Overexpression, Purification, and Characterization of Isochorismate Synthase (EntC), the First Enzyme Involved in the Biosynthesis of Enterobactin from Chorismate†

Jun Liu,‡,§ Nina Quinn,§ Glenn A. Berchtold,§ and Christopher T. Walsh*,‡

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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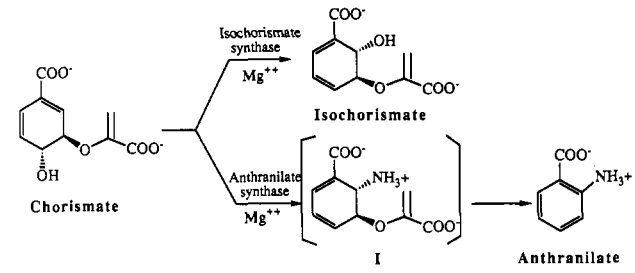
ABSTRACT: Isochorismate synthase (EC 5.4.99.6), the *entC* gene product of *Escherichia coli*, catalyzes the conversion of chorismate to isochorismate, the first step in the biosynthesis of the powerful iron-chelating agent enterobactin. A sequence-specific deletion method has been used to construct an EntC overproducer, which allows for the purification and characterization of the *E. coli* isochorismate synthase for the first time. The N-terminal sequence and the subunit molecular weight (43 000) of the polypeptide derived from SDS–polyacrylamide gel electrophoresis agree with those deduced from DNA sequence data. The enzyme is an active monomer with a native molecular weight of 42 000. It was shown that EntC alone is fully capable of catalyzing the interconversion of chorismate and isochorismate in both directions and the associated activity is not affected by EntA of the same biosynthetic pathway as has recently been speculated [Elkins, M. F., & Earhart, C. F. (1988) *FEMS Microbiol. Lett. 56*, 35; Liu, J., Duncan, K., & Walsh, C. T. (1989) *J. Bacteriol. 171*, 791; Ozenberger, B. A., Brickman, T. J., & McIntosh, M. A. (1989) *J. Bacteriol. 171*, 775]. The kinetic constants were determined with K_m = 14 μM and k_{cat} = 173 min^{-1} for chorismate in the forward direction and K_m = 5 μM and k_{cat} = 108 min^{-1} for isochorismate in the backward direction. The equilibrium constant for the reaction derived from the kinetic data is 0.56 with the equilibrium lying toward the side of chorismate, corresponding to a free energy difference of 0.36 kcal/mol between chorismate and isochorismate. The equilibrium constant was also determined independently by NMR experiments, and it was in agreement with that obtained from kinetic analysis. An $H_2{}^{18}O$ labeling experiment established that the incoming hydroxyl group during the reaction was from water rather than by intramolecular transfer from substrate.

Under iron-deficient conditions *Escherichia coli*, as well as some other enteric bacteria, synthesizes and secretes into the environment a variety of iron-chelating agents called siderophores to sequester the limited source of iron for their survival and growth. Among bacterial siderophores known to date, one stands out as the most powerful iron chelator, with an estimated association constant of 10^{52} (Harris et al., 1979). This siderophore is known as enterobactin (or enterochelin), a cyclic trimeric lactone of (2,3-dihydroxybenzoyl)serine. Since it was discovered some 20 years ago independently by two groups (Pollock & Neilands, 1970; O'Brien & Gibson, 1970), its structure and function have been studied extensively (McMurry et al., 1989; Raymond et al., 1988, 1984). It has also stirred the imagination of synthetic organic chemists, several groups having succeeded in its total synthesis (Corey & Bhattacharyya, 1977; Rastetter et al., 1980; Shanzer & Libman, 1983). In contrast, the molecular studies of its biosynthesis have lagged behind mainly because of the lack of availability of the enzymes and certain metabolic intermediates. From early genetic studies, it is known that the biosynthesis of enterobactin involves at least seven gene products, namely, EntA–G. The *entC*, *entB*, and *entA* gene products catalyze the sequential conversion of chorismate, the last common intermediate in the biosynthetic pathways to aromatic amino acids, folate, and ubiquinone, to 2,3-dihydroxybenzoate. The *entE* and *entF* gene products activate the carboxyl groups of 2,3-dihydroxybenzoate and serine with ATP for amide and ester (Rusnak et al., 1989; Bryce & Brot, 1972) bond formation, respectively. The final construction of enterobactin has been considered to require two additional enzymes, the *entD* and *entG* gene products, but the way in which these final stages of enterobactin structure assembly take place remains largely unknown.

Scheme I



Fascinated by the way in which nature managed to design and make such a powerful iron-chelating agent from a limited source of molecular inventory, we recently initiated a project for molecular studies of the enterobactin biosynthetic pathway aimed at an understanding of the series of remarkable enzymes catalyzing the conversion of chorismate and serine to enterobactin. Of the first four biosynthetic genes (*entC*, *-E*, *-B*, *-A*) that have been sequenced so far, EntA and EntE have

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*Author to whom correspondence should be addressed.

‡Harvard Medical School.

§Massachusetts Institute of Technology.

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recently been purified to homogeneity and partially characterized (Liu et al., 1989; Rusnak et al., 1989). Herein, we report our studies on the *entC* gene product. The purification and characterization of isochorismatase, the *entB* gene product, is detailed in the following paper (Rusnak et al. 1990).

Isochorismate synthase encoded by *entC* is the first enzyme in the enterobactin biosynthetic pathway, and it catalyzes the intriguing stereo- and regiospecific double S_N2' displacement of the 4-hydroxyl group in chorismate by water in the presence of Mg^{2+} to give isochorismate (Scheme I). Although there exist some chemical and biological precedents for double S_N2' type reactions [for example, see DeMarinis et al. (1974) and Law et al. (1989)], very little is known about the mechanism. Furthermore, it has recently been demonstrated that EntC has significant homology with two other chorismate-utilizing enzymes, anthranilate synthase and *p*-aminobenzoate synthase, suggesting not only that these enzymes might have shared a common ancestor (Goncharoff & Nichols, 1984) but also an intimate relationship between their catalytic functions. In particular, for the Mg^{2+}-dependent anthranilate synthase, the first half-reaction proceeds via the conversion of chorismate to intermediate I [*trans*-6-amino-5-[(1-carboxyethenyl)-oxy]-1,3-cyclohexadiene-1-carboxylic acid], an amino version of isochorismate (Policastro et al., 1984; Teng & Ganem, 1984) (Scheme I). The second half-reaction is aromatization with loss of pyruvate. Thus, anthranilate synthase can be formally viewed as a bifunctional enzyme with its first function overlapping that of isochorismate synthase. Therefore, the study of the mechanism of the reaction catalyzed by isochorismate synthase and probes of its structure and function will not only be very interesting in their own right but should also shed significant light on those related aspects of anthranilate synthase as well as PABA synthase.

Isochorismate synthase was first detected by Young and Gibson in 1969 in crude cell extracts from *Klebsiella pneumoniae* and was partially purified by one ion-exchange column. The enzyme activity was assayed qualitatively by detecting the amount of chorismate remaining in the assay mixture after a heat decomposition method on the isochorismate product (Young & Gibson, 1969). It was not until very recently that the *entC* gene was cloned and sequenced (Ozenberger et al., 1989; Elkins & Earhart, 1988). In fact, the *entC* gene was originally thought to be *FepF*, and only after extensive homology was found between sequences of this gene and *trpE* and *pabB* was it reassigned as *entC*, the gene for isochorismate synthase. In order to explain the contradicting fact that an earlier *entC* mutation (Young et al., 1971) actually mapped to the structural gene of *entA* (Ozenberger et al., 1989), it has been hypothesized that there may be some interaction between the *entA* (Liu et al., 1989) and *entC* gene products which form a two-component isochorismate synthase. However, this hypothesis could not be tested without the purified *entC* gene product.

In this paper, we report the construction of an efficient expression system for the *entC* gene and the first purification and initial characterization of the *entC* gene product.

Materials and Methods

Materials and Substrates. The natural enantiomer of chorismate was isolated by the procedure of Gibson (1970). Racemic isochorismate was synthesized as described (Busch & Berchtold, 1983). 2,3-Dihydroxybenzoic acid and anhydrous magnesium chloride were purchased from Aldrich, Milwaukee, WI. $H_2{}^{18}O$ (97–98%), D_2O (99.9%), and NaOD (40% w/w in D_2O) were from Cambridge Isotope Laboratories, Woburn, MA. Phenyl-Sepharose CL-4B, DEAE-Sepharose CL-6B, FPLC Mono-Q 16/10, and Sephadex G-100 were obtained from Pharmacia, Piscataway, NJ. RedA dye affinity gel was from Amicon, Lexington, MA.

Plasmids, Strains, and Media. The plasmid pITS557 with a 2.1-kb *Ava*I–*Hpa*I fragment containing *entC* cloned into *Ava*I–*Hin*cII-digested pGEM3Z was kindly provided by Prof. M. A. McIntosh of the University of Missouri—Columbia. The plasmid pJLT4023 was constructed by subcloning the *Kpn*I–*Pst*I fragment from pITS557 into pIBI30 (International Biotechnologies, Inc., New Haven, CT) in which an *Xba*I site is present in front of the *entC* gene. The T7 expression vector pT7-7 and K38 (HfrC λ) harboring pGP1-2 were from Dr. Stan Tabor of the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School. *E. coli* JM101 [*SupE thi*Δ (*lac-proAB*) (F′ *traD36 proA*$^+$ *proB*$^+$ *LacI*^{q} *lacZ*ΔM15)], JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) (F′ *traD36 proA*$^+$ *proB*$^+$ *LacI*^{q} *lacZ*ΔM15)], and the replicative form of M13mp19 were from New England Biolabs, Beverly, MA. Enriched "T7 media" (Tabor & Richardson, 1985) were used for the growth of the overproducer strain.

DNA Manipulations. DNA manipulations were performed as described in either Maniatis et al. (1982) or Ausubel et al. (1989). Enzymes were obtained from a number of suppliers and were used according to the instructions of the manufacturers. Oligonucleotide deletion primers were synthesized by Genetic Research, Huntsville, AL.

Construction of entC Overproducers. The closest useful restriction site available for subcloning *entC* gene from the plasmid pJLT4023 is an *Ava*I site which is 100 bp upstream from the *entC* ATG start codon (Ozenberger et al., 1989). Being the first gene in an operon (Ozenberger et al., 1989; Elkins & Earhart, 1988) regulated by iron and the Fur protein (Bagg & Neilands, 1987), this 100 bp fragment contains a promoter element and a Fur operator site (called iron box) which prevents the *entC* gene from being expressed at normal iron concentration. To overexpress the *entC* gene product, it was deemed necessary to remove the regulatory element preceding the *entC* gene before it is placed under the control of the Tac or T7 promoter. Also, a site for commonly used restriction enzymes is desired in front of the *entC* structural gene so that the subsequent cloning into an expression vector would be more convenient. The restriction enzyme *Xba*I was chosen since it does not exist within the *entC* structural gene and it has been placed in the polylinker region of pT7-7 downstream from the T7 promoter (Stan Tabor, personal communication). In the first attempt to construct an *entC* overproducer, we used a site-specific deletion method developed in this lab (Liu et al., 1989) to delete the 100 bp upstream sequence and to simultaneously introduce an *Xba*I site in front of the *entC* gene [a variation of this method has been independently developed by Chang et al. (1988) under a different context].

(*i*) *Subcloning of a 2.1 kb SacI–SphI entC-Containing Fragment from PJLI4023 into M13mp19 and Preparation of Single-Stranded DNA for the Deletion.* The plasmid pJLI4023 was digested with restriction enzymes *Sac*I and *Sph*I. The 2.1-kb *Sac*I–*Sph*I *entC*-containing fragment was isolated from a 2.0% low melting point agarose gel (NuSieve, FMC BioProducts, Rockland, ME) and ligated into a *Sac*I–*Sph*I-digested replicative form of M13mp19 as previously described (Maniatis et al., 1982; Ausubel et al., 1989). Single-stranded DNA template containing the insert was prepared by the method of Dale et al. (1985), and it was designated MJL4050.

(*ii*) *Site-Specific Deletion of MJL4050 (Figure 1a).* A mixture containing 2 μL of MJL4050 (1 mg/mL), 2.5 μL of 10 × reaction buffer (330 mM Tris–acetate, 660 mM KOAc, and 100 mM $MgOAc_2$), 1 μL of a linearization primer (5′-ATCCTCTAGAGTCGACCTGCAGGGG-3′), 1 μL of 100 mM dithiothreitol (DTT), 1 μL of *Xba*I (20 units/μL), and 12.5 μL of H_2O was incubated at 42 °C for 1 h. The restriction enzyme was inactivated by heating at 65 °C for 10 min. To the reaction mixture was added 2 μL of a deletion primer (0.02 mg/mL, 5′-TCTAGATTTTGTGGAGGATGATATGGAT-3′) followed by heating at 65 °C for 10 min and then cooling to room temperature over a period of 30 min. The deletion was accomplished by addition of 2.5 μL of 100 mM DTT, 1 μL of BSA (10 mg/mL), 1 μL of 2 mM thymidine triphosphate, and 2 μL of T4 DNA polymerase (2 units/μL) and incubation at 37 °C for 20 min. The enzyme was inactivated by heating at 65 °C for 10 min, and the mixture was cool to room temperature. The deleted DNA was religated by addition of 3 μL of 10 mM ATP, 4 μL of H_2O, and 1 μL of T4 DNA ligase (2 units/μL) and incubation at room temperature for 1 h.

(*iii*) *Transformation and Screening for Deleted Clones.* The ligation mixture from (ii) was transformed into competent *E. coli* JM101 and plated out with fresh JM101. After overnight incubation at 37 °C, a number of plaques appeared on the plate. Four plaques were used for single-stranded DNA preparation and sequencing. Two of them have the correct deleted sequence, and they were designated MJL4075.

(*iv*) *Subcloning of the Deleted XbaI–PstI Fragment into pT7-7.* The deleted 2.0-kb *Xba*I–*Pst*I DNA fragment was isolated from replicative form of MJL4075 on a 2.0% low melting point agarose gel and ligated into *Xba*I–*Pst*I-digested pT7-7. This gave pJLT5004, the first overproducer for *entC*, whose ribosome binding site is shown in Figure 1b-I.

(*v*) *Construction of the Second entC Overproducer pJLT5053.* The second overproducer was constructed to improve the expression of *entC* gene by changing the sequence of the ribosome binding site. In the first step, an *Nde*I restriction site was introduced in front of *entC* gene by using the site-specific deletion method. Thus, 2 μg of MJL4050 was linearized with *Eco*RI and the RD20mer used in the Dale method (Dale et al., 1985). After an *Nde*I deletion primer (5′-GAATTCATATGGATACGTCACTGGCT-3′) was annealed, the deletion reaction was carried out with 9 units of T4 DNA polymerase in the presence of 0.2 mM dGTP at 37 °C for 12 min. This is followed by religation and transformation similar to those in the first deletion, and the desired clones were screened by sequencing. Subsequently, the *Nde*I–*Pst*I fragment was subcloned into pT7-7 digested with the same enzymes to afford the second *entC* overproducer pJLT5053, in which the whole ribosome binding site of the native *entC* gene has been replaced by that of pT7-7 (Figure 1b-II). The second overproducer was indeed a much better overproducer for *entC* and was used for EntC purification.

Enzyme Purification (Table I). (*i*) *Crude Cell Extract.* A 3-L culture of *E. coli* strain K38/pGP1-2/pJLT5053 was grown in T7 media at 30 °C with shaking. When the OD_{595} reached 1.5, the temperature was raised to 42 °C. After 30 min, the temperature was lowered to 35 °C and the incubation was continued for another 4 h. All subsequent operations were carried out at 4 °C. The cells were harvested, washed with 200 mL of 10 mM potassium phosphate buffer (KP_i, pH 7.5), and resuspended in 100 mL of the same buffer. The cells were lysed by two passages through a French press at 12 000 psi at the orifice. The lysate was centrifuged at 10000*g* for 25

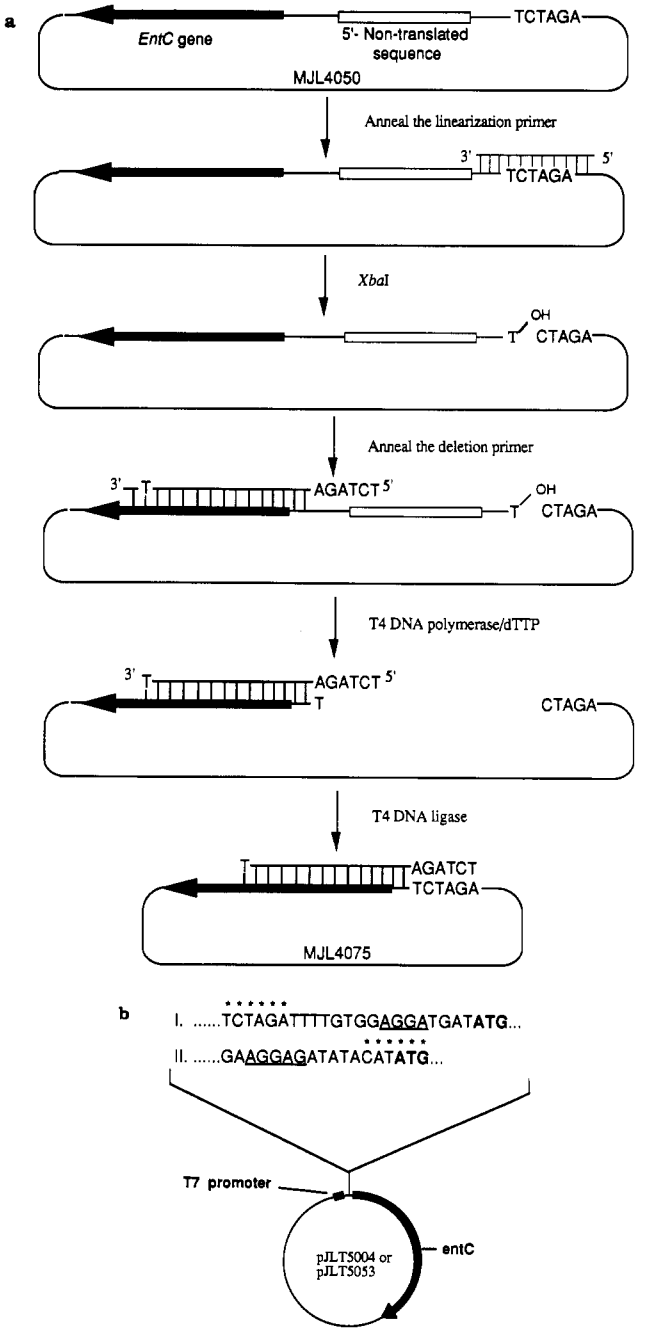


FIGURE 1: (a) Site-specific deletion of MJL4050 for the construction of the first *entC* overproducer pJLT5004. Starting from single-stranded DNA MJL4050, it was linearized with *Xba*I after the linearization primer was annealed to the *Xba*I site to generate a short double-stranded region. The 3′ to 5′ deletion of the linearized MJL4050 was stopped at the deletion primer binding site (T) where the 3′ to 5′ exonuclease activity and 5′ to 3′ polymerase activity of T4 DNA polymerase compete in the presence of the substrate thymidine triphosphate (dTTP). The 5′ overhang of the deletion primer was designed to complement the 5′ end of the single-stranded DNA template generated in the linearization step so that the religation will occur to place an *Xba*I site at the newly generated end, which is eight base pairs upstream of the Shine/Dalgarno sequence of *entC*. Highlighted are the *Xba*I site used for linearizing the DNA, the 5′ nontranslated sequence, the *entC* structural gene, and the 5′ overhang of the deletion primer. The sequences of the two primers used are shown in the text. (b) Structure of pJLT5004 and pJLT5053 with their ribosome binding site sequences (rbs) highlighted. (I) Sequence of rbs for pJLT5004; (II) sequence of rbs for pJLT5053. In each sequence, the restriction site generated by the sequence-specific deletion–insertion method is starred. The Shine/Dalgarno sequences are underlined, and the starting ATG codons are in boldface. The relative locations of T7 promoter and the *entC* structural gene are indicated. There is also a β-lactamase gene divergently placed on the same plasmid which is not shown.

Table I: Purification of Isochorismate Synthase

step	protein			enzyme			
	volume of fraction (mL)	concn (mg/mL)	total amount (mg)	specific activity (units/mg)	total amount (units)	yield (%)	purification factor (x-fold)
crude extract	100	11.6	1160	0.33	383	100	1
15–85% ammonium sulfate	45	20.2	909	0.47	427	111	1.4
phenyl-Sepharose	52	4.4	229	1.51	346	90	4.6
DEAE-Sepharose CL-6B	16	6.4	102	1.63	166	43	4.9
Red A	9.0	4.2	38	3.91	149	39	11.8
Mono-Q 16/10	3.1	3.4	11	4.00	44	11	12.1

min, and the supernatant (100 mL) was separated. Nucleic acid was removed by addition of 20 mL of 2% protamine sulfate solution followed by centrifugation at 10000g for 30 min.

(*ii*) *Ammonium Sulfate Precipitation.* To the 110 mL of crude extract obtained in (i) was added 9.24 g of ammonium sulfate (15% saturation). After stirring for 20 min, the supernatant was separated by centrifugation at 12000g for 30 min. Ammonium sulfate was added to the supernatant to 80% saturation (50.16 g), and the protein pellet was separated by centrifugation at 12000g for 30 min. The pellet was dissolved in 40 mL of 0.5 M of ammonium sulfate in 50 mM KP_i buffer.

(*iii*) *Phenyl-Sepharose Chromatography.* A column of phenyl-Sepharose CL-4B (12 × 1.2 cm) was equilibrated with 300 mL of 50 mM KP_i (pH 7.5) containing 0.5 M ammonium sulfate. The 40-mL protein solution from (ii) was applied to the column, and the column was washed with 200 mL of the equilibrating buffer. The enzyme was eluted with 300 mL of a linear gradient from 0.5 to 0 M ammonium sulfate in 50 mM KP_i. Fractions containing isochorismate synthase activity were pooled and concentrated by an Amicon ultrafiltration cell (PM30) to a final volume of 52 mL. The concentrated protein solution was dialyzed overnight against 2 L of 20 mM Tris-HCl (pH 7.5).

(*iv*) *DEAE-Sepharose Anion-Exchange Chromatography.* The dialyzed protein solution from (iii) was applied to a 2.2 × 12 cm DEAE-phenyl-Sepharose CL-6B column equilibrated with 300 mL of 20 mM Tris-HCl (pH 7.5). The column was washed with 200 mL of the Tris-HCl buffer, and the enzyme was eluted with a linear gradient of 0–0.4 M NaCl. The fractions containing IS activity were combined, concentrated, and dialyzed against 2 L of 20 mM Tris-HCl.

(*v*) *Dye Ligand Chromatography.* The 16 mL of dialyzed protein solution from (iv) was applied to a column of Dyematrex Red A–agarose (6 × 1 cm), and the column was washed with 100 mL of starting Tris-HCl buffer. The enzyme was eluted with 200 mL of a linear gradient of 0–1 M of NaCl. The fractions containing IS activity were pooled, concentrated, and dialyzed against 2 L of 20 mM Tris-HCl.

(*vi*) *FPLC Mono-Q Anion-Exchange Chromatography.* The pooled and dialyzed solution from (v) was applied to an FPLC Mono-Q column (16/10) and washed with 100 mL of 20 mM Tris-HCl (pH 7.5). The protein was eluted with a 300-mL linear gradient of 0–0.2 M NaCl. Fractions containing IS activity were combined and concentrated to 3 mL. This solution was dialyzed overnight against a storage buffer consisting of 20 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM DTT, and 40% (v/v) glycerol and stored at −20 °C.

Analyses of Protein. Protein concentration was determined by the method of Bradford (1976) using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). The native molecular weight of the enzyme was determined by chromatography on an FPLC Superose 6 column (Pharmacia) from a plot of logarithm of molecular weight vs K_{av}. The molecular weight standards used included blue dextran (M_r = 2000000), thyroglobulin (M_r = 669000), ferritin (M_r = 440000), catalase (M_r = 232000), aldolase (M_r = 158000), bovine serum albumin (M_r = 68000), ovalbumin (M_r = 43000), chymotrypsinogin A (M_r = 25000), and vancomycin (M_r = 3300). The subunit molecular weight was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis performed as previously described (Laemmli, 1970). The N-terminal sequence was determined on an 890M microsequencer by Drs. W. Lane and D. Andrews at the Harvard University microchemistry facility, Cambridge, MA.

Enzyme Assays. All kinetic experiments were performed at 37 °C. Absorption spectra and steady-state reaction rates were measured on a Perkin-Elmer 554 spectrophotometer with a circulating thermostat.

(*i*) *Forward Direction.* The forward reaction rate was measured by monitoring the absorbance change at 275 nm in a coupled assay with an excess amount of isochorismatase (Rusnak et al., 1990). The initial rate was calculated on the basis of the extinction coefficients of the substrate (chorismate, λ_{max} = 273 nm, ϵ_1 = 2630 M^{-1} cm^{-1}) (Heyde & Morrison, 1978) and the product (2,3-dihydro-2,3-dihydroxybenzoate, λ_{max} = 278 nm, ϵ_2 = 8150 M^{-1} cm^{-1}) (Young et al., 1969) by the equation

$$V_i = \frac{\Delta A}{\Delta t}\frac{10^6}{\epsilon_2 - \epsilon_1} = \frac{\Delta A}{\Delta t}\frac{10^6}{5520}\ (\mu M/\text{min})$$

The assay mixture contained 100 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 100 nM isochorismate synthase, and 200 nM isochorismatase. The concentration of chorismate was determined by using the extinction coefficient at 273 nm.

(*ii*) *Reverse Direction.* The reaction rate in the reverse direction was measured by coupling with an excess amount of anthranilate synthase (Walsh et al., 1987) and lactate dehydrogenase. The rate was calculated on the basis of the decrease in absorbance at 340 nm from the conversion of NADH to NAD^+, with the change in extinction coefficient at this wavelength taken to be 6220 M^{-1} cm^{-1}. The assay mixture (1 mL total volume) consisted of 100 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM glutamine, 0.2 mM NADH, 76 nM isochorismate synthase, 100 nM lactate dehydrogenase, and 80 nM anthranilate synthase and varying amounts of racemic isochorismate. Isochorismate concentrations were determined by the amount of NADH consumed after complete conversion of isochorismate to 2,3-dihydro-2,3-dihydroxybenzoate and pyruvate with excess amounts of isochorismatase and lactate dehydrogenase.

NMR Analysis. NMR spectra were recorded on a 500-MHz Varian VXR-500 spectrometer. Water suppression was employed, and all spectra were taken in a buffer of 50 mM KP_i (pH 7.5) and 5 mM $MgCl_2$ in D_2O. The spectra of pure

chorismate and isochorismate were obtained by dissolving 2 mg of each in 0.7 mL of buffer. For the enzyme-catalyzed interconversion, isochorismate synthase was first exchanged into D_2O buffer by three successive dilution (with about 2 mL of the deuteriated buffer) and ultrafiltration cycles. Either chorismic acid or isochorismic acid neutralized with 2 equiv of 1 M KOD was dissolved in the buffer and equilibrated at 37 °C for 4 min before isochorismate synthase in deuteriated buffer was added. Subsequently, a spectrum was scanned every 4 min. The ratio between chorismate and isochorismate was calculated from the integrations of the most downfield protons in each compound, i.e., the C-6 proton in chorismate and the C-2 proton in isochorismate.

Oxygen-18 Labeling Experiment and Mass Spectrometry. To 1 mL of a mixture consisting of 100 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 30 mM NAD^+, 1 mM isochorismatase, 0.63 mM 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase, and 6.5 mM chorismate (1.5 mg) in 50% $H_2{}^{18}O$ was added 5 μL of isochorismate synthase (7.0 mg/mL; final concentration 0.81 mM). The mixture was incubated at 37 °C for 25 min until no further absorbance change at 340 nm occurred. The mixture was acidified with 70 μL of 1 M trifluoroacetic acid (TFA), filtered through an 0.22-μm membrane and injected into a Brownlee C-18 column (5 μm, 250 × 4.6 mm) on a Waters HPLC system. The column was eluted with 30% MeOH–70% H_2O containing 0.01 M TFA at a flow rate of 0.6 mL/min with the detector set at 314 nm. The 2,3-dihydroxybenzoate eluted from the column at 20 min was collected. The eluant was freeze-dried, and a white powdery solid was obtained which was used for MS analysis. In one control experiment, 100% $H_2{}^{16}O$ was used in place of 50% $H_2{}^{18}O$. In a second control experiment, 13 mM synthetic isochorismate (3.0 mg) was employed in place of chorismate and isochorismate synthase was omitted from the incubation mixture.

The mass spectra were recorded by E. Takach using electron ionization on a Finnigan MAT 8200 mass spectrometer at 32 eV.

Results

Subcloning, Expression, and Purification of Isochorismate Synthase under T7 Promoter. The 2.1-kb fragment from pJLT4023 contained, in addition to the *entC* structural gene, the regulatory region for the *entCEBA(P15)* regulon which includes the promoter and the Fur protein regulated operator (Ozenberger et al., 1989; Elkin & Earhart, 1988; Bagg & Neiland, 1987). In the first attempt to construct an *entC* overproducer, about 100 base pairs of DNA upstream of the *entC* structural gene were deleted and an *Xba*I restriction site was simultaneously introduced eight base pairs upstream of the *entC* Shine/Dalgarno (S/D) sequence (Figure 1b-I) to yield the strain K38/pGP1-2/pJLT5004. While elevated expression was observed from protein gel analyses as compared to pJLT4023 in which the whole 2.1-kb fragment was placed under the control of the T7 promoter, when a purification of the enzyme was attempted, pure enzyme was obtained in only small amounts (2 L of culture gave less than 1 mg of pure enzyme).

A closer examination of the ribosome binding site of the native *entC* gene showed that the S/D sequence and the start ATG codon were separated by only four base pairs as opposed to seven to nine base pairs typical of *E. coli* genes. It was reasoned that the *entC* expression from the first overproducer construct pJLT5004 might be limited at the translational level due to the unusually short distance separating the S/D sequence and the ATG start codon. A second overproducer construct was made by replacing the whole native ribosome binding site of *entC* with that from pT7-7, increasing the distance between the S/D sequence and the ATG start codon from 4 bp (in the wild type and in pJLT5004) to 8 bp. This is completed in two steps. First, a sequence-specific deletion method was used to delete the upstream *entC* regulatory region and, in addition, to change the G at −3 to C, allowing the introduction of an *Nde*I restriction site (5′-CATATG-3′) in front of *entC* gene. Subsequently, the 2-kb *Nde*I–*Pst*I fragment was subcloned into pT7-7 to afford the second overproducer pJLT5053 (Figure 1b-II). When the new overproducer (K38/pGP1-2/pJLT5053) was induced by heating at 42 °C, much higher expression was observed although a substantial amount of isochorismate synthase was found in the cell debris after the cells were opened. This strain was used for purification of isochorismate synthase.

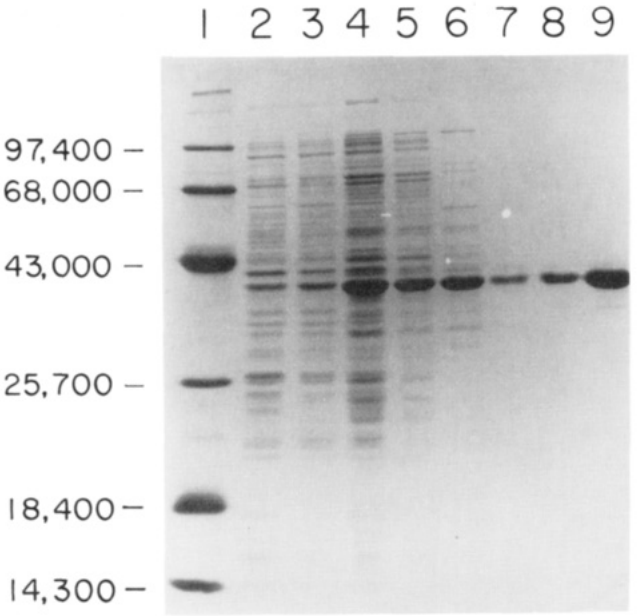


FIGURE 2: Twelve percent sodium dodecyl sulfate–polyacrylamide gel electrophoresis of isochorismate synthase at different stages of purification. Lanes: 1, marker proteins; 2, crude cell extract; 3, 15–85% ammonium sulfate pellet after dialysis; 4, phenyl-Sepharose column fractions; 5, DEAE-Sepharose CL-6B column fractions; 6, Red A column fractions; 7, Mono-Q 16/10 fractions (2 μg); 8, Mono-Q 16/10 fractions (8 μg); 9, Mono-Q 16/10 fractions (35 μg). Molecular weight standards used were phosphorylase (97 400), bovine serum albumin (68 000), ovalbumin (43 000), α-chymotrypsinogen (25 700), β-lactoglobulin (18 400), and lysozyme (14 300).

With the overproducer strain *E. coli* K38/pGP1-2/pJLT5053, the isochorismate synthase constitutes about 8% of overall soluble cellular proteins (as judged by the specific activity) despite the large amount of enzyme precipitation that followed heat induction. After a substantial survey of separation techniques, the optimized purification consisted of four chromatographic steps. One unusual feature about isochorismate synthase is that it is not subject to fractionation by ammonium sulfate as the specific activities at different percent saturations were similar (data not shown). As shown in Figure 2, after FPLC Mono-Q anion-exchange chromatography, the protein was essentially pure. From 3 L of cells, 11 mg of pure enzyme was obtained.

From SDS–polyacrylamide gel analyses, the enzyme's subunit molecular weight was determined to be 43 000; this matches the 42 000 native molecular weight determined by chromatography on a Superose 6 gel filtration column. Both of these values are in agreement with that derived from predicted amino acid sequence of 42 900 (Ozenberger et al., 1989;

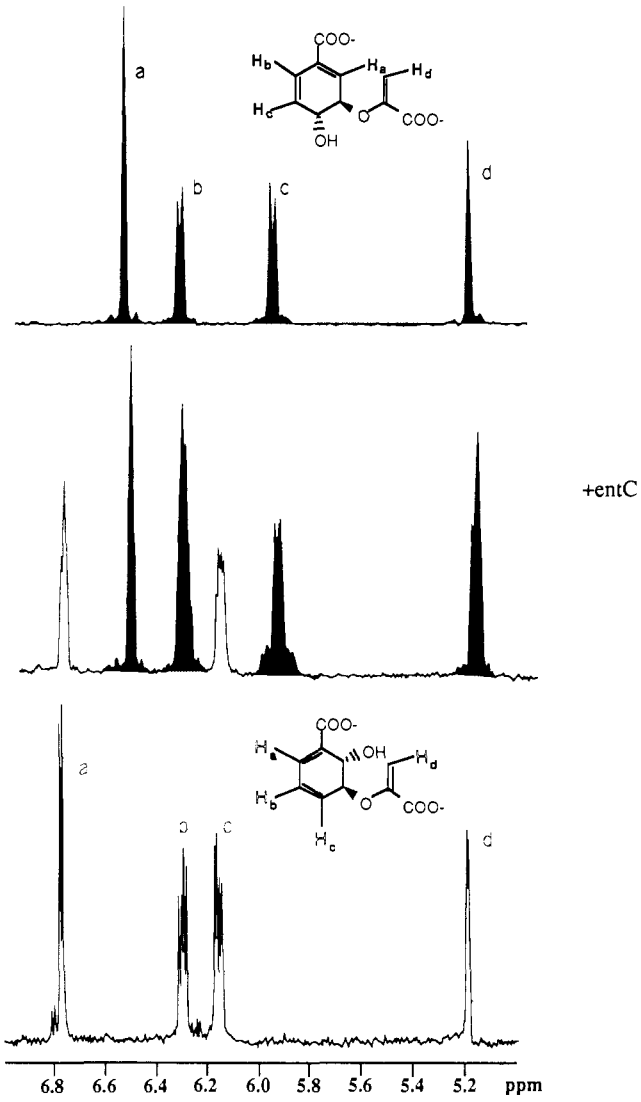
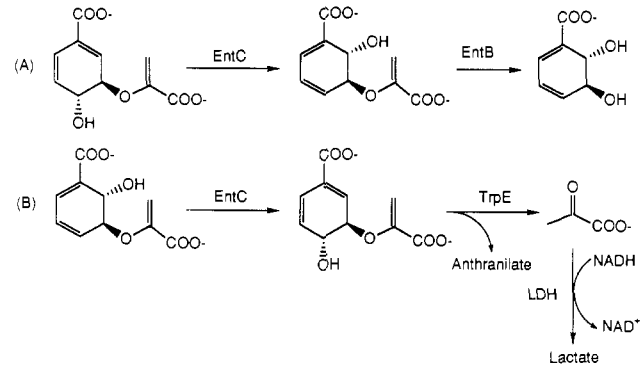


FIGURE 3: ^{1}H NMR spectra of chorismate (top), isochorismate (bottom), and their equilibrium mixture in the presence of isochorismate synthase (middle). Only the vinylic regions (5.0–8.0 ppm) of the NMR spectra were displayed. The peaks arising from chorismate are filled in black, those from isochorismate are not filled, and the overlapping peaks from both are shaded. The four vinyl protons H_a, H_b, H_c, and H_d and the corresponding peaks a, b, c, and d from both chorismate and isochorismate are labeled.

Elkins & Earhart, 1989). The amino-terminal sequence of the purified enzyme was determined to be M-D-T-S-L-A-E-E-V-Q-Q-T, which is identical with that deduced from the DNA sequence.

Determination of V_{max} and K_m for Chorismate and Isochorismate and K_{eq}. The purified isochorismate synthase catalyzes the readily reversible interconversion of chorismate and isochorismate. To obtain steady-state kinetic constants, assays were devised with coupling enzymes that permitted continuous spectrophotometric monitoring in either the forward direction (isochorismatase) or reverse direction (anthranilate synthase and lactate dehydrogenase) (parts A and B, respectively, of Scheme II). For chorismate, K_m = 14 μM and k_{cat} = 173 min^{-1}; for isochorismate, K_m = 5 μM and k_{cat} = 108 min^{-1}. This indicates that isochorismate synthase has similar binding affinity for both substrates (or products). The two rates for the interconversion are also very close. It should be pointed out that isochorismatase also uses chorismate as an alternative substrate (Rusnak et al. 1990), but its K_m for chorismate is so high compared to the substrate concentrations used in the isochorismate synthase assay that the EntB-catalyzed conversion of chorismate is negligible.

Scheme II



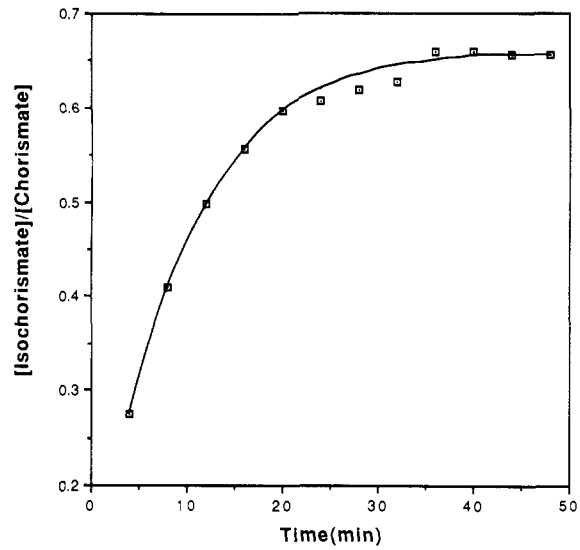


FIGURE 4: Time-dependent conversion of chorismate to isochorismate as observed by NMR spectroscopy. The ratios between the concentrations of chorismate and isochorismate are calculated on the basis of the integration intensities of the two most downfield proton peaks (H_a's) from chorismate and isochorismate as in Figure 3. The equilibrium constant derived from this curve is 0.66.

Given the ready reversibility, the K_{eq} could then be obtained by two independent routes. The first was by calculation from the steady-state kinetic constants using the Haldane equation (eq 1), which allows the calculation of K_{eq} from K_m and V_{max}

$$K_{eq} = \frac{V_{max,f}}{V_{max,r}} \frac{K_{m,r}}{K_{m,f}} \quad (1)$$

values in each direction (Briggs & Haldane, 1925). By this route, the equilibrium constant was calculated to be 0.56, corresponding to a ΔG value of 0.36 kcal/mol between the chorismate and isochorismate molecules.

To corroborate the above results, an NMR experiment not only served as an independent way to determine the equilibrium constant but also made it possible to observe directly the enzymatic interconversion of chorismate and isochorismate. When pure isochorismate synthase was added to an NMR tube containing chorismate and Mg^{2+} (as magnesium chloride), a time-dependent conversion of chorismate into isochorismate was observed by monitoring the vinyl proton regions in the 500-MHz ^{1}H NMR spectrum (Figure 3). Accumulation of spectra over time allows measurement of an approach to equilibrium, as shown for the chorismate to isochorismate direction in Figure 4. By integration of the two unique olefinic protons from chorismate and isochorismate, the equilibrium

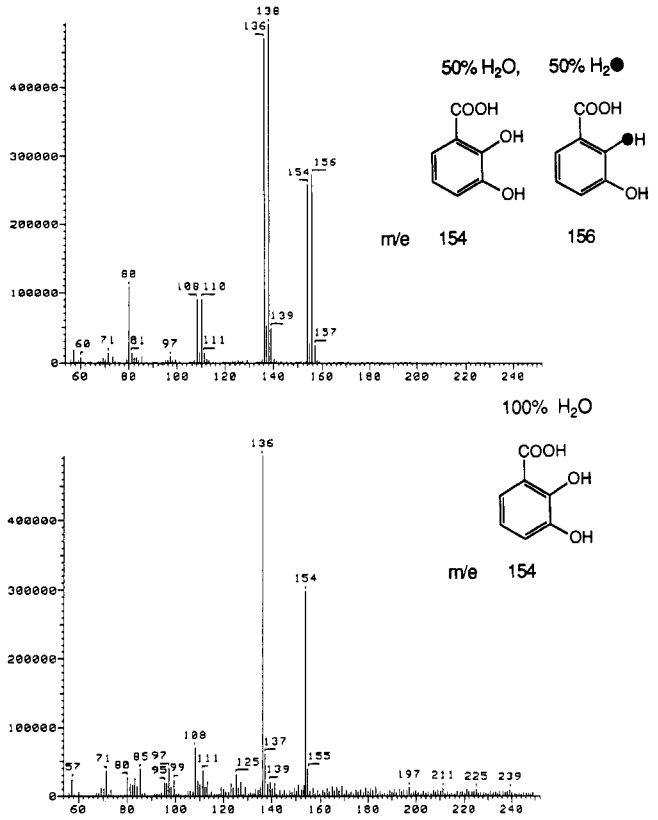


FIGURE 5: Mass spectroscopy of 2,3-dihydroxybenzoate (2,3-DHB) isolated from oxygen-18 labeling experiments. Top: Spectrum for 2,3-DHB isolated from a reaction mixture containing 50% $H_2{}^{18}O$. Bottom: Spectrum of 2,3-DHB separated from a control experiment where 100% $H_2{}^{16}O$ was used.

constant can be calculated and was determined from this experiment to be 0.66. This is in reasonable agreement with the value of 0.56 from the Haldane equation.

Oxygen-18 Labeling Experiment. While the chorismate to isochorismate transformation is in a formal sense a one substrate/one product reaction, it seemed likely that there would not be conservation of the -OH group from chorismate to isochorismate, and this was tested explicitly by an incubation in $H_2{}^{18}O$. The most obvious method to detect oxygen-18 incorporation seemed to be the examination of the molecular ion peak of the isochorismate product by mass spectrometry. However, the susceptibility of isochorismate to heat-induced decomposition ($t_{1/2}$ = 6 h at 30 °C in KP_i buffer in D_2O at pD 7.4; Berchtold, unpublished results) rendered it necessary to find a more stable derivative. Thus, 2,3-dihydroxybenzoate was chosen since it was observed to give a strong parent ion peak in mass spectrometry and we had available pure EntB (Rusnak et al., 1990) and EntA (Liu et al., 1989). These enzymes could be used in conjunction with isochorismate synthase to convert isochorismate to 2,3-dihydroxybenzoate, which could be isolated by HPLC and subjected to MS analysis. As shown in Figure 5, when the EntC, -B, and -A reaction was conducted in $H_2{}^{16}O$, only a single peak corresponding to a molecular weight of 154 was seen, while in 50% $H_2{}^{16}O$–50% $H_2{}^{18}O$, an additional peak with about equal intensity appeared at 156. In contrast, in a control experiment in which isochorismate was used in 50% $H_2{}^{16}O$–50% $H_2{}^{18}O$, no incorporation of ^{18}O was observed in the parent ion of 2,3-dihydroxybenzoate with EntB and EntA only (data not shown). The ^{18}O incorporation is therefore clearly a result of EntC-catalyzed conversion of the chorismate to isochorismate and not from subsequent steps catalyzed by EntB or EntA.

Possible Interaction between Isochorismate Synthase (EntC) and 2,3-Dihydro-2,3-dihydroxybenzoate Dehydrogenase (EntA). From a recent genetic study on the enterobactin biosynthetic genes, it was found that an earlier genetic lesion corresponding to isochorismate synthase activity, *entC401*, lies within the *entA* structural gene (Ozenberger et al., 1989). To test for a possible modifying or regulatory interaction between *entC* and *entA* gene products in vitro, we measured the isochorismate synthase activity of *entC* gene product in the absence and presence of purified EntA and vice versa. EntA has no observable effect on the EntC activity, nor does EntC have any detectable effect on EntA activity, prompting the conclusion that isochorismate synthase and 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase are two independent enzymes encoded by two separate genes. Independent experiments by Tummuru et al. (1989) with partially purified EntC and EntA has reached the same conclusion.

Discussion

The *entC* gene product, isochorismate synthase, is of interest both as the first step in the iron-regulated enterobactin biosynthetic pathway and as an enzyme catalyzing an unusual 1,5-addition/elimination sequence in the conversion of the central metabolite chorismate to the less well-known isochorismate. Following its initial assignment genetically as *entC* and assays in crude bacterial extracts to indicate isochorismate as product, essentially no further molecular information has been available for this enzyme. Until very recently, the gene map order was in error and it was only via DNA sequencing that the gene order of an *entCEBA* regulon has now been established (Elkins & Earhart, 1988; Liu et al., 1989; Ozenberger et al., 1989). We have recently confirmed that EntE, -B, and -A do indeed encode 2,3-dihydroxybenzoate–AMP ligase, isochorismatase, and 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase by overproducing each of these enzymes, purifying them to homogeneity, and establishing their catalytic activities (Liu et al., 1989; Rusnak et al., 1989, 1990). We report similar results for *entC* in this work including the first purification to homogeneity of isochorismate synthase.

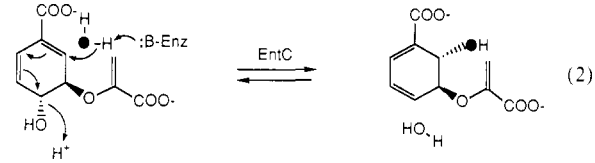
With the gene order *entCEBA* established, it was possible to subclone *entC* with a T7 expression system by placing an *Xba*I restriction site eight base pairs upstream of the *entC* S/D system by a site-specific subcloning procedure. While overproduction from the construct pJLT5004 was detected at 0.5 mg of enzyme/L of culture, the rather low level prompted closer examination of the upstream sequence of the structural gene. Subcloning to yield pJLT5004 removed the "iron box" in the 5′-upstream sequence that normally mediates Fe^{2+}-directed repression of the *entCEBA* regulon (Ozenberger et al., 1989; Elkin & Earhart, 1988; Bagg & Neiland, 1987). However, only four base pairs separate the consensus S/D sequence and the *entC* ATG initiation codon. This is at the short extreme of all such distances known in *E. coli*, where a range of 4–18 nucleotides has been cataloged (Stormo et al., 1982; Dreyfus, 1988) with typical distances of 7–9 nucleotides. This suggests that *entC* expression may be limited at the translational level even when the operon is induced under iron deficiency. This may be understandable given that chorismate is an essential metabolite for Phe, Trp, Tyr, folate, and ubiquinone synthesis, so diverting a large flux to isochorismate may compromise more central interests of the cell. To test whether alteration of the ribosome binding site to start codon distance might remove the putative translational constraint, the site-specific deletion method was used to construct pJLT5053 with an 8 bp distance between the S/D sequence and the initiation ATG codon. As a result, about 8% of the

soluble cell protein was the desired isochorismate synthase. Thus, engineering of both the distance from the promoter and the distance from the S/D sequence to the ATG start codon dramatically increased the *entC* expression.

With pure protein in hand, convenient reliable assays for isochorismate synthase have been developed, taking advantage of the contemporaneous purification of EntB and prior purification of anthranilate synthase (Walsh et al., 1987). There had been a prior hint of reversibility of isochorismate synthase in crude extract assays (Young & Gibson, 1969), but this was not quantifiable and pure isochorismate was not available. We note that while racemic isochorismate has been synthesized (Busch & Berchtold, 1983), a sample of which was invaluable in the initial characterization of both EntC and EntB activities, optically pure isochorismate can now be prepared readily by incubation of chorismate with purified EntC followed by HPLC isolation.

The chorismate–isochorismate interconversion catalyzed by isochorismate synthase is readily reversible, with comparable V_{max} values of 173 min^{-1} vs 108 min^{-1} for forward vs backward reaction, respectively, and comparable K_m values of 14 μM for chorismate and 5 μM for isochorismate. This leads to $V_{max}/K_{m(chorismate)}$ of 2×10^5 M^{-1} s^{-1} vs $V_{max}/K_{m(isochorismate)}$ of 2.5×10^5 M^{-1} s^{-1}. These V/K catalytic efficiency values are so similar that they suggest very similar pseudosymmetric recognition of the two isomers by the enzyme. The acceleration over the nonenzymic interconversion is not known since chorismate and isochorismate break down by other routes (e.g., by 3,3-rearrangement or by aromatization) before detectable interconversion. Given that the Mg^{2+}-dependent isochorismate synthase equilibrates the two isomers, chorismate and isochorismate, in a formal one substrate/one product reaction, one can use the steady-state K_m and V_{max} values in each direction to calculate via the Haldane equation a K_{eq} value, much as one would for an epimerase or a racemase, or the well-characterized triose-P isomerase (Alber et al., 1987). The K_{eq} of 0.56 provides for the first time a thermodynamic comparison of the much less well-studied metabolite isochorismate to its more celebrated isomer, chorismate. Under the indicated physiological conditions, chorismate is more stable than isochorismate by only 0.36 kcal/mol. The K_{eq} value was confirmed independently by monitoring the approach to equilibrium from the chorismate direction via integration of the chorismate and isochorismate C-6 and C-2 vinylic hydrogens in the 500-MHz ^1H NMR spectrometer. The NMR analysis also validated the absence of any other side products during the enzyme-catalyzed reaction despite the known propensity of both chorismate and isochorismate to undergo facile nonenzymic 3,3-rearrangement (Andrews et al., 1973), because enough of the overproduced pure enzyme was available to make the reaction approach equilibrium in 20–30 min. The K_{eq} of 0.5–0.6 is for all conformations of chorismate and isochorismate in solution. It has recently been determined by NMR that chorismate exists with about 10% trans-diaxial and 90% diequatorial conformers in H_2O. The corresponding ratio for isochorismate conformers, however, has not been determined, and it is not yet known if isochorismate synthase shows a preference for trans-diaxial substrate conformers, as postulated for chorismate mutase (Copley & Knowles, 1987).

With the enzyme overproduced and purified in quantity, assays established, and steady-state kinetic constants in both directions measured, the stage is now set for examination of mechanistic issues. The chorismate–isochorismate isomeric interconversion is an unusual transformation and is likely related to initial steps in anthranilate synthase and PABA synthase catalysis where aminocyclohexadiene intermediates arise by nucleophilic additions of NH_3 into the homoallylic alcohol system of chorismate (Policastro et al., 1984; Teng & Ganem, 1984; Teng et al., 1985; Walsh et al., 1987). By analogy, one anticipates that, while the chorismate–isochorismate enzymatic isomerization is in a formal sense a one substrate/one product interconversion, a solvent water molecule (or OH^- equivalent with enzymic general-base catalysis) may act as nucleophile in a net 1,5-addition/elimination mechanism. By use of a mixed $H_2{}^{16}O/H_2{}^{18}O$ solvent and trapping the resultant isochorismate product via in situ conversion by EntB and EntA to 2,3-dihydroxybenzoate which gave a usable molecular ion peak, mass spectroscopic analysis established that the hydroxyl group of isochorismate is derived from solvent water rather than a net intramolecular hydroxyl group transfer from chorismate. This result serves as an initial mechanistic constraint and establishes the net 1,5-addition/elimination sequence of eq 2. It is also worth pointing out



Chorismate Isochorismate (2)

that in the conversion of chorismate to ^{18}O-labeled 2,3-dihydroxybenzoate we have in effect reconstituted in vitro the first three steps in the enterobactin biosynthesis. Whether the enzyme indeed catalyzes a synchronous double S_N2' sequence or involves stepwise displacements, e.g., by Micheal type addition of OH^- to an acicarboxylate species or by an X-group addition sequence to the C2–C3 double bond with concomitant C4-OH elimination, can now be investigated and may indeed reveal the catalytic strategy of the related anthranilate and PABA synthases.

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

Frank Rusnak,‡ Jun Liu,‡,§ Nina Quinn,§ Glenn A. Berchtold,§ and Christopher T. Walsh*,‡

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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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

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* Author to whom correspondence should be addressed.
‡ Harvard Medical School.
§ Massachusetts Institute of Technology.

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