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 μM 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 $[1,7-^{13}C_2]$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*

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**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 $[^{14}C]$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 μM, 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 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...
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*-3'F and *ubiC*-R, see below) which was treated with Klenow polymerase and T4 kinase prior to ligation (Sure Ligation Kit, Pharmacia) into the *Sma*I 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 were 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 μl sodium acetate buffer (0.75 M, pH 4.0) were extracted twice with 1 ml Et,O. 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 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<sub>600</sub> 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'-TTCAGCTCAAAATCTCA-3'; *ubiC*-2F, 5'-GTTGGGTGTGTGGC-3'; *ubiC*-3F, 5'-TGTAACGGAGAGTTCGG-3'; *ubiC*-R, 5'-ATTCTGGCCTCAGACTCC-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 μl 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 °C, followed by 35 cycles of 1 min annealing at 52 °C, 3 min chain elongation at 72 °C, and 1 min denaturation at 95 °C. The reaction was finished after a final elongation step of 10 min at 72 °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 μg DNA (plasmid or PCR fragments) including <sup>[35]S</sup>methionine for 2 h at 37 °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-Omar AR film. For measurement of enzyme activity, 2 μg supercoiled plasmid DNA or 4 μg PCR fragments were included in a non-radioactive transcription/translation assay (2 h at 37 °C). After addition of

![Image of synthesis of ubiquinone](https://example.com/ubiquinone-synthesis.png)
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 NaHCO₃ (1 M). The NaHCO₃ layer was acidified (HCl) and extracted with 3 x 500 ml Et₂O. The combined Et₂O layers were dried (Na₂SO₄) and evaporated to dryness. The residue was subjected to preparative TLC (SiO₂; solvent: n-hexane/ethyl acetate/methanol/formic acid, 70:28:1:1, by vol.). The spot of PHB (Rf 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 ml) 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 pBluescriptKSI-1, by standard transformation methods. pALMU3 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.

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.
Enzyme activity was determined as described in Methods.

<table>
<thead>
<tr>
<th>Addition</th>
<th>PHB formation</th>
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<tr>
<td></td>
<td>pmol</td>
</tr>
<tr>
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<td>823</td>
</tr>
<tr>
<td>MgCl₂ (1 mM)</td>
<td>871</td>
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<tr>
<td>MgCl₂ (10 mM)</td>
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</tr>
<tr>
<td>EDTA (25 mM)</td>
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<td>NaCl (600 mM)</td>
<td>758</td>
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<tr>
<td>Tris/HCl (410 mM)</td>
<td>1056</td>
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</table>

**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 PENNZYME 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 super-coiled as well as from PstI-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 ubiC/A 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 Pla, 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 Pl 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 ± 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 ubiC/A 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 ubiC437 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 PstI-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\textsuperscript{13}C\textsubscript{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 \textsuperscript{13}C atoms, one in the carboxyl group and one in the adjacent ring position (Cho et al., 1992). One

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{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 PstI-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.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{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.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig5}
\caption{\textsuperscript{13}C-NMR spectra of PHB: authentic PHB (upper panel), and PHB formed by E. coli AN385 after feeding of [1,7\textsuperscript{13}C\textsubscript{2}]shikimate (lower panel). NMR: 100 MHz, inverse gated decoupling; CD\textsubscript{3}OD, \textsuperscript{13}C-depleted.}
\end{figure}
coli 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 doublet rather than a singlet signal, due to $^{13}$C-$^{13}$C coupling, i.e. due to either parallel or antiparallel spin of two $^{13}$C atoms in adjacent positions, they give a 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 shikimate 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-amino-4-deoxybenzoate in vitro. 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 $\mu$M, which is in reasonable agreement with the previously published value of 9.7 $\mu$M (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), antranilate 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-deoxy-chorismate to 4-amino-4-deoxybenzoate.

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 (Pla 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
ACKNOWLEDGEMENTS

We are grateful to Dr I. G. Young (Canberra) for providing us with the *E. coli* AN244 and AN92 strains, to Dr G. Unden (Dusseldorf) for *E. coli* AN385, to K. Schmidt (Bonn) for a sample of isochorismic acid, and to Drs J. Schroder and G. Schröder for their help in the investigations of the *ubiC* gene. Special thanks to Dr A. Behchhold for cloning of the *ubiC* gene and helpful discussion and to Dr U. May for the development of the enzyme purification procedure. This work was supported by a Heisenberg scholarship, the Alfried Krupp-Förderpreis für junge Hochschullehrer, research grants from the Deutsche Forschungsgesellschaft, and by the Fonds der Chemischen Industrie (grants to L.H.).

REFERENCES


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

Formation of 4-hydroxybenzoate in *E. coli*


Received 13 August 1993; revised 6 October 1993; accepted 26 October 1993.