Formation of 4-hydroxybenzoate in *Escherichia coli*: characterization of the *ubiC* gene and its encoded enzyme chorismate pyruvate-lyase

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Chorismate pyruvate-lyase from *Escherichia coli* converts chorismate to 4-hydroxybenzoate. The enzyme was enriched 3000-fold by overexpression and chromatographic purification. It has an apparent K_m value for chorismate of $6.1 \mu\text{M}$ and an isoelectric point of pH 6.45. The enzyme activity did not require metal cofactors. Promoter sequences in the 5' flanking sequences of the *ubiCA* operon were localized by transcription and translation of active chorismate pyruvate-lyase *in vitro* from different PCR fragments. Sequencing of the *ubiC* gene of the mutant strain AN244 revealed a G → A transition resulting in a change from glutamic acid to lysine. A feeding experiment with [1,7- $^{13}\text{C}_2$]shikimate confirmed the chorismate pyruvate-lyase as the sole enzymic source of 4-hydroxybenzoate *in vivo*.

Keywords: ubiquinone, chorismate, 4-hydroxybenzoate, chorismate pyruvate-lyase, *Escherichia coli*

INTRODUCTION

4-Hydroxybenzoate (PHB) is a key intermediate in the biosynthesis of ubiquinones in all living organisms. It is formed in plants and animals from chorismate via phenylpropanoid intermediates (see Fig. 1) (Pennock & Threlfall, 1983). In bacteria, the contribution of this pathway to the biosynthesis of PHB is not clearly established. The apparent incorporation of [U- ^{14}C]tyrosine into ubiquinone, observed in *Rhodospirillum rubrum*, was largely, but not entirely due to contamination of the labelled tyrosine with labelled 4-hydroxybenzaldehyde (Parson & Rudney, 1965). Conversion of tyrosine to PHB was observed in cell-free enzyme extracts from *Aerobacter aerogenes* (*Klebsiella pneumoniae*), but not from *Escherichia coli* (Cox & Gibson, 1966). On the other hand, a direct conversion of chorismate to PHB was observed in crude *E. coli* extracts, and a mutant unable to carry out this reaction was isolated (Gibson & Gibson, 1964; Lawrence *et al.*, 1974). This led to the proposal that PHB is produced by a one-step reaction catalysed by the enzyme chorismate pyruvate-lyase (CPL), the translation product of the gene *ubiC* (see Fig. 1).

Recently, this hypothesis has been confirmed by the cloning of *ubiC* and the purification of CPL, independently

by Nichols & Green (1992) and by our laboratory (Siebert *et al.*, 1992). The *ubiC* gene is situated in an operon structure together with *ubiA*; the latter gene encodes the enzyme PHB polyprenyltransferase, the subsequent enzymic step in ubiquinone biosynthesis. Although CPL has been purified to homogeneity (Nichols & Green, 1992) as a monomeric polypeptide of approximately 19 kDa, the only biochemical data reported up to now are the K_m value for chorismate, determined as $9.7 \mu\text{M}$, and the turnover number, calculated as 49 min^{-1} .

In this study, we present further biochemical characterization of CPL and confirm the role of the CPL reaction as the sole source of PHB *in vivo*. *In vitro* transcription/translation experiments with *UbiC* gave evidence for the localization of the promoter region. Furthermore, we have sequenced the mutated *ubiC* gene of *E. coli* AN244 and we discuss the role of the affected amino acid residue in the reaction mechanism.

METHODS

Chemicals and enzymes. Chorismate was purchased as the barium salt from Sigma. [^{35}S]dATP αS and [^{35}S]methionine were from Amersham. *Taq* polymerase was obtained from Promega or Pharmacia. PCR primers were synthesized by MWG Biotech.

Bacteria, media, plasmids, and DNA manipulation. *E. coli* AN244, AN92, AN385 and SG13009 are described by Lawrence *et al.* (1974), Young *et al.* (1971), Wallace & Young (1977), and

Abbreviations: PHB, 4-hydroxybenzoate; CPL, chorismate pyruvate-lyase; Et₂O, diethyl ether.

Fig. 1. Biosynthesis of ubiquinone: (a) in plants and animals (upper pathway) and (b) in bacteria (lower pathway).

Gottesman *et al.* (1981), respectively. pBluescriptIIKS+ (Short *et al.*, 1988) and *E. coli* XL1Blue were obtained from Stratagene, pTZ19R and pUC18 from Pharmacia, and pREP4 from Qiagen. pALMU1 is described by Siebert *et al.* (1992); pALMU3 contains a 1.86 kb *SacI* fragment from the pALMU1 insert, ligated into the *SacI* site of pTZ19R. pUBIC contains the PCR-amplified *ubiC* gene (primer *ubiC*-3F and *ubiC*-R, see below) which was treated with Klenow polymerase and T4 kinase prior to ligation (Sure Ligation Kit, Pharmacia) into the *SmaI* site of pUC18. The accuracy of the amplification was confirmed by sequencing. Sequencing reactions were done using the USB Sequencing Kit in accordance with the manufacturer's instructions. Cultivation of bacteria, cloning and DNA manipulation were carried out according to standard procedures (Sambrook *et al.*, 1989). DNA and protein sequence analysis was performed using the HUSAR program package at the DKFZ Heidelberg, FRG.

Assays for enzyme activity. Commercially available barium chorismate contains, besides other impurities, a considerable amount of PHB. Therefore it had to be purified for the enzyme assay using the following procedure. Six milligrams of barium chorismate dissolved in 100 μ l sodium acetate buffer (0.75 M, pH 4.0) were extracted twice with 1 ml Et_2O . The organic layer was discarded, and the aqueous phase adjusted to pH 8.0 with Tris buffer (0.5 M, pH 8.0) and 0.1 M NaOH. Unless stated otherwise, the assay for CPL activity was carried out according to Siebert *et al.* (1992) with an incubation time of 8 min and 50 μ M chorismate. PHB was extracted from the reaction mixture and assayed by HPLC, using 3-hydroxybenzoic acid as an internal standard. Values for enzymic PHB formation are corrected taking into account the PHB formed by chemical decomposition of chorismate during incubation. The reaction was linear with time for 15 min and with protein concentration between 30 and 300 μ g crude protein preparation ml^{-1} . For the determination of the K_m value, incubations were carried out for 3 min at 37 $^{\circ}\text{C}$, with 1.4 μ g purified enzyme (see below) and chorismate concentrations between 2.1 and 33.8 μ M. Isochorismate was tested as a substrate at a concentration of 47 μ M, with detection of salicylic acid formation by HPLC. For the investigation of the inhibitory effect of PHB, a coupled photometric assay was used; in this case, the incubation mixture

contained, in a total volume of 1 ml, 50 μ mol Tris/HCl pH 8.0, 200 μ mol NaCl, 500 nmol chorismate, 1.4 μ g purified enzyme, 5.5 U lactate dehydrogenase, and 100 nmol NADH. The decrease in absorbance was monitored at 340 nm. Induction experiments were carried out by adding IPTG to a final concentration of 1 mM to the bacterial culture at an OD_{600} of about 0.7.

Enzyme purification. This was carried out according to Siebert *et al.* (1992), using Sephadex G-75 for the gel chromatographic purification step.

PCR and *in vitro* transcription and translation. Primers used for PCR were: *ubiC*-1F, 5'-TTCAGCTCCAAATCTCA-3'; *ubiC*-2F, 5'-GTTGCGTTTTTGTTCGC-3'; *ubiC*-3F, 5'-TGTAACGGAGAGTTCGG-3'; *ubiC*-R, 5'-ATTCTGCGTCAGACTCC-3'. A schematic view of their locations is given in Fig. 2. PCR was performed with minor modifications to the method of Lesley *et al.* (1991) in 100 μ l final volume with 20 ng pALMU1 as template, 50 pmol of each primer, 0.1 mM of each dNTP, and 1 U *Taq* polymerase. Reactions were started by 5 min denaturation at 95 $^{\circ}\text{C}$, followed by 35 cycles of 1 min annealing at 52 $^{\circ}\text{C}$, 3 min chain elongation at 72 $^{\circ}\text{C}$, and 1 min denaturation at 95 $^{\circ}\text{C}$. The reaction was finished after a final elongation step of 10 min at 72 $^{\circ}\text{C}$, followed by extraction with phenol. Excess primers were removed by chromatography on Sephadex G-50 and ethanol precipitation. Redissolved DNA fragments were analysed by gel electrophoresis and their concentration estimated by comparison with a standard of known concentration.

In vitro transcription and translation was done using the S-30 extract for linear DNA from Promega essentially as described by Lesley *et al.* (1991) and in the manufacturer's instructions. For visualization of protein bands the extract was incubated with approximately 2 μ g DNA (plasmid or PCR fragments) including [^{35}S]methionine for 2 h at 37 $^{\circ}\text{C}$. Translation products were precipitated with acetone, redissolved in SDS loading buffer and separated on a 12% (w/v) polyacrylamide gel (Laemmli, 1970). The gel was dried and exposed to Kodak X-Omat AR film. For measurement of enzyme activity, 2 μ g supercoiled plasmid DNA or 4 μ g PCR fragments were included in a non-radioactive transcription/translation assay (2 h at 37 $^{\circ}\text{C}$). After addition of

Fig. 2. Localization of the potential promoter regions. (a) Schematic overview of the *ubiC* sequence, which is available in the EMBL data library (accession number X66619) and its flanking regions. The locations of the putative promoter structures are marked P1 and P2. Primers used for the *in vitro* transcription/translation experiment: 1F, *ubiC*-1F; 2F, *ubiC*-2F; 3F, *ubiC*-3F; R, *ubiC*-R (the lengths of the arrows indicating the primer locations are not to scale). (b) Sequence comparison of the *E. coli* consensus and the putative *ubiC* promoters. Numbering refers to the original sequence published by Siebert *et al.* (1992).

10% (v/v) glycerol, the reaction mixtures could be stored at -20°C or were used directly for the enzyme assay.

Generation of single-stranded DNA by PCR. Generation of single-stranded DNA was performed in two steps. First, 1 μg genomic DNA, extracted from *E. coli* AN244 by a standard procedure (Sambrook *et al.*, 1989), was used for the symmetrical amplification using the protocol described above with the primers *ubiC*-2F and *ubiC*-R and 2 U *Taq* polymerase. Then the asymmetrical PCR reaction was performed with 1 μl of the first reaction mixture (containing approximately 50 ng double-stranded DNA) and 1 and 50 pmol of the respective primers. The amplified DNA was purified of *Taq* polymerase, excess primers and dNTPs by microconcentration (Microcon 100, Amicon).

Direct sequencing of PCR-amplified DNA. Ten picomoles (1 μl) of the limiting primer of the asymmetrical PCR reaction (*ubiC*-2F, *ubiC*-3F, or *ubiC*-R) were annealed to about 100 ng single-stranded DNA (in 9 μl $1\times$ sequencing buffer) by warming the sample to 65°C for 2 min and cooling over a period of 30 min to room temperature. The sequencing reactions were performed using the USB kit according to the manufacturer's instructions with minor changes in labelling time (3 min) and buffer composition (Mn^{2+} instead of Mg^{2+}).

Feeding of [1,7- $^{13}\text{C}_2$]shikimic acid. To 10 flasks each containing 1 l of a minimal medium with 10 mM citrate and 30 mM glucose (Wallace & Young, 1977; Stroobant *et al.*, 1972), a total of 1 mmol (176.3 mg) [1,7- $^{13}\text{C}_2$]shikimic acid (Cho *et al.*, 1992) was added, and the flasks were each inoculated with 50 ml of a preculture (overnight) of *E. coli* AN385 in the same medium.

After shaking for 11 h at 37°C and 60 strokes min^{-1} (reciprocal shaker, 100 mm stroke), cells were centrifuged off (5000 g) and

discarded. The medium was adjusted to pH 1 with HCl and extracted with 3 l ethyl acetate. The organic layer was extracted with 500 ml of aqueous NaHCO_3 (1 M). The NaHCO_3 layer was acidified (HCl) and extracted with 3×500 ml Et_2O . The combined Et_2O layers were dried (Na_2SO_4) and evaporated to dryness. The residue was subjected to preparative TLC (SiO_2 ; solvent: n-hexane/ethyl acetate/methanol/formic acid, 70:28:1:1, by vol.). The spot of PHB (R_f 0.23) was scraped off, eluted with methanol and subjected to reverse-phase column chromatography (Lobar RP-8; solvent: methanol/water, 1:1, v/v). Fractions containing PHB were combined and evaporated, and PHB was crystallized from water (75 μl) with a yield of 4.89 mg.

RESULTS

Overexpression of CPL

In cell-free extracts of wild-type *E. coli* strains, chorismate is predominantly metabolized to prephenate by the chorismate mutase reaction (Lawrence *et al.*, 1974), preventing the detection of CPL activity (data not shown). We therefore used the *E. coli* mutant AN92, which is blocked in chorismate mutase as well as in anthranilate synthase (Young *et al.*, 1971), for detection of CPL activity in crude extracts.

Overexpression of CPL was achieved by transformation of strain AN92 with plasmids pALMU1, pALMU3 or pUBIC. pALMU1 and pALMU3 both contain the entire *ubiCA* operon with its 5' flanking sequences, pALMU1 in the vector pBluescriptKSII+, pALMU3 in the vector pTZ19R (see Methods).

Transformation of strain AN92 with pALMU1 or pALMU3 resulted in an approximately 38-fold increase of CPL activity in crude enzyme extracts, compared to the activity of AN92 transformed with the empty vectors. CPL activity could not be stimulated by the addition of IPTG to the culture medium. This indicates that in both vectors the gene is under control of its own promoter rather than under the *lacZ* promoter of the vector. This assumption is also supported by the expression of the UbiC protein in *E. coli* minicells from pTZ19R vectors, containing the pALMU3 insert in either orientation (Siebert *et al.*, 1992) and by the *in vitro* transcription/translation experiments described below.

Plasmid pUBIC contains only the *ubiC* structural gene, obtained by PCR amplification and ligated into the vector pUC18 (see Methods). From this recombinant plasmid, UbiC is synthesized as a LacZ fusion protein under the control of the *lacZ* promoter. By transformation of strain AN92 with this plasmid, a 1400-fold overexpression of CPL activity was achieved. Since strain AN92 contains only low amounts of *lac* repressor, no IPTG induction of enzyme activity was observed. However, in strain SG13009/pREP4, which overproduces the Lac repressor protein, CPL activity was, upon transformation with pUBIC, inducible by IPTG as expected. Strains containing these overexpression vectors showed detectable CPL activity even in the presence of intact chorismate mutase.

Table 1. Effects of ions on CPL activity

Enzyme activity was determined as described in Methods.

Addition	PHB formation	
	pmol	Percentage of control
None	823	100
MgCl ₂ (1 mM)	871	106
MgCl ₂ (10 mM)	1318	160
EDTA (2.5 mM)	980	119
EDTA (25 mM)	1280	156
NaCl (200 mM)	1239	151
NaCl (600 mM)	758	92
Tris/HCl (410 mM)	1056	128

Properties of CPL

From strain XL1Blue, transformed with pALMU1, CPL was purified 87-fold as described previously (Siebert *et al.*, 1992). Considering the 38-fold overexpression of CPL in XL1Blue harbouring pALMU1, an approximately 3000-fold enrichment was achieved, compared with extracts from wild-type strains. In the presence of 10 % glycerol, the purified enzyme could be stored at -20°C for several months without significant loss of activity.

The enzymic reaction was not dependent on metal cofactors (Table 1). The observed stimulation of the activity by 10 mM MgCl₂ was apparently an effect of ionic strength, since a similar stimulation could be achieved by EDTA, NaCl or Tris/HCl. The addition of 160–200 mM NaCl to the reaction buffer (50 mM Tris/HCl) increased the activity by 50 %. In the absence of NaCl, the optimal concentration of Tris/HCl was 200–400 mM.

A strong product inhibition was observed in the presence of PHB. Using a chorismate concentration of 500 μM , the addition of 25 μM or 100 μM PHB reduced the reaction velocity by 51 or 83 %, respectively. Addition of an excess of chorismate reversed the inhibition, suggesting a competitive mechanism. In contrast, pyruvate did not show an inhibitory effect at concentrations up to 50 mM, using the HPLC assay for the measurement of enzyme activity.

Using a buffer containing citrate, borate and phosphate, each at 40 mM, the pH optimum of the CPL reaction was determined as 7.5, with half-maximal reaction velocities at pH 5.7 and 9.2. The enzyme showed an isoelectric point of pH 6.45, determined by the method of Harzer (1970), contrasting with the value of 8.08 calculated from the amino acid sequence using the HUSAR program.

The apparent K_m value for chorismate was calculated as 6.1 μM with a standard deviation of 12 %, using the PENNYME program (Eisenthal & Cornish-Bowden, 1974; Cornish-Bowden & Eisenthal, 1974; Cornish-Bowden, 1977). For these experiments, the incubation was shortened to 3 min in order to measure accurately the

initial reaction velocities and to minimize product-inhibition effects. When chorismate was replaced with 47 μM isochorismate (Young *et al.*, 1969) as substrate, no formation of salicylic acid could be detected, showing this pyruvate-lyase reaction to be specific for chorismate.

As expected, a reverse reaction could not be observed, using PHB concentrations up to 25 mM and pyruvate concentrations up to 250 mM.

Localization of the promoter region

In vitro transcription/translation experiments from supercoiled as well as from *Pst*I-linearized pALMU3 yielded a prominent protein band of 19 kDa (Fig. 3). Because in the latter case the plasmid *lacZ* promoter is dissected from the *ubiC* gene, *ubiC* must be expressed from its own promoter.

As reported previously (Siebert *et al.*, 1992), sequence analysis revealed two possible promoter regions for the *ubiCA* operon (see Fig. 2). The putative upstream promoter, designated P2, shows a perfect -10 box but weak homology to the -35 consensus sequence. The potential downstream promoter, designated P1a, has well-conserved -35 and -10 boxes which are, however, separated by an unprecedentedly long spacing of 24 bp.

To examine the localization of sequences with promoter activity, we undertook *in vitro* transcription/translation experiments with PCR-amplified DNA fragments containing different portions of the *ubiC* upstream region. Radioactive labelling detected no translation products from a DNA fragment lacking both putative promoters (using the 5' primer *ubiC*-3F). In contrast, the region of 104 bp upstream of the start codon (obtained with the primer *ubiC*-2F) containing only the P1 region is sufficient for protein expression (Fig. 3).

The above results were confirmed by measurement of the enzyme activity of the CPL formed by *in vitro* transcription/translation. S-30 extracts incubated with the empty vector pTZ19R or the promoterless gene *ubiC* (obtained with the primer *ubiC*-3F) showed little CPL activity: 0.16 ± 0.06 and 0.29 ± 0.04 nmol PHB min⁻¹ (mg protein)⁻¹, respectively (means $\pm$ SD, $n = 4$). This residual activity might have been attributable to endogenous CPL present in the S-30 extracts. Presence of the putative promoter region P1 in the *ubiC* fragment (obtained with the primer *ubiC*-2F) clearly enhanced this activity, to 0.68 ± 0.11 nmol min⁻¹ mg⁻¹. The activity was further increased by more than 50 %, to 1.06 ± 0.15 nmol min⁻¹ mg⁻¹, when additionally the upstream region containing the potential promoter P2 was present, using the DNA fragment generated with primer *ubiC*-1F. The activity obtained with pALMU3, which contains the entire *ubiCA* operon in pTZ19R, was 1.12 ± 0.22 nmol min⁻¹ mg⁻¹.

Mutation of the *ubiC* gene in *E. coli* AN244

E. coli AN244 carrying the *ubiC*₄₃₇ mutant allele (Lawrence *et al.*, 1974) is unable to convert chorismate into PHB. This mutant strain was originally obtained using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as mutagen.

Fig. 3. *In vitro* translation products of plasmids and PCR fragments containing the *ubiC* gene. *In vitro* expression and gel electrophoresis was carried out as described in Methods. Lanes 1 and 2, expression from pALMU3 in supercoiled or *Pst*I-restricted form, respectively. Lanes 3 to 5, translation products from PCR fragments generated with the 3' primer *ubiC*-R and the 5' primers *ubiC*-1F, *ubiC*-2F, and *ubiC*-3F, respectively. The arrow indicates the band formed by the *ubiC* gene product.

We analysed the entire *ubiC* gene of AN244 by PCR direct sequencing. The only change detected within the gene in comparison with the wild-type was a point mutation located at position 466 near the carboxy-terminus of the gene. It shows a transition from G to A (Fig. 4), which leads to a change of amino acid from glutamic acid (GAA) to lysine (AAA). Since direct PCR sequencing was employed, rather than sequencing of a cloned PCR fragment, the nucleotide change cannot be attributed to a PCR artifact.

Feeding experiment with [1,7- $^{13}\text{C}_2$]shikimate

The two possible biosynthetic pathways to PHB, i.e. via phenylpropanoid compounds or via the CPL reaction, differ in the fate of the carboxyl group of shikimate (Fig. 1): it is lost in the aromatization of prephenate on the route via phenylpropanoids, but gives rise to the carboxyl group of PHB in the CPL reaction. The relative contribution of the two pathways to the formation of PHB can be assessed by using as a precursor a shikimate sample carrying an isotope label in the carboxyl group and a reference label in the ring.

We have synthesized a double-labelled shikimic acid, carrying two ^{13}C atoms, one in the carboxyl group and one in the adjacent ring position (Cho *et al.*, 1992). One

Fig. 4. Location of the mutation in the *ubiC* gene from *E. coli* AN244, which is blocked in the CPL reaction. The region from nucleotides 463 to 477 is shown (numbering refers to 1 as the translation start). (a) Wild-type sequence; (b) mutated sequence. The arrow indicates the exchange of the respective nucleotide. Amplification and sequencing reactions are described in Methods.

Fig. 5. ^{13}C -NMR spectra of PHB: authentic PHB (upper panel), and PHB formed by *E. coli* AN385 after feeding of [1,7- $^{13}\text{C}_2$]shikimate (lower panel). NMR: 100 MHz, inverse gated decoupling; CD_3OD , ^{13}C -depleted.

millimole (176.3 mg) of this compound was fed to the *E. coli* mutant AN385, which is unable to convert PHB into ubiquinone (Wallace & Young, 1977) and therefore accumulates low amounts of PHB in the culture medium (Young *et al.*, 1972); this PHB was extracted and purified (see Methods). In ^{13}C NMR analysis, each carbon of a molecule gives a signal due to the natural abundance of ^{13}C (1.1 % of the predominant isotope ^{12}C). Incorporation of isotope label from a ^{13}C -labelled precursor results in increased signals of the respective carbon. If the precursor contains two ^{13}C atoms in adjacent positions, they give a doublet rather than a singlet signal, due to ^{13}C – ^{13}C coupling, i.e. due to either parallel or antiparallel spin of the neighbouring ^{13}C . In ^{13}C NMR the PHB isolated from the feeding experiment showed coupled signals due to the incorporation of ^{13}C both into the carboxyl group (a) and into the neighbouring ring position (b) (Fig. 5, bottom). Incorporation of the shikimic acid with loss of the carboxyl group, in contrast, would have resulted in an increase of the singlet signal of the ring position (b) alone. Total incorporation was 14.8 %, and the detection limit for loss of C-7 was calculated as 1 % of the total incorporation. Therefore, incorporation of shikimate into PHB proceeded with complete (> 99 %) retention of the carboxyl group, i.e. exclusively via the CPL reaction.

DISCUSSION

CPL could be overexpressed 1400-fold in *E. coli* strains harbouring the plasmid pUBIC. The overexpressed enzyme is a fusion protein comprising the original CPL with 17 additional amino acids of the LacZ protein at its N-terminus. Since these additional amino acids might influence the properties of the enzyme, we used for the characterization experiments the unmodified CPL, which could be obtained in approximately 3000-fold enrichment by transformation of *E. coli* strains with plasmid pALMU1 and subsequent chromatographic purification.

The purified CPL did not accept isochorismate as substrate; this substance differs from chorismate by carrying the hydroxyl group in the 2-position instead of the 4-position. On the other hand, Nichols & Green (1992) presented evidence which indicates that CPL can convert 4-amino-4-deoxychorismate to 4-aminobenzoate *in vivo*. Therefore a hydroxy or amino function in 4-position appears to be essential for the reaction.

We determined the K_m value of CPL for chorismate as 6.1 μM , which is in reasonable agreement with the previously published value of 9.7 μM (Nichols & Green, 1992). The latter value was obtained with 30 min incubations, which in our hands proved to be beyond time-linearity of the reaction, probably due to the strong product inhibition by PHB which we have observed.

Some of the chorismate-metabolizing enzymes, i.e. isochorismate synthase (EntC), anthranilate synthase (TrpE subunit) and aminodeoxychorismate synthase (PabB subunit), show homologies to each other, especially at the carboxy-terminus of the proteins (Ozenberger *et al.*, 1989), and all three enzymes require Mg^{2+} ions as cofactors. Walsh *et al.* (1990) have proposed that the role

of the Mg^{2+} ion is to chelate the 4-hydroxy group of chorismate, making it a better leaving group. In contrast, the 4-hydroxy group is not removed from chorismate in the CPL and the chorismate mutase reactions. These two enzymes do not require Mg^{2+} ions and do not show homology to the other three chorismate-metabolizing enzymes nor to each other. As reported previously (Nichols & Green, 1992), there is also no homology between UbiC and PabC, which catalyses the pyridoxal phosphate-dependent conversion of 4-amino-4-deoxychorismate to 4-aminobenzoate.

The *ubiC* gene is, together with *ubiA*, part of a typical operon structure (Siebert *et al.*, 1992). However, no sequence element with high homology to the *E. coli* consensus promoter and a conventional spacing of the –10 and –35 box could be detected within the 570 bp directly upstream of the ATG start codon. Nevertheless, a region containing the 104 bp 5' of the start codon was sufficient to express the UbiC protein *in vitro* detected as radioactively labelled translation product and as active enzyme. This contrasts with the work of Nichols & Green (1992), who found 169 bp of the upstream sequence not to be sufficient for generation of measurable enzyme activity, whereas 243 or more bp of this flanking region resulted in CPL formation. The difference may be caused by their method of expressing the enzyme *in vivo* using plasmids with a T7 promoter. The 104 bp which we identified as sufficient for gene expression contain a –10 and a –35 box strongly homologous to the *E. coli* consensus promoter (P1a in Fig. 2) but their extremely long spacing of 24 bp (compared to 17 bp in average) has not been documented for functional *E. coli* promoters (Lisser & Margalit, 1993). Because many promoters show only a rather weak homology to the consensus (Lisser & Margalit, 1993), especially to the –35 box (O'Neill, 1989), we assume that the low-consensus –35 box depicted in Fig. 2 as P1b is the functional promoter element. However, exact localization of the promoter would require the determination of the transcription start site and mutational analysis of the putative promoter sequences. Usage of a longer fragment of 313 bp of the region upstream of the ATG start codon increases the *ubiC* expression, probably due to the activity of an additional promoter element in this fragment as mentioned above.

Sequence analysis of the entire *ubiC* gene, amplified from the *ubiC* mutant *E. coli* AN244, revealed a point mutation causing a change of an amino acid residue from glutamate to lysine near the carboxy-terminus (position 156). The Chou–Fasman (Chou & Fasman, 1978) and the Garnier–Osguthorpe–Robson (Garnier *et al.*, 1978) algorithms for the calculation of the secondary structure for the wild-type and the mutant UbiC yielded divergent results. While the former predicted a change from a helical structure (wild-type) to a β -sheet (mutant) in the affected region (amino acid residues 150 to 159), the latter showed no conformational difference. On the other hand, the change of glutamate to the positively charged lysine residue with the concomitant loss of enzyme activity may indicate that the negatively charged glutamate residue is essential for

Fig. 6. Hypothetical reaction mechanism of the conversion of chorismate to PHB by CPL involving the glutamate residue.

the enzymic mechanism. Walsh *et al.* (1990) proposed a 1,2-elimination for the aromatization step in the CPL reaction starting with an initial abstraction of the C₄-H of chorismate followed by the loss of the C₃-enolpyruvyl group (see Fig. 6). For the first step a nucleophilic group is essential for acceptance of the proton. This group could be represented by glutamate but not by lysine. The proton needed for the pyruvate abstraction in the second step could be supplied by a basic residue, for example lysine or arginine. Both amino acids are located close to the site of the mutation. Further site-directed mutational analysis of this putative active site of the enzyme is required to confirm this assumption.

A major difficulty in the cloning of the *ubiC* gene was the leaky phenotype of the *ubiC* mutant strain AN244. Despite the mutation, ubiquinone is formed by this strain in an amount as high as 13% of the value obtained with external PHB supplementation (Lawrence *et al.*, 1974). In the hands of Nichols & Green (1992), this leakiness prevented the use of phenotypic complementation as a cloning strategy for *ubiC*. A possible explanation for this phenomenon could be that CPL is the major, but not the only, biosynthetic source of PHB, and that a pathway similar to the eukaryotic one, i.e. via phenylpropanoid intermediates (Fig. 1), can be used to some extent. Our feeding experiment with [1,7-¹³C₂]shikimate proved, however, that under the experimental conditions employed more than 99% of PHB is formed by the CPL reaction. Therefore, a phenylpropanoid pathway to PHB is not used to a significant extent. The leakiness of the phenotype may be due to a residual CPL activity of the mutated *UbiC* gene product or, more likely, to the observed chemical decomposition of chorismate to PHB (Gibson & Gibson, 1964; Siebert *et al.*, 1992).

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