larger than the k_{cat} for isochorismate by 1.6 and 2.2 times, respectively. It is interesting to note that the absence of the C_2 hydroxyl group in compounds **6–8** does not seem to appreciably affect either substrate binding or the rate of the enzyme-catalyzed reaction, indicating that this functionality is not strictly required by the enzyme. The larger values of K_m for compounds **6** and **7** may relate to the increased size of the ring in compound **6** and the altered geometry (from trigonal to tetrahedral about C_1 with the carboxyl group now attaining a position above the plane of the ring) in compound **7**. Note that K_m values for compounds **5–8** are again determined by measuring the amount of pyruvate produced and, thus, the reported K_m values represent only those isomers of the racemic mixtures that are utilized by the enzyme.

Table IV[a]

compound	no.	K_i (μM)	type
	**13**	NI	NA
	**14**	130	comp
	**15**	2000	comp

[a] Abbreviations: NI, not inhibitory; NA, not applicable; comp, competitive.

The second group of substrate analogues, exemplified by chorismate (**2**) and compounds **9–12**, appear to be "poor" substrates when the relative values of k_{cat} are compared. These compounds also have K_m values at least 3 orders of magnitude larger than the K_m value for isochorismate (**1**) (data not shown). This decreased binding affinity most likely relates to the presence of the hydroxyl group at C_4 in all of these compounds (except **12** which has two methyl substituents at C_5). It is also of interest that the k_{cat} for chorismate (**2**), when extrapolated to infinite chorismate concentration, is over 2 times larger than k_{cat} for isochorismate (**1**). Thus, the 1400-fold difference in k_{cat}/K_m between isochorismate and chorismate is due solely to the decreased affinity of the enzyme for chorismate.

It is important to emphasize that there is a larger uncertainty in the calculated kinetic constants of the second group of compounds **2** and **9–12** than in those compounds that are better substrates (compounds **1** and **5–8**). The reason for this is twofold. First, except for chrorismate, which has a well-known extinction coefficient (Addadi et al., 1983), the concentration of the substrates **9–12** was determined gravimetrically and thus there is a larger uncertainty in the concentration of these compounds. Second, the velocity versus concentration curve for compounds **2** and **9–12** does not show complete saturation over the practical range of substrate concentrations used in these experiments, and thus the values of k_{cat} and K_m computed for these compounds are probably lower limits. For example, for the assay concentrations of chorismate from 50 μM to 10 mM, the velocity ranges from 0.075 to 8.2 μmol of NADH oxidized min^{-1} mg^{-1} (data not shown) and is practically linear throughout this range. As a result, we are reluctant to draw conclusions about the reaction from kinetic data obtained with the "poor" substrates. Thus, although it is interesting to note the range of k_{cat} values calculated within this group of similar compounds (**2**, **9–12**), the uncertainties of these values makes it difficult to attribute these differences to effects such as ring conformations or the configuration of the carboxyl group at C_1.

A number of compounds that contained an enolpyruvyl group at C_3, in addition to those shown in Table III, were not utilized by isochorismatase; i.e., if any pyruvate was released, it was ≤1 nmol in a 10-min period. Table IV documents two that were competitive inhibitors. 4,5-Dihydroisochorismate (**5**) was chosen as the substrate in the inhibitor studies since these studies require extensive amounts of substrate and **5** is

more stable than isochorismate. Dihydroisochorismate (**5**) is also an excellent substrate for the isochorismatase reaction (Table III). Of the compounds listed in Table IV, the best inhibitor is the aromatic compound **14**, which acts as a competitive inhibitor with K_i = 135 μM. The similar aromatic compound **15** with the additional hydroxyl function at C_2 was a poorer competitive inhibitor with K_i = 2.04 mM. The dihydro amino analogue of isochorismate, **13**, is not inhibitory. Thus, the presence of the amino group at C_2 evidently prevents productive binding. Other compounds that were not utilized by isochorismatase and were not inhibitory included the chorismate analogues **16** and **17** (data not shown). For these, compound **16** contains a methoxyl group at C_4 and compound **17** is missing the C_1 carboxyl group. That these compounds are not inhibitory is not surprising given the very high K_m observed for chorismic acid (**2**) (they all have substituents at C_4).

Progress of the Isochorismatase-Catalyzed Reaction Followed by NMR Spectroscopy. The processing of (±)-isochorismate (**1**) by isochorismatase was followed by ^{1}H NMR spectroscopy. The results, illustrated in Figure 3, clearly show the conversion of isochorismate to 2,3-dihydro-2,3-dihydroxybenzoate (**3**). The NMR spectrum of (±)-isochorismate at 30 °C in 50 mM potassium phosphate, pD = 6.81 buffer is shown in Figure 3A. The resonances appearing in the 5.0–7.0 ppm region include the three vinyl protons of the ring (δ 6.14, dd, H_4; δ 6.28, dd, H_5; δ 6.76, d, H_6) and the enolpyruvyl ether side chain proton trans to the ether oxygen (δ 5.17, s, H_A). Protons H_2 and H_3, and the other enolpyruvyl ether side-chain proton, H_B, resonate upfield near the water proton frequency and are not observable under water suppression conditions. Since these upfield resonances are suppressed, they do not contribute to scalar coupling, explaining the coupling pattern of those protons shown in Figure 3.

Upon addition of enzyme, several changes occur in the vinyl proton resonances over a period of about 12 min (Figure 3B, 8.3 min after adding enzyme): (1) In addition to the loss of intensity of the doublet due to H_6 at δ 6.76, a well-resolved doublet at δ 6.79 appears; (2) the 6.10–6.40 ppm region appears more complex as additional multiplets appear at δ 6.12 and 6.24; (3) the intensity of the enolpyruvyl ether proton resonance (H_A, δ 5.17) decreases; (4) an additional intense resonance assigned to the methyl protons of pyruvate appears at δ 2.30 (not shown). These changes are consistent with the reaction shown in Scheme I in which hydrolysis of the enolpyruvyl ether side chain of isochorismate occurs to form 2,3-dihydro-2,3-dihydroxybenzoate and pyruvate. Evidence that the new resonances appearing in the 5.0–7.0 ppm region are due to 2,3-dihydro-2,3-dihydroxybenzoate is obtained by comparing the spectrum in Figure 3B with a spectrum of authentic (±)-2,3-dihydro-2,3-dihydroxybenzoate shown in Figure 3C.

After 12 min, no further changes in the NMR spectrum are observed; the NMR spectrum after completion of the reaction shows an approximately equimolar mixture of isochorismate (**1**) and 2,3-dihydro-2,3-dihydroxybenzoate (**3**) (spectrum not shown). The fact that only half of the (±)-isochorismate is consumed in the reaction implies that only one isomer of the racemic compound is metabolized by the enzyme. Since the (+)-2*S*,3*S* isomer of isochorismate has been isolated and shown to be metabolized by partially purified extracts of iron-repressed *K. pneumoniae* (Young et al., 1969a), it is presumed that the 2*R*,3*R* isomer of isochorismate accumulates.

The hydrolysis of the enolpyruvyl ether side chain of (−)-chorismate (**2**) by isochorismatase was also followed by

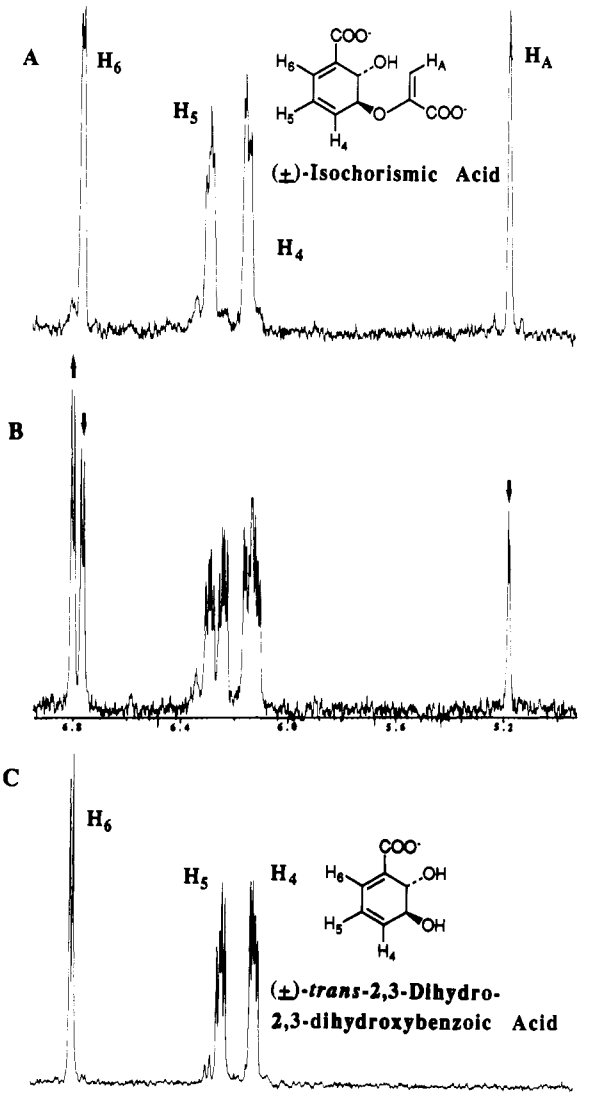


FIGURE 3: Progress of the isochorismatase-catalyzed isochorismate to 2,3-dihydro-2,3-dihydroxybenzoate conversion as followed by ^{1}H NMR spectroscopy. (A) 30 °C spectrum of (±)-isochorismate, 1.5 mg/mL, in 50 mM potassium phosphate buffer, pD = 6.81. Only the 5.0–7.0 ppm region is illustrated. (B) Isochorismate sample shown in (A), 8.3 min after adding ≈100 μg of isochorismatase. Arrows show resonances that change in intensity as isochorismate is converted into 2,3-dihydro-2,3-dihydroxybenzoate. (C) NMR spectrum of (±)-2,3-dihydro-2,3-dihydroxybenzoate in the same buffer as in (A). The proton resonances of isochorismate and 2,3-dihydro-2,3-dihydroxybenzoate are numbered according to the scheme for isochorismate shown in Table III.

NMR spectroscopy with formation of the expected products 3,4-dihydro-3,4-dihydroxybenzoate (**4**) and pyruvate (Figure 4A,B). Thus, in addition to changes in the 5.8–6.3 ppm region, the resonance at δ 6.51 (s) of the H_1 proton of chorismate decreases and a resonance at δ 6.43 ppm (s) appears while the intensity of the enolpyruvyl ether side-chain proton H_A resonance (δ 5.15, s) decreases and a resonance due to pyruvate appears at δ 2.30. A spectrum of authentic 3,4-dihydro-3,4-dihydroxybenzoate is shown in Figure 4C for comparison. The reaction is about 80% complete after 28 min, with *all* of the natural (−)-chorismate eventually converted into 3,4-dihydro-3,4-dihydroxybenzoate.

Discussion

The *entB* gene, coding for the enzyme isochorismatase, has been subcloned into the multicopy plasmid pKK223-3 and overproduced in a strain of *E. coli*. The resulting clone is an

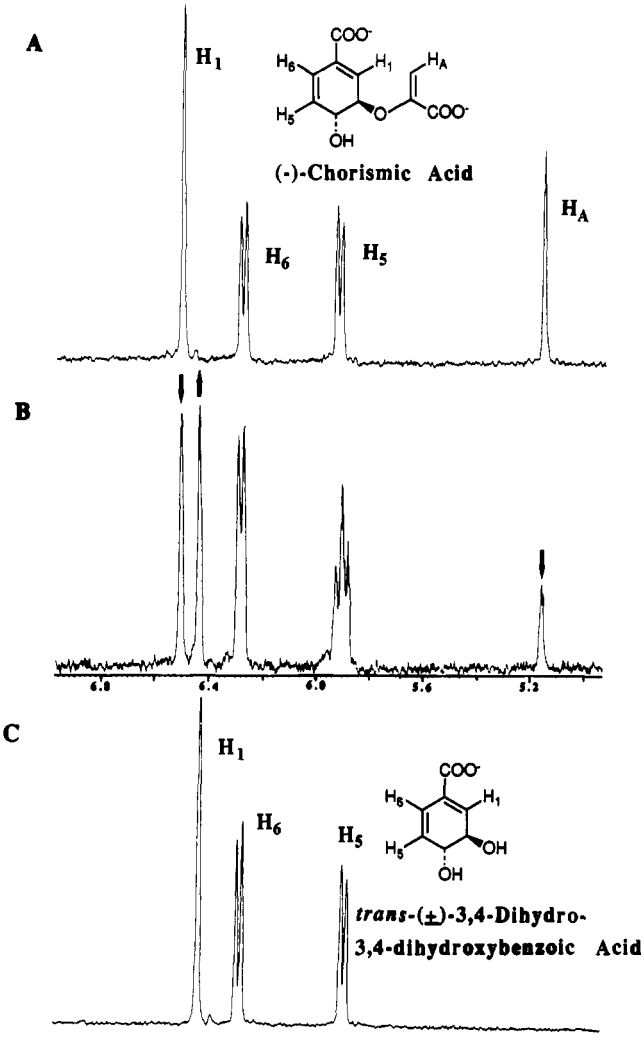


FIGURE 4: Conversion of (−)-chorismate to 3,4-dihydro-3,4-dihydroxybenzoate catalyzed by isochorismatase as followed by ^{1}H NMR spectroscopy. (A) (−)-Chorismic acid, 2.0 mg/mL in 50 mM potassium phosphate buffer, pD = 6.81. (B) Sample shown in (A), 12.3 min after adding ≈100 μg of isochorismatase. (C) Spectrum of authentic (±)-3,4-dihydro-3,4-dihydroxybenzoate in the same buffer as in (A).

excellent overproducer, with isochorismatase accounting for over 50% of the total cellular protein, permitting its purification to homogeneity for the first time. As a result, 80 mg of pure enzyme is available from as little as 1 L of culture (2.8 g of cells) after a simple purification procedure involving ammonium sulfate fractionation and two chromatographic steps. Thus, all four of the essential enterobactin biosynthetic enzymes (EntC, -E, -B, and -A) necessary for the biosynthesis and activation of 2,3-dihydroxybenzoate from chorismate have now been overproduced and purified separately (Liu et al., 1989, 1990; Rusnak et al., 1989) in quantities sufficient for enzymological characterization.

We have developed a convenient coupled assay for isochorismatase (Scheme I) using the respective dehydrogenases for the products of the reaction, pyruvate and 2,3-dihydro-2,3-dihydroxybenzoate. These assays have been used to determine kinetic constants for the substrate isochorismate as well as a number of analogues that act as either substrates or inhibitors. The K_m and k_{cat} values for natural isochorismate in the coupled assay with L-lactate dehydrogenase at 37 °C are 14.2 μM and 600 min^{-1}, respectively. Synthetic (±)-isochorismate yielded similar values for K_m and k_{cat} of 10.6 μM and 520 min^{-1}, respectively, based on the utilization of only one isomer of the racemic mixture. Within experimental error, the values for K_m and k_{cat} are the same for natural and synthetic isochorismate.

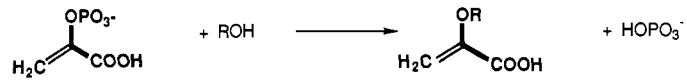
A number of compounds have been shown to act as alternate substrates of isochorismatase (Table III). There does not seem to be a strict requirement for the diene system of isochorismate since many of the compounds in Table III which were hydrolyzed have only one double bond in the ring (e.g., compounds **5** and **7**–**12**) or a diene system different from isochorismate (e.g., compounds **2** and **6**). The lack of the hydroxyl group at C_2 also does not affect the enzyme-catalyzed reaction. A substituent at C_4 does, however, alter the binding properties of the substrate to isochorismatase. This is manifested in a series of chorismate analogues (compounds **2** and **9**–**12**) which have a hydroxyl group at C_4 or large substituents at C_5 and whose K_m values have increased over 1000-fold. This is supported by the lack of inhibition of compounds **16** and **17**. The fact that the planar aromatic compounds **14** and **15** were not hydrolyzed suggests that the side chain of these compounds may not move into the proper orientation in the active site. Curiously, two compounds which have an oxygen atom in the ring (at the 6-position, compounds **11** and **12**) can be hydrolyzed by isochorismatase.

Of all compounds tested, perhaps the most surprising compound that was hydrolyzed by the enzyme was chorismate (**2**), the common precursor of the aromatic amino acids and a substrate for isochorismate synthase, the enzyme preceding isochorsmatase in the pathway to 2,3-dihydroxybenzoate (Young et al., 1971). This result explains an earlier report which found that chorismate is metabolized to 3,4-dihydro-3,4-dihydroxybenzoate by extracts of *K. pneumoniae* (Young & Gibson, 1969) grown under iron-deficient conditions (i.e., derepressing conditions for the *entCEBA* regulon). That the vinyl ether side chain of chorismate is hydrolyzed by isochorismatase seems counterproductive for the efficient production of enterobactin. However, this may not be a problem in vivo since the K_m for chorismate is quite large (≥37 mM) and the intracellular concentration of chorismate may not become high enough for this (presumably adventitious) reaction to occur appreciably.

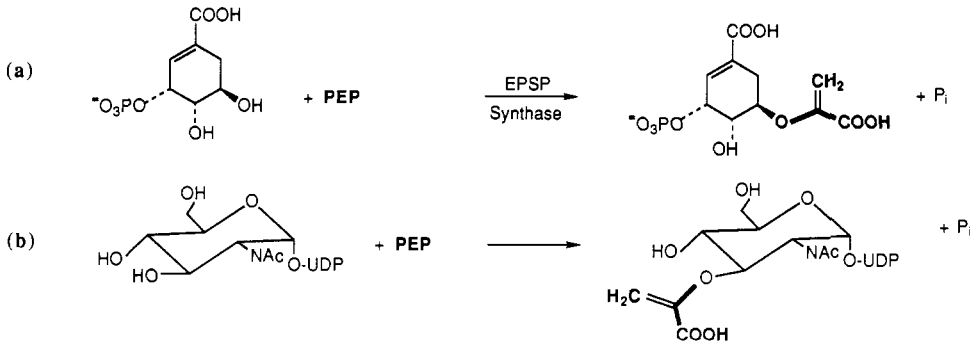
The enzyme isochorismatase is an example of a group of enzymes in which an enol ether group is involved in an enzyme-catalyzed group-transfer reaction. These enzymes can be classified into two subcategories, which we refer to as type I and type II. Type I enzymes use phosphoenolpyruvate as the donor to effect the addition of the enolpyruvyl group to an alcohol function to form a vinyl ether bond (Scheme II). Two noted examples of the type I enzymes are 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, in which the enolpyruvyl group is added to shikimate-3-phosphate in the biosynthesis of chorismate (Bondinell et al., 1971), and the enzyme in peptidoglycan cell wall biosynthesis which adds the enolpyruvyl group to the sugar 3′-OH of UDP-*N*-acetylglucosamine (Cassidy & Kahan, 1973; Zemell & Anwar, 1975a,b). A tetrahedral intermediate in the EPSP synthase reaction formed by the nucleophilic attack of C_5 hydroxyl group on the C_2 position of phosphoenolpyruvate has been detected recently by NMR spectroscopy (Anderson et al., 1988). A similar intermediate may accumulate in the analogous reaction with UDP-*N*-acetylglucosamine pyruvyltransferase, although a mechanism different from the EPSP synthase mechanism involving a covalent enolpyruvyl enzyme has been proposed (Zemell & Anwar, 1975b).

Type II enzymes, of which isochorismatase is an example, function as hydrolases to cleave a vinyl ether bond, releasing both alcohol and carbonyl products (Scheme III). Besides

Scheme II: Type I—Enolpyruvyl Ether Synthesis

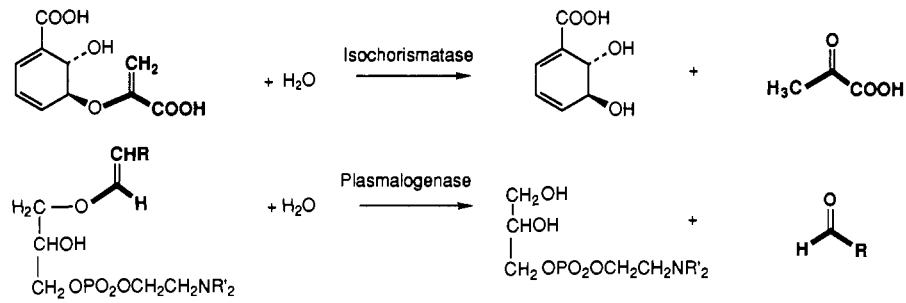


Examples:



Scheme III: Type II—Enol Ether Hydrolysis

Examples:



R = $C_{16}H_{33}$
Alkenyl glyceryiphosphorylcholine, R' = CH_3
Alkenyl glyceryiphosphorylethanolamine, R' = H

isochorismatase, the only other example we are aware of at this time is the enzyme plasmalogenase, present in liver microsomes (Warner & Lands, 1961; Ellingson & Lands, 1968; Gunawan & Debuch, 1981) and oligodendroglial brain cells (Freeman et al., 1984). Plasmalogenase hydrolyzes the alkenyl ether bond of 1-*O*-(1′-alkenyl)-2-*O*-acyl-*sn*-glycero-3-phosphoethanolamine to produce a long-chain aldehyde and the 2-acylglycerophosphoethanolamine. The mechanism for plasmalogenase has not been worked out in detail but may involve a tetrahedral intermediate similar to the one described above for the type I enzymes. The principal difference between the proposed intermediates formed in the type I and type II enzymes is that, in type I enzymes, deprotonation of the transiently formed methyl group leads to elimination of phosphate from the intermediate in the forward direction. In the type II enzymes, deprotonation would occur from the hydroxyl group originally derived from solvent water with the elimination of an alcohol group. The deprotonation in the type I enzymes probably involves an active site base to assist in catalysis. Deprotonation of the tetrahedral intermediate in the type II enzymes may well be spontaneous, owing to the inherent instability of the transiently formed hemiacetal/hemiketal.

Although a comparison of the DNA/protein sequence of isochorismatase failed to turn up any homologies, particularly when compared to the sequence of *E. coli* EPSP synthase (Duncan et al., 1984), it remains to be determined whether there are any homologies between isochorismatase and either UDP-*N*-acetylglucosamine enolpyruvyltransferase or the more mechanistically similar enzyme plasmalogenase; a gene or protein sequence is not known for either of these two enzymes.

Acknowledgments

We thank Dr. I. G. Young for providing us with the plasmid pMS111.

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