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# Monofunctional Chorismate Mutase from *Bacillus subtilis*: Purification of the Protein, Molecular Cloning of the Gene, and Overexpression of the Gene Product in *Escherichia coli*†

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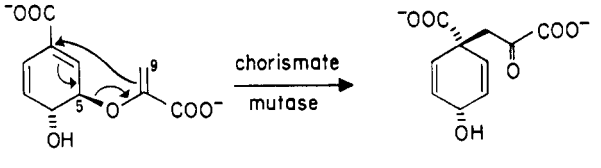
ABSTRACT: The monofunctional chorismate mutase from *Bacillus subtilis* has been purified 2200-fold to homogeneity. The enzyme is a homodimer of subunit $M_r$ = 14 500 and is the smallest natural chorismate mutase that has been characterized. The purified enzyme follows Michaelis–Menten kinetics with a $K_m$ of 100 $\mu$M and a $k_{cat}$ of 50 $s^{-1}$, carries no other associated enzymic activities, and is unaffected by any of the aromatic amino acids. The N-terminal amino acid sequence of the protein has been determined, and this information has been used to construct a precise oligonucleotide probe for the gene by means of in vitro DNA amplification from total chromosomal DNA by the polymerase chain reaction. The cloned *aroH* gene encodes a protein of 127 amino acid residues and is expressed in *Escherichia coli*. The cloned gene product is indistinguishable from that purified from *Bacillus*. The *aroH* coding region was directly subcloned into a phagemid expression vector by means of the polymerase chain reaction. The resulting construct, with the *aroH* gene positioned behind efficient transcription and translation initiation sequences of *E. coli*, results in the production of the monofunctional mutase at levels of 30–35% of the soluble cell protein in *E. coli* transformants. Chorismate mutases comprise a set of functionally related proteins that show little sequence similarity to each other. This diversity stands in contrast to other chorismate-utilizing enzymes.

Chorismate mutase catalyzed the rearrangement of chorismate (**1**) to prephenate (**2**), and the enzyme lies at the branch point of the biosynthetic pathway leading to the three aromatic amino acids, phenylalanine, tyrosine, and tryptophan. The




chorismate **1** prephenate **2**

catalyzed reaction is formally a Claisen rearrangement and is the only example of what appears to be a pericyclic process in primary metabolism.

The uncatalyzed reaction occurs readily, though this transformation is accelerated by the enzyme by more than $10^6$ at 25 °C (Andrews et al., 1973; Görisch, 1978). The nonenzymic process has been extensively studied and is believed to proceed via a concerted, asynchronous reaction (Dewar, 1984) in which bond breaking is far in advance of bond making at the transition state (Addadi et al., 1983). In contrast, little is known about the mechanism of the enzyme-catalyzed rearrangement. The absence of secondary tritium kinetic isotope effects at C-5 and C-9 of chorismate (**1**) suggests that some transition state other than that involving the chemical transformation is rate limiting (Addadi et al., 1983). This conclusion leaves obscure the origins of the enzyme-mediated rate enhancement. The stereochemical course of the enzymic rearrangement has been established (Sogo et al., 1984) and involves a transition state of chairlike geometry, as has also been found for the nonenzymic process (Copley & Knowles, 1985).

The most thoroughly studied chorismate mutases are the P and T proteins of *Escherichia coli*, which are both bifunctional enzymes of subunit $M_r$ near 40 000, the mutase activity

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being associated either with that of prephenate dehydratase (in the P protein) or with that of prephenate dehydrogenase (in the T protein), respectively (Koch et al., 1971; Davidson et al., 1972). Early work on each of these bifunctional proteins suggested some interdependence between the two active sites. For chorismate mutase-prephenate dehydrogenase (the T protein), chemical modification by various reagents has been shown to destroy both the mutase and the dehydrogenase activities in parallel (Hudson et al., 1984; Heyde, 1979), and only recently has it proved possible to separate these functions by the expression of deletion mutants of the gene (*tyrA*) that encodes this protein (Maruya et al., 1987). For chorismate mutase-prephenate dehydratase (the P protein), the two active sites have been said to be functionally distinct (Schmit et al., 1970), though with "some degree of interconnection" (Baldwin & Davidson, 1983).

The existence of multiple (never mind interacting) binding sites for the substrates or products of these bifunctional enzymes would compromise many direct approaches to the study of the interactions between the mutase and its ligands. In search of a simpler system we have elected to purify and characterize the chorismate mutase of the Marburg strain of *Bacillus subtilis* (Llewellyn et al., 1980). This enzyme is known to be monofunctional, and its activity is unaffected by the end-product aromatic amino acids (Lorence & Nester, 1967). We report herein the purification of this enzyme to homogeneity and show that it is devoid of other enzymic activities and that it possesses kinetic parameters typical of other mutases that have been isolated. The *Bacillus* enzyme is a homodimer of subunit $M_r$ = 14 500 and represents the smallest natural chorismate mutase so far characterized. We further report the cloning and sequencing of the gene (*aroH*) that encodes this enzyme and the overexpression of the gene product in *E. coli*. The cloned chorismate mutase is indistinguishable from the purified enzyme from *B. subtilis*.

## Experimental Procedures

*Strains and Media.* The prototrophic Marburg strain of *B. subtilis* (ATCC 6051) was used both as the source of the enzyme and as the donor strain for chromosomal DNA. *E. coli* strain DH5$\alpha$ was used as the host strain for library construction and screening. *E. coli* strain KB357 ($\Delta tyrA$, $\Delta pheA$) (a generous gift of Dr. Keith Backman, Biotechnica International, Cambridge, MA) was used as the host strain for the purification of the cloned gene product and for the large-scale preparation of plasmid DNA for sequencing. Overexpressing constructs were maintained in *E. coli* strain XL1-Blue (Bullock, 1987). *B. subtilis* (ATCC 6051) was grown in Spizizen's minimal medium (Spizizen, 1958) at 37 °C after inoculation with overnight starter cultures that had been grown in nutrient broth at 30 °C. Cells were harvested at an $A_{550nm}$ of 1.5, and the cell paste was stored frozen at −70 °C. Transformants of the *E. coli* strains were grown in LB medium supplemented with ampicillin (200 $\mu$g/mL) and/or tetracycline (20 $\mu$g/mL), as appropriate.

*Protein Determination.* Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as the calibration standard.

*Enzyme Assays.* Chorismate mutase activity was determined by monitoring the disappearance of chorismate spectrophotometrically at 274 nm in 50 mM potassium phosphate buffer, pH 7.5, at 30 °C. Prephenate dehydratase activity was assayed by quantifying, at 320 nm in NaOH (1.4 N), the amount of phenyl pyruvate produced after incubation of the enzyme with prephenate (300 $\mu$M) at 30 °C for 30 min in *N*-ethylmorpholine-2-*N*-morpholinoethanesulfonic acid buffer (200 mM), pH 7.5. Prephenate dehydrogenase activity was assayed by monitoring the conversion of $NAD^+$ (2 mM) to NADH at 340 nm in 50 mM potassium phosphate buffer, pH 7.5, after the addition of prephenate (280 $\mu$M). All spectrophotometric measurements were made on a Hewlett-Packard 4582A diode array spectrophotometer.

*Enzyme Purification from B. subtilis.* Frozen cell paste (30 g) of *B. subtilis* (ATCC 6051) was thawed and suspended in ice-cold buffer A [50 mM potassium phosphate, pH 7.5, containing dithioerythritol (1 mM), phenylmethanesulfonyl fluoride (1 mM), 2-propanol (5% v/v), and glycerol (10% v/v)] (60 mL). The cells were lysed by a single pass through a French pressure cell at 20 000 psi. Cell debris was removed by centrifugation at 30000*g* for 30 min at 4 °C. The supernatant was diluted to 300 mL with buffer A and loaded onto a column (550 mL) of DEAE-cellulose (DE52) that had been preequilibrated with the same buffer. The column was developed isocratically with buffer A. Fractions containing chorismate mutase activity were pooled, and the pH was adjusted to 8.9 with NaOH (2 N). The enzyme sample was then loaded onto a column (115 mL) of DEAE-cellulose equilibrated with buffer B [50 mM glycine–NaOH buffer, pH 8.9, containing dithioerythritol (1 mM), phenylmethanesulfonyl fluoride (1 mM), 2-propanol (5% v/v), and glycerol (10% v/v)]. The column was washed with 200 mL of buffer B, and the protein was eluted by a linear gradient (1500 mL plus 1500 mL) of NaCl (0–0.3 M) in the same buffer. Fractions containing chorismate mutase activity were pooled and concentrated by ultrafiltration through PM10 membranes (Amicon Corp., Danvers, MA). The concentrated protein solution was then applied to a Protein Pak 300SW HPLC gel filtration column (7.8 × 300 mm, from Waters Associates, Medford, MA) preequilibrated with buffer A. The column was eluted isocratically with buffer A at a flow rate of 0.5 mL/min. The pooled fractions containing mutase activity were concentrated by centrifugation using Centriprep 10 concentrators (Amicon). This solution was then applied to a Mono Q HR 5/5 HPLC anion-exchange column (5 × 50 mm, from Waters) preequilibrated with buffer C [50 mM Tris-HCl buffer, pH 8.2, containing dithioerythritol (1 mM), phenylmethanesulfonyl fluoride (1 mM), 2-propanol (5% v/v), and glycerol (10% v/v)]. The column was washed with buffer C (5 mL) and eluted with a linear gradient (15 mL plus 15 mL) of NaCl (0–0.5 M) in the same buffer. The active fractions were collected and concentrated as above. The protein was then applied to a Protein Pak 125 HPLC column (7.8 × 300 mm, from Waters) preequilibrated with buffer A. This column was developed isocratically with buffer A at a flow rate of 0.5 mL/min. The active fractions were collected and concentrated as described above.

The molecular weight of the purified enzyme was estimated from the elution volume (relative to standards of known $M_r$) from a Protein Pak 125 HPLC column run under the conditions described above. The subunit molecular weight was estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970).

Amino acid sequencing was done by automated Edman degradation, by Dr. Robert Mattaliano (Biogen Inc., Cambridge, MA).

*Plasmids, Oligonucleotide Synthesis, and DNA Manipulations.* Cloning was performed in the plasmid pUC13 constructed by Messing and co-workers (Yanisch-Perron et al., 1985). Subcloning was performed in the phagemid pBSX1c (Hermes et al., 1989). Oligonucleotides were synthesized on a MilliGen-Biosearch 7500 DNA synthesizer (MilliGen Corp.,

Bedford, MA). Oligonucleotides were purified either from polyacrylamide gels or by using oligonucleotide purification cartridges (Applied Biosystems, Foster City, CA). Nitrocellulose HATF filters were from Millipore Corp. (Bedford, MA). Site-directed mutagenesis was performed by the method of Eckstein (Taylor et al., 1985a,b; Nakamaye & Eckstein, 1986; Sayers et al., 1988), using materials from Amersham Corp. (Arlington Heights, IL). Restriction endonucleases and DNA modification enzymes were from New England Biolabs (Beverly, MA) or Boehringer Mannheim Biochemicals (Indianapolis, IN). All radioactive materials were from Amersham. DNA manipulations were performed according to the methods of Maniatis et al. (1982) and Ausubel et al. (1987).

*Synthetic Oligonucleotides.* The oligonucleotides synthesized were

01 5′-TTCTTCTGTATCTCGTCCAACTGTTGTTG-CTCCTCGAATGCCTCGAATCATCAT-3′
02 5′-GAAATTCTGCAGAAAACAAAACAGCTG-CTGGAAAAAATTATTGAAGAAAAC-3′
03 5′-ATGATGATTCGAGGAATTCGAGGCGCM-ACMAC-3′
04 5′-TTCAATAATTTTTTCCTGCAGCTGTTTM-GTMTT-3′
05 5′-ATGATGATTCGAGGAATTCGAGGCGC-3′
06 5′-AGTTGAACGGGATACTGAAGAAGA-3′
07 5′-GATTGGAGGAGACACCATGGTGATTCG-CGGATTCGC-3′
08 5′-TTTCTAAGCTGTTCTTCTGCAGTTACAAT-TCAGTATT-3′

where M specifies a mixed position containing equimolar amounts of each of the four deoxyribonucleotides.

*Preparation of Chromosomal DNA from Bacillus.* Frozen cells (250 mg) were thawed and suspended in 0.1 M Tris-HCl buffer, pH 8.0, containing EDTA (10 mM) (9.5 mL). Chromosomal DNA was prepared by the method of Murray and Thompson (1980) (Ausubel et al., 1987), modified as follows. After treatment of the cell suspension with lysozyme (10 mg/mL) at 37 °C for 45 min with gentle agitation, the solution was repeatedly frozen and thawed. The inclusion of this step was found to facilitate cell lysis. The isolated DNA was purified by ultracentrifigation in a cesium chloride density gradient and was analyzed by electrophoresis through 0.3% agarose gels.

*Southern Blotting and Hybridization.* Samples of chromosomal DNA (1 μg per lane) that had been digested to completion with a restriction endonuclease (*Hin*dIII, *Eco*RI, or *Pst*I) were subjected to electrophoresis in 0.7% agarose gels. The DNA was transferred to Zeta-Probe nylon membranes (Bio-Rad, Richmond, CA) by the alkaline-transfer method. Oligonucleotide probes were 5′-end labeled with $^{32}P$ (>5000 Ci/mmol) by using T4 DNA kinase. The labeled probes were used without further purification and the filters probed as directed by the manufacturer.

*Polymerase Chain Reaction.* The reactions were performed in 30 mM Tris–acetate and 60 mM sodium acetate buffer, pH 7.9, containing $Mg(OAc)_2$ (10 mM), dATP (1 mM), dGTP (1 mM), dCTP (1 mM), dTTP (1 mM), priming oligonucleotides (1 μM), and *B. subtilis* chromosomal DNA (1 μg). Amplification reactions were carried out in a total volume of 100 μL. A typical temperature cycle was 2 min at 95 °C, 2 min at 37 °C, and a further 2 min at 37 °C following the addition of DNA polymerase I (Klenow fragment). Fresh polymerase was added during each cycle as follows: 0.5 unit for cycles 1–7, 1 unit for cycles 8–16, 2.5 units for cycles 17–24, and 5 units for the subsequent five cycles. The amplified product was purified on a nondenaturing polyacrylamide gel. A further 10 cycles of in vitro amplification were performed on this product as described above, with 5 units of fresh DNA polymerase 1 (Klenow fragment) per cycle. This reamplified product was isolated, and double-strand sequencing was performed directly with a $^{32}P$-end-labeled primer (>1000 Ci/mmol), using the template denaturation protocol of Wrischnik et al. (1987) and modified T7 DNA polymerase, as directed by United States Biochemical Corp. (Cleveland, OH).

*Subgenomic Library Construction.* Chromosomal DNA from *B. subtilis* (ATCC 6051) was digested to completion with *Hin*dIII and fractionated in a 0.7% agarose gel. DNA fragments of 2–3 kb were excised and purified by using GeneClean (Bio 101 Inc., La Jolla, CA). These fragments were then ligated into pUC13 that had been digested with *Hin*dIII and subsequently dephosphorylated by using calf intestinal alkaline phosphatase. Supercompetent *E. coli* strain DH5α, prepared by the method of Hanahan (1983), was transformed directly with the ligation mixtures.

*Library Screening by Colony Hybridization.* Transformation mixtures were plated at a colony density of approximately 1000 per plate (150 mm). Multiple nitrocellulose filter replicas were prepared and probed as described by Woods (1984).

*Purification of Cloned Chorismate Mutase from E. coli KB357(pJG23).* Cell paste (100 mg) of *E. coli* KB357-(pJG23) was thawed and suspended in ice-cold buffer C (2 mL). Cells were lysed in two passes of this suspension through a French pressure cell at 20 000 psi. Cell debris was removed by centrifugation, and the supernatant (1 mL) was loaded onto a Mono Q HR 5/5 FPLC column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) preequilibrated with buffer C. The column was washed with buffer C (5 mL) and eluted with a linear gradient (7.5 mL plus 7.5 mL) of NaCl (0–0.3 M) in the same buffer. Active fractions were pooled and concentrated by centrifugation. Concentrated protein was loaded on a Superose 12 FPLC gel filtration column (Pharmacia) preequilibrated with buffer A. The column was developed in the same buffer at a flow rate of 0.4 mL/min.

*Sequencing aroH.* DNA sequencing was performed by the dideoxy chain termination method (Sanger et al., 1977). Supercoiled template DNA was prepared by the alkaline lysis method. The template was denatured in alkali and then sequenced by using modified T7 DNA polymerase (United States Biochemical Corp.).

*Subcloning of aroH.* The polymerase chain reaction was performed with DNA polymerase 1 (Klenow fragment), as described above. Only six cycles of amplification were performed by using supercoiled pJG23 plasmid (5 μg) as template. During each cycle, the same amount of polymerase (5 units) was added with subsequent incubation at 37 °C for 15 min. The product was isolated from a 1.5% agarose gel. After digestion with *Nco*I and *Pst*I restriction endonucleases, the fragment was ligated into the large *Nco*I–*Pst*I fragment of phagemid pBSX1c. The ligation mixture was used to transform *E. coli* XL1-Blue. The cloned insert was sequenced by using modified T7 DNA polymerase (United States Biochemical Corp.). Site-directed mutagenesis was performed on the single-stranded phagemid DNA prepared as directed by Stratagene (La Jolla, CA).

*Purification of Cloned Chorismate Mutase from E. coli XL1-Blue(pBSCM2).* Cell paste (15 g) of *E. coli* XL1-Blue(pBSCM2) was thawed and suspended in ice-cold buffer A (150 mL). Cells were lysed in two passes through a French

Table I: Purification of Chorismate Mutase from *B. subtilis*

| | total catalytic act. ($\mu$mol min$^{-1}$) | unit yield (%) | sp catalytic act. ($\mu$mol min$^{-1}$ mg$^{-1}$) | purifn factor | total protein (mg) |
|---|---|---|---|---|---|
| crude lysate | 191 | 100 | 0.092 | 1 | 2070 |
| DEAE-cellulose (pH 7.5) | 146 | 76 | 3.8 | 41 | 38 |
| DEAE-cellulose (pH 8.9) | 35 | 18 | 27 | 290 | 1.3 |
| gel filtration | 15 | 8 | 31 | 340 | 0.49 |
| Mono Q | 10 | 5.2 | 160 | 1700 | 0.062 |
| gel filtration | 7.5 | 3.9 | 200 | 2200 | 0.038 |

pressure cell at 20 000 psi. Cell debris was removed by centrifugation. The supernatant was diluted to 300 mL with buffer A and loaded onto a column (500 mL) of DEAE-cellulose (DE52) that had been preequilibrated in the same buffer. The column was developed isocratically with buffer A. Fractions containing chorismate mutase activity were pooled and concentrated to 50 mL by ultrafiltration through PM10 membranes (Amicon Corp.). The buffer was changed to buffer C, again by ultrafiltration. The resulting solution (100 mL) was loaded onto a column (300 mL) of QAE-Sephadex (50–120 $\mu$m) preequilibrated with buffer C. The column was washed with buffer C (100 mL) and then eluted with a linear gradient (1.5 L plus 1.5 L) of NaCl (0–0.5 M) in the same buffer. Active fractions were pooled and concentrated by ultrafiltration.

*Data Bank Searches and Protein Sequence Alignments.* GenBank and EMBL databases were searched at the Molecular Biology Computer Research Resource of the Dana Farber Cancer Research Institute. Detailed pairwise sequence alignments were performed by using the ALIGN program (B. C. Orcutt, M. O. Dayhoff, D. G. George, and W. C. Barker, Protein Identification Resource, National Biomedical Research Foundation, Georgetown University Medical Center, Washington, DC, 20007).

## Results and Discussion

*Purification of Chorismate Mutase from B. subtilis.* Two unlinked genetic loci each of which encodes a chorismate mutase enzyme have been identified in *B. subtilis* (Nasser & Nester, 1967; Hoch & Nester, 1973). The *aroG* locus encodes a 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase-chorismate mutase bifunctional enzyme (Huang et al., 1974), which is present in strains 23 and 168 and their derivatives (Lorence & Nester, 1967), the most commonly used laboratory strains of *B. subtilis*. The bifunctional enzyme is not present in the wild-type Marburg strain (Llewellyn et al., 1980), and it has been suggested that this chorismate mutase activity evolved from a monofunctional 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase by mutation of its allosteric binding site for prephenate (Llewellyn et al., 1980). In contrast, the *aroH* locus, which is closely linked to a number of genes that encode other enzymes of aromatic amino acid biosynthesis, specifies a monofunctional chorismate mutase (Lorence & Nester, 1967). This enzyme is present in the Marburg strain and also in strain 23 and is believed to be the "natural" mutase (Llewellyn et al., 1980). In our search for a simple monofunctional chorismate mutase, we chose to purify and characterize the latter enzyme from the Marburg strain of *B. subtilis*.

The purification scheme is summarized in Table I. The enzyme was purified to homogeneity, with an overall purification factor of about 2200. The purified enzyme was found to be a homodimer having a subunit $M_r$ of 14 600. The protein appears to be a monofunctional chorismate mutase and contains undetectable levels of prephenate dehydratase or prephenate dehydrogenase activity. The enzyme follows classical

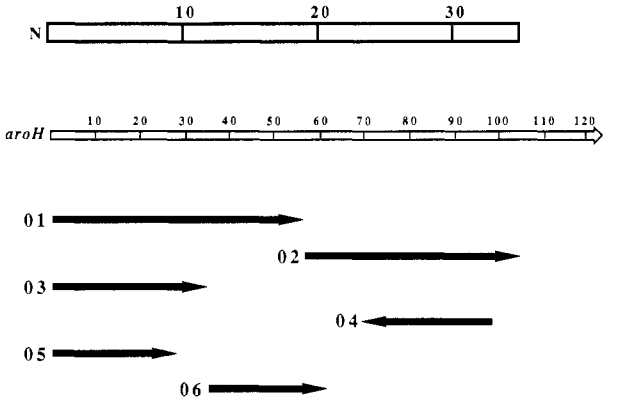



FIGURE 1: Oligonucleotides used in the cloning of the *aroH* gene and their relationship to the N-terminal 35 amino acid sequence of the protein and to the corresponding 5′-region of the gene. The arrowheads represent the positions of the 3′-ends of the oligonucleotides.

Michaelis–Menten kinetics with a $K_m$ of 100 $\mu$M and a $k_{cat}$ of 50 s$^{-1}$ per subunit. These kinetic parameters are typical of other well-characterized bacterial chorismate mutases (SampathKumar & Morrison, 1982; Görisch & Lingens, 1974). The N-terminal amino acid sequence was found by sequential Edman degradation to be

MMIRGIRGATTVERDTEEEILQKTKQLLEKIIEEN

*Cloning of the Chorismate Mutase Gene (aroH).* On the basis of the N-terminal amino acid sequence above, two long oligonucleotides, designated 01 (a 54-mer) and 02 (a 51-mer), were synthesized as probes for the 5′-end of the *aroH* gene (see Figure 1). These oligonucleotides were nonoverlapping, and their sequences were based upon the known codon preferences of *B. subtilis* (Piggot & Hoch, 1985). These oligonucleotides were used to probe chromosomal DNA (Southern, 1975) that had been digested to completion with various restriction endonucleases. Surprisingly, the two probes hybridized specifically to different DNA fragments under the most stringent washing conditions employed. Common bands were observed only at low wash stringency when both probes hybridized relatively nonspecifically to many genomic DNA fragments. This behavior cast doubt on which, if either, of the probes was specific for the *aroH* gene. Since the gene could not be located by this experiment, a precise probe was produced by enzymic amplification of a portion of the 5′-end of the gene from chromosomal DNA using the polymerase chain reaction (Oste, 1988; Mullis & Faloona, 1987), as described below. This experiment both provided a precise probe and verified the correctness of the N-terminal amino acid sequence.

Two oligonucleotides, a 32-mer and a 33-mer, were synthesized to correspond to each end of the known N-terminal amino acid sequence. These oligonucleotides, 03 and 04, were designed to prime DNA synthesis toward each other across a 34-bp region of the 5′-end of the *aroH* gene (see Figure 1). The most probable base, identified from the known codon usage frequencies, was incorporated at each position, though

near the 3′-end of each oligonucleotide, all four bases were incorporated at positions of uncertainty. The latter feature ensures efficient priming by at least a subset of each synthetic oligonucleotide. The polymerase chain reaction was performed directly on chromosomal DNA isolated from *B. subtilis* Marburg. When *Thermus aquaticus* DNA polymerase was used in the polymerase chain reaction, no amplification products were detected. It seemed probable that the reaction temperature of *T. aquaticus* DNA polymerase (72 °C) was higher than the melting temperature of one or both of the priming oligonucleotide–target DNA hybrids. In support of this view, the major product from 35 cycles of amplification at 37 °C using the Klenow fragment of DNA polymerase I was 99 bp in length, corresponding precisely to the size of the expected fragment. This product was isolated and then used as template for a further 10 cycles of in vitro amplification. This 99-bp fragment was sequenced directly by using as primer a shortened oligonucleotide, 05, corresponding to 03 but lacking the 3′-end region containing the mixed bases. The nucleotide sequence of the amplified region was in exact agreement with the N-terminal amino acid sequence of the protein. By use of a precise probe (06) based upon the amplified sequence, it was shown that, of the first pair of long "guessmers", 01 identifies the correct fragment of genomic DNA.

From the Southern blot analysis with 01 as the probe, it was clear that the 5′-end region of the *aroH* gene was located on a 2.5-kb *Hin*dIII fragment of the *B. subtilis* chromosome. A subgenomic library, containing 2–3-kb *Hin*dIII fragments of the *Bacillus* chromosome, was therefore constructed in the vector pUC13. This library was screened by colony hybridization with oligonucleotide 01. Two positive colonies were detected from 2000 screened, each of which harbored plasmids of approximately 10 kb that contained multiple *Hin*dIII inserts of the expected size range. Analysis of Southern blots showed that each plasmid carried a 2.5-kb *Hin*dIII fragment to which 01 specifically hybridized, one of which, designated pJG23, was used in further work.

To determine if pJG23 drives the synthesis of chorismate mutase in *E. coli*, transformants of *E. coli* KB357 (a strain that produces no endogenous chorismate mutase) were examined. The *Bacillus* chorismate mutase was found to comprise approximately 5% of soluble cell protein, this estimate being based on the known specific catalytic activity of the protein purified from *B. subtilis*. To characterize the cloned chorismate mutase, the enzyme was purified from extracts of *E. coli* KB357(pJG23). After chromatography on a column of Mono Q followed by gel filtration, homogeneous enzyme was obtained. Analysis by denaturing polyacrylamide gel electrophoresis gave a single protein band of $M_r$ = 14600. From gel filtration chromatography, the native $M_r$ is about 30000. The enzyme therefore appears to be an $\alpha_2$ dimer. The steady-state kinetic parameters of the cloned enzyme were indistinguishable from those of the wild-type protein. Plasmid pJG23 evidently carries the intact *aroH* gene, which is efficiently expressed in *E. coli*.

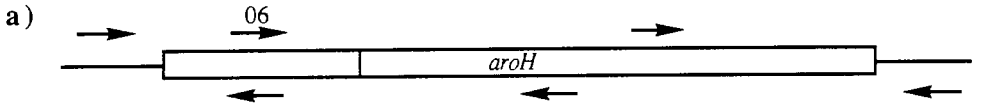
*Sequencing the aroH Gene.* The precise oligonucleotide probe, 06, derived from the polymerase chain reaction described above, not only allowed the resolution of the initial probe ambiguity but also constituted a primer to initiate the immediate sequencing of the *aroH* gene. Direct double-strand sequencing was performed on supercoiled pJG23 by using this oligonucleotide and its complement as primers. The resulting sequence information was used to design new primers, until the entire gene sequence had been obtained (see Figure 2a). The nucleotide sequence and the derived amino acid sequence for the open reading frame are presented in Figure 2b. The *aroH* gene encodes a protein of $M_r$ = 14479 having 127 residues. The derived sequence of the N-terminal 35 amino acid residues is in exact agreement with that determined for the purified protein from *Bacillus*. The codon usage pattern for the gene is typical of *B. subtilis* (Piggot & Hoch, 1985).

The sequence of the regions flanking *aroH* shows that the gene lies within a large cluster of aromatic amino acid biosynthesis genes, the so-called aromatic "supra-operon" of *B. subtilis* (Zalkin & Ebbole, 1988). This cluster now appears to consist of 12 cotranscribed genes arranged in the order *aroFBHtrpEDCFBAhisHtyrAaroE* (Gray and Knowles, unpublished results).

Knowledge of the complete gene sequence allows us to examine the basis for the ambiguity in the results of probing genomic DNA with the long oligonucleotides 01 and 02. Oligonucleotide 01 proved to be 89% complementary to its target sequence, a level that is consistent with its being a specific probe (Lathe, 1985). The mismatches were distributed evenly throughout the sequence. The oligonucleotide itself is moderately A/T rich (57%), as is the target sequence of the gene (54%). Oligonucleotide 02 proved to be 82% correct, the mismatches again being evenly distributed across the probe. This oligonucleotide should, in principle, constitute a specific probe for the 5′-end of the *aroH* gene (Lathe, 1985). However, this oligonucleotide is very A/T rich (69%), as is the corresponding region of the *aroH* gene (76%). The high A/T content of the target sequence combined with the lower complementarity between the oligonucleotide 02 and the *aroH* gene accounts for the observed low melting temperature of the resulting hybrid. The origin of the higher stability of the artifactual complex between 02 and the uncharacterized chromosomal sequence remains unknown.

*Subcloning and Overexpression of aroH.* When the plasmid pJG23 and used to transform *E. coli* strain DH5$\alpha$, cell growth was so slow that efforts to obtain a subclone that contained only the 2.5-kb *Hin*dIII fragment harboring the *aroH* gene was frustrated. Growth of pJG23 transformants of *E. coli* strains TG1 and KB357 was more rapid, yet all constructs carrying the *aroH* gene in these strains were unstable in liquid culture. The best strain for harboring pJG23 was found to be *E. coli* XL1-Blue, the growth properties of which were little affected by transformation.

The *aroH* gene was subcloned directly into a phagemid expression vector of high copy number. This transformation was accomplished in two steps and required prior knowledge of the sequence of the *aroH* gene and of its immediate flanking regions only. First, polymerase chain reaction amplification was used to excise the open reading frame from double-stranded pJG23 and simultaneously to introduce convenient restriction sites at each end of the gene. The primers for the amplification were designed to introduce an *Nco*I restriction site at the initiation codon (primer 07) and a *Pst*I restriction site immediately following the stop codon of the *aroH* gene (primer 08). The introduction of the *Nco*I restriction site necessitated the mutation of the second encoded amino acid residue from methionine to valine. This change was subsequently corrected (see below). The polymerase chain reaction was performed with both *T. aquaticus* DNA polymerase and DNA polymerase I (Klenow fragment). When *T. aquaticus* DNA polymerase was used, the sole amplified product was of the correct size, approximately 422 bp. Of the two amplified products obtained when DNA polymerase I (Klenow fragment) was used, the major product was of the correct size.




b)

```
                                                        10          20          30
                                                        *           *           *
-GAGAGCTGGCTGAACAAATGGCGATTGGAGGAGACATC ATG ATG ATT CGC GGA ATT CGC GGA GCA ACT
                                        Met Met Ile Arg Gly Ile Arg Gly Ala Thr

            40          50          60          70          80          90
            *           *           *           *           *           *
ACA GTT GAA CGG GAT ACT GAA GAA GAA ATT TTA CAA AAA ACT AAA CAG CTG TTA GAG AAA
Thr Val Glu Arg Asp Thr Glu Glu Glu Ile Leu Gln Lys Thr Lys Gln Leu Leu Glu Lys

           100         110         120         130         140         150
            *           *           *           *           *           *
ATC ATA GAA GAA AAT CAT ACA AAA CCG GAA GAT GTT GTT CAA ATG CTT CTG TCG GCT ACA
Ile Ile Glu Glu Asn His Thr Lys Pro Glu Asp Val Val Gln Met leu leu Ser Ala Thr

           160         170         180         190         200         210
            *           *           *           *           *           *
CCT GAT TTG CAC GCT GTT TTC CCG GCA AAA GCT GTT CGC GAG CTT TCA GGA TGG CAG TAT
Pro Asp Leu His Ala Val Phe Pro Ala Lys Ala Val Arg Glu Leu Ser Gly Trp Gln Tyr

           220         230         240         250         260         270
            *           *           *           *           *           *
GTA CCG GTA ACA TGT ATG CAG GAA ATG GAC GTA ACA GGC GGT CTG AAA AAG TGC ATA AGA
Val Pro Val Thr Cys Met Gln Glu Met Asp Val Thr Gly Gly Leu Lys Lys Cys Ile Arg

           280         290         300         310         320         330
            *           *           *           *           *           *
GTC ATG ATG ACG GTC CAG ACA GAT GTC CCT CAG GAT CAG ATC AGA CAT GTA TAT TTA GAA
Val Met Met Thr Val Gln Thr Asp Val Pro Gln Asp Gln Ile Arg His Val Tyr Leu Glu

           340         350         360         370         380
            *           *           *           *           •
AAA GCT GTC GTA TTG AGG CCC GAT TTA TCA TTG ACA AAA AAT ACT GAA TTG TAA TACGATA
Lys Ala Val Val Leu Arg Pro Asp Leu Ser Leu Thr Lys Asn Thr Glu Leu Och

AGAACAGCTTAGAAATACACAAGAGTGTGTATAAAGCA-
```

FIGURE 2: (a) Schematic representation of the positions of the sequencing primers relative to the *aroH* gene. (b) Nucleotide sequence of the *aroH* gene and of its immediate flanking regions. The nucleotide numbering begins at the initiation codon of the reading frame. The translated amino acid sequence of the gene is displayed beneath the corresponding codons. The underlined amino acids represent those determined both from the nucleotide sequence of the gene and by Edman degradation of the purified protein.

Table II: Purification of Chorismate Mutase from *E. coli* XL1-Blue(pBSCM2)

| | total catalytic act. ($\mu$mol min$^{-1}$) | unit yield (%) | sp catalytic act. ($\mu$mol min$^{-1}$ mg$^{-1}$) | purifn factor | total protein (mg) |
|---|---|---|---|---|---|
| crude lysate | $9.5 \times 10^4$ | 100 | 64 | 1 | 1500 |
| DEAE-cellulose (pH 7.5) | $4.6 \times 10^4$ | 48 | 180 | 2.9 | 246 |
| QAE-Sephadex (pH 8.2) | $3.9 \times 10^4$ | 41 | 210 | 3.3 | 183 |

Because of the lower in vitro error rate of DNA synthesis when DNA polymerase I (Klenow fragment) is used (Oste, 1988), the product derived from amplification using this enzyme was chosen. To minimize further the chances of incorporating an error into the *aroH* gene during amplification, only six cycles were performed, with a large initial concentration of supercoiled pJG23 template. The 422-bp amplification product was purified, digested with *Nco*I and *Pst*I restriction endonucleases, and ligated into the appropriate fragment of the phagemid pBSX1c (Hermes et al., 1989). The resulting vector (pBSCM1) contains the *aroH* open reading frame positioned behind the *trc* promoter and the *lacZ* Shine–Dalgarno sequence derived from the plasmid pX1 (Straus & Gilbert, 1985). This construct is stable in *E. coli* XL1-Blue and gives high levels of chorismate mutase activity (approximately 50 units/mg of soluble cell protein). The mutation at the second amino acid residue evidently has little effect on the catalytic activity of the enzyme. Nevertheless, in the second step of the subcloning, the gene was reverted to wild type by a single site-directed mutagenesis resulting in the construct pBSCM2. The entire gene insert was then sequenced to establish that no changes had been introduced during the subcloning. On the basis of the specific catalytic activity of the purified *aroH* gene product, transformants of *E. coli* XL1-Blue with pBSCM2 produce the *B. subtilis* chorismate mutase at 30–35% of soluble cell protein. This expression level is stably maintained in liquid culture.

The purification scheme from *E. coli* XL1-Blue(pBSCM2) is summarized in Table II. This represents an improvement of nearly $10^4$-fold in the yield of chorismate mutase.

*Database Searches and Sequence Alignments.* The derived amino acid sequence of the *B. subtilis* chorismate mutase has no significant similarity (Doolittle, 1986) to any sequence in the GenBank and EMBL databases. Detailed pairwise alignments with the amino acid sequences of the chorismate mutases from *Saccharomyces cerevisiae* (Schmidheini et al., 1989) and from *E. coli* (Hudson & Davidson, 1984) were performed. The *B. subtilis* enzyme was found to be marginally similar to a short region of the N-termini of the two *E. coli* bifunctional enzymes. This similarity is illustrated in Figure 3. However, no similarity was detected with the monofunctional mutase from yeast. This is not surprising, since it is

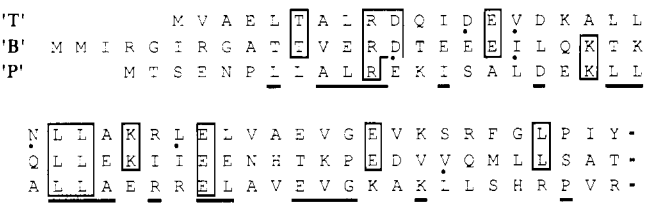


FIGURE 3: Amino acid sequence alignments of the N-terminal regions of the two *E. coli* bifunctional chorismate mutase enzymes with that of the monofunctional chorismate mutase from *Bacillus*. The *Bacillus* sequence (B) is in the middle of the three, that of chorismate mutase-prephenate dehydrogenase (T) being on the top and that of the chorismate mutase-prephenate dehydratase (P) being on the bottom. Exact matches with the *Bacillus* sequence are boxed. Similar amino acids are indicated by a dot. The positions of exact matches between the two *E. coli* sequences are indicated by the underlines.

known that the yeast sequence is not similar to the N-terminus of either of the *E. coli* mutases. This absence of similarity stands in contrast to other enzymes of the aromatic amino acid biosynthesis pathway in these organisms, for which considerable pairwise similarities between corresponding genes have been noted (Henner et al., 1984, 1986; Zalkin et al., 1984; Schmidheini et al., 1989). Furthermore, no significant sequence similarity was detected in pairwise alignments with any of the other chorismate-utilizing enzymes, which are known to form a set of strikingly similar proteins (Elkins & Earhart, 1988; Ozenberger et al., 1989). This singularity might suggest that an unusually wide variety of primary structures can serve as effective catalysts for the mutase transformation. This view is reinforced by the possibility that a chorismate mutase has evolved from the allosteric binding site for prephenate in 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase (Burkholder & Giles, 1947; Llewellyn et al., 1980), and the idea gains further support from the recent isolation of monoclonal antibodies with impressively high catalytic potency for the mutase reaction (Jackson et al., 1988; Hilvert et al., 1988). While it is surely too simplistic to suggest that chorismate mutase only provides a relatively rigid binding site for the single transition state of the rearrangement, it is certainly true that in this case there is no formal requirement for the presence of a complex constellation of catalytic functionality.

These arguments notwithstanding, the availability of large amounts of a simple, small, monofunctional mutase allows the more detailed characterization of this enzyme-catalyzed reaction than has been yet possible, and opens the door to physicochemical studies on this intriguing enzyme.

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**Registry No.** DNA (*Bacillus subtilis* gene aroH), 124098-28-8; chorismate mutase (*Bacillus subtilis* reduced), 124098-41-5; chorismate mutase, 9068-30-8; oligonucleotide 01, 124098-32-4; oligonucleotide 02, 124098-31-3; oligonucleotide 05, 124098-25-5; oligonucleotide 06, 124098-24-4; oligonucleotide 07, 124098-26-6; oligonucleotide 08, 124098-27-7.

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# On the Molecular Weight and Subunit Composition of Calf Thymus Ribonuclease H1†

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ABSTRACT: We have reinvestigated the molecular weight and subunit composition of calf thymus ribonuclease H1. Earlier studies suggested a variety of molecular weights for the enzyme in the range of 64K–84K and reported that the enzyme either was a single polypeptide of 74 kDa or consisted of from two to four subunits in the range of 21–34 kDa. Although we too find bands in this lower molecular weight range in our highly purified preparations following SDS–PAGE, our data suggest that the native structure of RNase H1 is a dimer of 68-kDa subunits. The evidence includes the following: (1) Western blot analysis of fractions taken at various stages of the purification indicates that the predominant antigenic form of the enzyme in crude extracts has a molecular weight of 68K but that during purification in the absence of sufficient protease inhibitors a variety of lower molecular weight forms appear concomitant with the disappearance of the 68-kDa band. (2) Activity gel analysis of the highly purified enzyme prepared in the presence of a battery of protease inhibitors reveals that the 68-kDa band (as well as several bands of lower molecular weight) possesses RNase H activity. (3) The 68-kDa band recognized by Western blotting with anti-RNase H immune sera is not detected by using preimmune sera. Furthermore, when immune sera are used, a trace of a 140–150-kDa antigenic form can sometimes be detected, consistent with the existence of a dimeric form of the enzyme. (4) Gel filtration analysis of highly purified RNase H1 reveals that nearly all the observed RNase H activity is found at an elution volume corresponding to a molecular weight in the range of 140K–150K, although under the conditions of sucrose gradient centrifugation, the majority of the enzyme appears to sediment as a 68-kDa monomer. We therefore suggest that the proteolytic lability of RNase H1 may account for earlier reports that the enzyme contains low molecular weight subunits and that the enzyme is composed of a 68-kDa polypeptide which can dimerize and remain active.

Ribonucleases H (RNases H)[1] are enzymes that specifically degrade the RNA strand of RNA/DNA hybrids. They are not active against the DNA of the hybrid or against single- or double-stranded RNAs (Crouch & Dirksen, 1982). In bacteria, RNase H plays an important role in determining whether DNA replication proceeds either from the normal origin or from cryptic origins that are activated in the absence of RNase H (Kogoma, 1986), and also aids in removing the RNA primers from Okazaki pieces formed during lagging-strand DNA synthesis (Ogawa & Okazaki, 1984). The enzyme also plays an important role in the control of col E1 plasmid replication (Polisky, 1989). The role(s) of RNases

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton(s); GdnHCl, guanidine hydrochloride; NP-40, Nonidet P40; PMSF, phenylmethanesulfonyl fluoride; RNase H, ribonuclease H; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SRB, standard renaturation buffer; TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; 2-MCE, 2-mercaptoethanol.

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