Escherichia coli para-aminobenzoate synthase component I : cloning, sequencing and in vitro mutagenesis of pabB

ESCHERICHIA COLI PARA-AMINOBENZOATE SYNTHASE COMPONENT I

CLONING, SEQUENCING AND IN VITRO MUTAGENESIS OF PABB.

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR AAN DE TECHNISCHE UNIVERSITEIT EINDHOVEN, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF.IR. M.TELS, VOOR EEN COMMISSIE AANGEWEZEN DOOR HET COLLEGE VAN DEKANEN, IN HET OPENBAAR TE VERDEDIGEN OP VRIJDAG 27 JANUARI 1989 TE 14.00 UUR

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Contents.

Abbreviations.

AA	anthranilic acid
Ala	alanine
AMP	adenosine 5'-monophosphate
Arg	arginine
AS	anthranilate synthase (E.C.4.1.3.27)
ATP	adenosine 5'-triphosphate
CCC DNA	covalently closed circular DNA
Ch	chorismic acid
CoI	component I
CoII	component II
Cys	cysteine
D	dalton (gr/mol)
2-D	two-dimensional
3-D	three-dimensional
DEAE cellulose	diethylaminoethyl cellulose
DON	6-diazo-5-norleucine
DTE	dithioerythritol
DTT	dithiothreitol
eda	gene coding for 2-keto-3-deoxygluconate 6-phosphate aldolase (E.C.4.1.2.14)
EDTA	ethylenediaminetetraacetate
fadD	gene coding for acylCoA synthetase (E.C.6.2.1.3)
fadL	gene coding for long chain fatty acid transport protein
fadR	gene coding for repressor protein (fad regulon and aceBA operon)
gap	gene coding for glyceraldehyde-3-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase (E.C.1.2.1.12)
GAT	glutamine amidotransferase
Gln	glutamine
Glu	glutamate
His	histidine
IPTG	isopropyl-β-D-thiogalactopyranoside
$lacI^q$	mutation in the gene coding for repressor protein (lac operon)
lambda cI	gene coding for repressor protein (lambda phage)
LCFA	long chain fatty acids
LDH	lactate dehydrogenase

Lys	lysine
MCFA	medium chain fatty acids
mutL, mutS	gene coding for protein involved in mismatch correction
NAD	nicotinamide adenine dinucleotide
OMP	outer membrane protein
PABA	para-amino benzoic acid
pabA	gene coding for p-aminobenzoate synthase CoII (E.coli)
pabB	gene coding for p-aminobenzoate synthase CoI (E.coli)
pabP	gene coding for p-aminobenzoate synthase (S.acrimycini)
pabS	gene coding for p-aminobenzoate synthase (S.griseus)
PABS	para-aminobenzoate synthase
PCS	phosphate control sequence
PEG	polyethylene glycol 6000
PEP	phosphoenolpyruvate
Phe	phenylalanine
PK	pyruvate kinase (E.C.2.7.1.40)
ptsM	gene coding for mannose phosphotransferase enzyme II
RBS	ribosome binding site
recA	gene coding for protein involved in recombination
RF DNA	replicative form DNA (M13 phage)
rnd	gene coding for ribonuclease D
rrnB T_1T_2	ribosomal RNA transcription terminators
S/D	Shine/Delgarno sequence
ss DNA	single strand DNA
TBE	Tris/Borate/EDTA electrophoresis buffer
TE	Tris/EDTA buffer
Tet	tetracycline
Tm	melting temperature
Tn10	transposon 10
Trp	tryptophan
Tyr	tyrosine
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter I.

Introduction.

I.1. General.

In living cells, the most important group of proteins exhibiting biological activity are the enzymes. These proteins are catalysts responsible for tremendous rate accelerations of organic reactions proceeding with high specificity and efficiency.

Ever since their discovery, continuous efforts have been made to elucidate the mechanisms enzymes employ to mediate catalysis. A traditional strategy to investigate enzyme catalysis is based upon the modification of the structures of amino acid functional groups, especially those present in the active site. Two conceptually similar, but experimentally different methods are applicable to achieve this goal.

The classical approach is chemical modification, the use of small molecules designed to react with a restricted class of functional groups. Due to some limitations of this technique, such as the lack of absolute specificity for active site amino acid residues, the uncertain impact of the steric and electrostatic properties of the modified amino acid on the reaction mechanism and the effect of the modification on the three-dimensional structure of the enzyme, biochemists have started to use an alternative approach in protein engineering developed in recent years.

With the introduction of recombinant-DNA techniques, it has now become feasible to change functional groups present in the active site of an enzyme genetically, by specific alteration of the codons for respective amino acids in a cloned gene coding for the enzyme. This technique, commonly referred to as site-directed mutagenesis, is considered to be a powerful tool for studying intrinsic features of any enzyme, since an almost inexhaustible number of mutant-enzymes, the kinetic properties of which are to be evaluated, can be acquired.

In this thesis, the results of a study concerning the cloning, sequencing and in vitro mutagenesis of the *Escherichia coli* gene coding for para-aminobenzoate synthase Component I will be reported.

A preliminary model for the reaction mechanism of the PABS holo-enzyme, based upon the kinetic parameters of mutant-enzymes constructed, will be presented.

I.2. The shikimic acid pathway.

In all microorganisms investigated to date, the shikimic acid pathway is utilized for the biosynthesis of the aromatic amino acids, the folate coenzymes and several other essential vitamins (1,2). An important common intermediate in this multibranched pathway is chorismic acid, which is converted by five separate enzymes to p-hydroxybenzoate, isochorismate, prephenate, anthranilate and p-aminobenzoate (Fig.I.1).

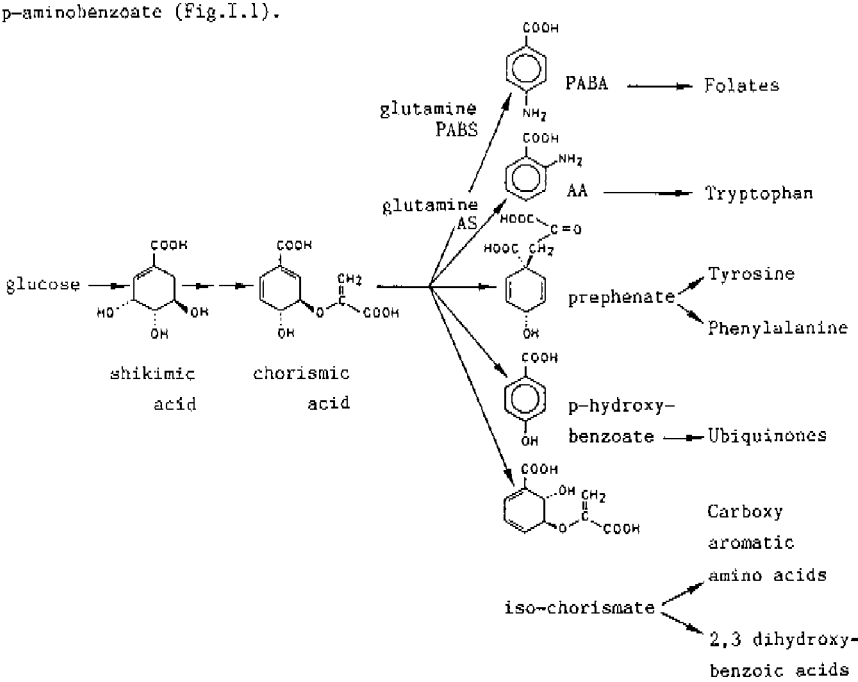


Fig.I.1 Shikimic acid pathway in microorganisms.

Considering the disparity of metabolic products synthesized from

chorismate, by their nature as well as by their quantity, it is obvious that the enzymes involved must be of particular design in order to satisfy the specific need for certain metabolites during cell growth and maintenance. Especially intriguing in the case of chorismate-converting enzymes is the way in which only slightly different precursors like o-aminobenzoate (anthranilate) and p-aminobenzoate (PABA) are produced from the common substrate, concomitantly preserving a considerable difference in the respective amounts synthesized. Therefore, research to elucidate underlying catalytic mechanisms of this class of enzymes not only will contribute to a better understanding of enzyme catalysis in general, but in addition will allow us to gain insight in the way fine-tuning of metabolism by differential substrate channeling is achieved.

I.3. p-Aminobenzoate synthase, evolution and genetic organisation.

In 1959, Weiss and Srinivasan (3) first showed that shikimate-5-phosphate plus glutamine can be converted to p-aminobenzoic acid by a yeast preparation. Somewhat later, they established, that the amino group of p-aminobenzoic acid in fact arises from the carbonamide nitrogen of glutamine (4). It was found by the group of Gibson et al. (5,6,7,8) that the bacterial growth factor PABA was formed together with other compounds, such as prephenic acid and anthranilic acid, from a common intermediate in aromatic biosynthesis. The intermediate was identified as chorismic acid (Fig.I.1). Evidence that chorismate was the precursor of PABA was the observation that neither p-hydroxybenzoate or prephenate could replace chorismate as a substrate. Furthermore, mild heat treatment of chorismate solutions, thereby destroying chorismate, also inactivated the capacity to function as substrate for PABA synthesis (9).

E.coli mutant strains requiring PABA for growth were isolated by Lampen et al. (10) and Huang & Pittard (11). The mutations were mapped by conjugation and transduction, and two genes concerned with the biosynthesis of PABA were identified, called pabA and pabB. These mutants map at two distinct loci; the pabA gene was found to be located at approximately 74 min., whereas the pabB gene was found to be located at approximately 40 min. on the *E.coli* genome. It was

shown, that there was no crossfeeding between strains mutated either in pabA or pabB (although Lingens (12) reported otherwise), but crossfeeding towards both types of mutants was pronounced in case wildtype *E.coli* strains were used. It was concluded that at least two enzymatic reactions are involved in the conversion of chorismate to PABA.

In *Bacillus subtilis*, p-aminobenzoate synthase (PABS) has the following properties: (i) in crude extracts synthesis of PABA is catalyzed in the presence of chorismate and either glutamine (amidotransferase activity) or ammonia (aminase activity); (ii) the enzyme is composed of two nonidentical subunits (12,13,14) designated as subunit A and subunit X (15); subunit X was later called subunit G (16) by analogy to subunit G from other organisms (17,18); subunit A has aminase activity but no amidotransferase activity and subunit G acts as a glutamine-binding protein; (iii) subunit G is bifunctional, since it is also a component of the anthranilate synthase (AS) complex in *B.subtilis*; therefore, in this organism the biosynthesis of the vitamin dihydrofolate is interlocked with tryptophan biosynthesis (19); (iv) subunit G can exist in two forms with respect to its sulfhydryl groups, oxidized or reduced, in which the reduced sulfhydryl group is not only essential for amidotransferase activity, but also for the formation of the AS aggregate; (v) due to the amphibolic nature of subunit G, the PABS enzyme of *B.subtilis* is readily associated and dissociated.

An investigation of the metabolism of chorismate by strains of *E.coli* carrying the pabA or the pabB alleles was first performed by Huang & Gibson (20). In order to study the enzymatic conversion of chorismate into PABA, it is necessary to use cell extracts from a strain unable to metabolize chorismate towards the three major end products derived from chorismate, namely phenylalanine, tyrosine and tryptophan. A triple aromatic auxotroph of *E.coli K12* was isolated (20), producing PABA when cell-free extract was incubated in a reaction mixture for a glutamine-dependent assay. Next step in mutant construction was the transfer of either a pabA or pabB mutated gene into this triple aromatic auxotroph, which was achieved by P_1-mediated transduction and a series of conjugation experiments. Upon incubation of a cell extract of the suitably blocked pabA strain or pabB strain

with the substrates necessary for PABA synthesis, no significant amount of PABA was formed. However, similar tests carried out with mixtures of cell extracts from pabA and pabB strains showed PABA synthesizing activity, although less than the triple blocked auxotrophic strain. These results again suggested that there are at least two reactions involved in the conversion of chorismate to PABA in *E.coli*, but no intermediate was detected, that could be converted to PABA by either mutant strain.

The next step in the study of PABA synthesis concerned fractionation of PABA synthesizing activity by gelfiltration on Sephadex G-100. No separate fraction showed any activity by itself, but activity was restored when two distinct fractions were mixed. These fractions each contained one of the components which together constitute the PABS holoenzyme, the pabB gene coding for Component I and the pabA gene coding for Component II. The instability of Component II was shown to be more pronounced than that of Component I upon storage.

Reiners et al. (21) used a radioimmunoassay to search for proteins that cross-react with antibodies to the subunits of the *E.coli* AS Components I and II. After chromatography of an *E.coli* extract on DEAE cellulose they reported an extremely labile PABS, the activity of which was no longer detectable after fractionation by gelfiltration. However, two peaks of protein were found that exhibited competition with ^{125}I-labeled AS for binding to either anti-AS CoI or anti-AS CoII. Since similarities in mechanism might accrue from similarities in structure, it appeared possible that glutamine amidotransferases may indeed employ similar mechanisms for glutamine binding and amide transfer (22-30).

Comparison of the amino acid sequences of the glutamine amidotransferase (GAT) subunits of enzymes of several microorganisms like *E.coli* PABS CoII (32), *E.coli* AS CoII (33,34), *E.coli* carbamoyl phosphate synthetase (35), *E.coli* GMP synthetase (36), *Salmonella typhimurium* AS CoII (32), *Serratia marcescens* AS CoII (32,37), *Pseudomonas putida* AS CoII (38), *Shigella dysenteria* AS CoII (34) and *Bacillus subtilis* AS CoII (39) reveals extensive homology suggesting that they arose from a common ancestor. A series of evolutionary stages has been postulated from these amino acid sequences (31). From

a primitive NH_3-dependent enzyme without GAT-activity, enzymes having GAT-subunits participating in two pathways might have evolved (*B.subtilis* (40), *Acetinobacter calcoaceticus* (41), *Pseudomonas acidovorans* (42)), followed by enzymes having their own exclusive GAT subunit (43). Repression control of bifunctional CoII by tryptophan in *B.subtilis* suggests its ancient origin as tryptophan-specific protein. Subsequent translocation of the trpG gene from the tryptophan operon may have been of selective advantage in order to ensure that a residual nonrepressible level of CoII could form vitamin amounts of folate, even in the presence of tryptophan. *E.coli* PABS and AS both belong to the class of amidotransferases without an interchangeable CoII, because in PABA synthesis, PABS CoI cannot function with AS CoII and similarly, in anthranilate synthesis, AS CoI cannot function with PABS CoII. This is inferred from the inability of wildtype trp genes to complement pabA and pabB mutants and the inability of pab genes to complement trpE and trp(G)D mutants (44).

The nucleotide sequence of *E.coli* pabA, coding for the GAT subunit of PABS, has been determined by Kaplan et al. (32). From this sequence the primary structure of PABS CoII was established, which predicted that a polypeptide product containing 187 amino acids and having a molecular weight of 20,752 is translated from the mRNA transcript of pabA. The molecular weight is twice that estimated from gel-permeation chromatography (20) and sodium dodecyl sulphate polyacrylamide gel-electrophoresis (21). This discrepancy could not readily be explained. The nucleotide sequence of *E.coli* trp(G)D, coding for AS CoII, has been determined also (33). Clearly, the coding regions of pabA and trp(G)D are homologous, suggesting that these two genes arose via the duplication of an ancestral gene. Moreover, Goncharoff & Nichols (45) have established the nucleotide sequence of *E.coli* pabB coding for PABS CoI and compared it with the trpE gene coding for AS CoI (46). In this case the amount of similarity suggested that these two genes have a common evolutionary origin also. Therefore, the existence of PABS and AS in *E.coli* is believed to be an actual case of acquisitive evolution which occurred after duplication of the genes encoding the initial synthase complex.

The extent of similarity between PABS CoII and AS CoII, 44% at the amino acid level, is greater than the 26% amino acid similarity

between PABS CoI and AS CoI. This is to be expected, since PABS CoII and AS CoII have identical roles of transferring the amino group from glutamine to the CoI subunit of each respective enzyme complex. According to Goncharoff & Nichols (45) the lower extent of similarity between PABS CoI and AS CoI can be ascribed to several factors. (i) AS CoI responds to feedback inhibition by tryptophan, whereas PABS CoI does not. The tryptophan binding area of AS CoI will therefore represent an area with no similarity to PABS CoI. The results of Matsui et al. (47), which show that the amino-terminal part of AS CoI is involved in allosteric regulation support this assumption, since this part of AS CoI showed no conservative amino acids when compared to PABS CoI. (ii) PABS CoI and AS CoI have slightly different catalytic functions. (iii) Subunit interaction areas are different in PABS CoI and AS CoI.

In all microorganisms mentioned so far, the PABS enzyme is composed of two nonidentical subunits, each coded for by a separate gene. In *Streptomyces griseus* however, PABA not only is the specific precursor of folate, but also of the aromatic p-aminoacetophenone moiety of candicidine, an antifungal antibiotic (48). Probably this explains why genetic organization and expression of PABS in *Streptomyces* is completely different from organisms not producing antibiotics, as described by the group of Gil et al. (49-53). Sulphonamide resistant clones were isolated. The resistance to sulphonamide apparently resides on over-production of PABA, shown by the ability of these strains to stimulate the growth of PABA-requiring mutants of *Streptomyces* by crossfeeding. A DNA fragment cloned from *S.griseus*, that conferred a prototrophic phenotype on PABA requiring auxotrophs of *Streptomyces lividans* and *Streptomyces coelicolor*, was able to ward off the metabolic anomaly in *E.coli* strains mutated either in pabA or pabB upon transformation. It was shown that in vivo deletion of the *S.griseus* promoter had occurred in the transformed *E.coli* strains. In contrast to *E.coli*, the genetic information for PABS in *S.griseus* evidently is not split between unlinked genes. The *S.griseus* gene was called pabS. This gene only exists in some strains of *S.griseus*, but not in *S.lividans* or *S.coelicolor*. A new pab gene, pabP has been cloned from a genomic library of *Streptomyces acrimycini*, carrying its own promoter recognized by *E.coli* RNA polymerase, in contrast to the

promoter of the pabS gene. PABS of *S.griseus* is subject to repression by aromatic amino acids and phosphate, effectors that control candicidin production. Regulation by phosphate does not exist in the expression in *E.coli* of a 3.5 kb fragment lacking the original *Streptomyces* promoter, suggesting the existence of a phosphate control sequence (PCS) in a 1 kb fragment deleted by *E.coli*. In *E.coli*, such a PCS does not exist (personal communication with P.Liras who tested the cloned pabB gene described in Chapter II.). Thus, in *Streptomyces* there seem to be specific genes involved in secondary metabolism (antibiotic synthesis), like pabS, with particular features and regulatory sequences (PCS) that may be absent from genes involved in primary metabolism, like pabA and pabB in *E.coli*.

I.4. p-Aminobenzoate synthase, mechanism of enzyme-catalysis.

I.4.1. Component I.

As shown in Fig.I.1, both PABS and AS catalyze complex reactions, involving the loss of an enolpyruvyl side chain, the loss of a hydroxyl substituent, the addition of an amide group, and the formation of an aromatic ring. Both holo-enzymes show amidotransferase as well as aminase activity in cell-free extracts. In *Bacillus subtilis*, mutants in trpG, coding for the bifunctional CoII, can utilize ammonia directly in an amination reaction under ordinary physiological conditions, showing that ammonia may serve in vivo as an amino donor for tryptophan synthesis. Gibson et al. (27) have shown, that in *E.coli* AS can generate anthranilate from chorismate and ammonia in whole cells. Whether or not PABS can generate PABA from chorismate and ammonia in vivo, by using the aminase activity of PABS CoI, has not been described in literature.

In the mid 1970's, Dardenne (55) and Haslam (56) independently suggested (Fig.I.2), that anthranilate and PABA both originated from chorismate (route a) or anthranilate arose from chorismate and PABA from isochorismate(5, route b) by parallel 1,5-addition/elimination reactions of ammonia or glutamine. Enzyme studies soon indicated, that isochorismate plays no part in the biosynthetic scheme (57,58). Compounds **2** and **6** have not been detected as bona fide intermediates

in PABA synthesis in *E.coli*, neither have mutants been isolated, reported to accumulate these compounds.

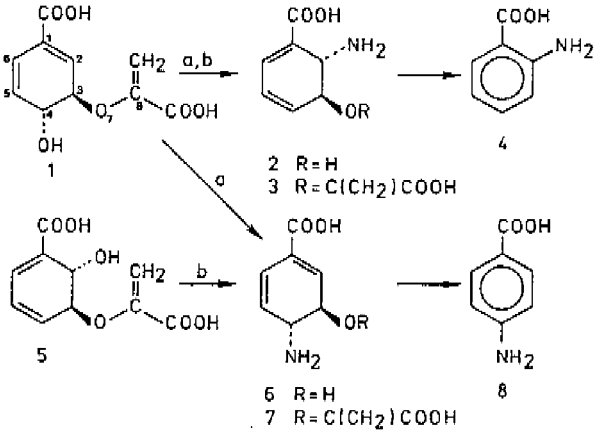
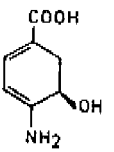


Fig.I.2

Conversion of chorismate to both anthranilate and p-aminobenzoate (route a) or conversion of chorismate to anthranilate, and isochorismate to p-aminobenzoate (route b) as proposed by Haslam (56) and Dardenne (55).

However, Altendorf et al.(12,59,60,61) did report the isolation of *Aerobacter aerogenes* mutant strains containing an additional genetic block prior to an intermediate which they called compound A and which could be converted to PABA. Based on spectrometric properties of compound A its structure was proposed as in Fig.I.3.



Compound A

Fig.I.3 Intermediate reported by Altendorf et al. (12, 59, 60, 61).

No pyruvate could be detected after conversion of compound A to PABA, either using lactic dehydrogenase or NMR spectroscopy. In aqeous solution compound A did not show fluorescence characteristics of PABA (excitation 290 nm, emission 340 nm versus anthranilate excitation

325 nm, emission 400 nm (62)), but three hours after addition of 0.1 N HCl fluorescence slowly arose from non-enzymatic conversion of compound A to PABA. Unless the pathway in *A.aerogenes* differs from that in *E.coli*, no explanation for the presence of compound A in *A.aerogenes* can be given.

In 1962, McCormick et al. (63) reported, that compound **2** was produced by a strain of *Streptomyces aureofaciens*, prompting the suggestion that **2** was a likely intermediate in anthranilate synthesis. The proposal of trans amino enol pyruvates **3** and **7** as possible intermediates in anthranilate (64) and PABA synthesis gained support when Teng & Ganem (65) and others (66) reported the conversion of **3** to anthranilate by AS CoI purified from *Serratia marcescens*. Moreover, Teng et al. (67) described the synthesis of 4-amino-4-deoxychorismate **7** and showed that **7** is a bonafide intermediate between chorismate and PABA by enzymic studies. In the absence of NH_4^+, **7** was converted to PABA by PABS CoI. Km(chorismate) was 12 μM and Km(**7**) was 16 μM. Vmax(**7**) was 12,8 times Vmax when chorismate was used as a substrate. By adding NH_4^+, Vmax(**7**) increased to 15.1 times Vmax(chorismate). Therefore, synthetic amino enol pyruvate **7** is a chemically and kinetically competent intermediate, although it has not been detected to accumulate during enzymatic processing of chorismate in any *E.coli* mutant strain.

Since the mechanism for the conversion of chorismate to **7** and the subsequent aromatization of **7** to PABA by elimination of pyruvate are not understood. Walsh et al. (68) recently performed a mechanistic study on *E.coli* PABS CoI which involved design, synthesis and testing of alternate substrates and inhibitors to probe for intermediates in the amination step and also to examine the effect of the leaving group at C-3 of chorismate on the rate of aromatization.

A variety of chorismate analogues were used as potential mechanism-based inactivators (by capture of reactive site nucleophiles prior to the addition of ammonia) or inhibitors of both AS and PABS. Inactivation was tested by preincubation of synthase with a chorismate analogue, establishing the remaining activity in normal assay thereafter. Inhibition was tested by determining synthase activity while simultaneously incubating chorismate and a chorismate analogue.

It was hoped for, that specific differences in inactivation or

inhibition behaviour between the two synthases might also yield information regarding the basis of regiospecificity in the amination reactions.

As far as inactivation is concerned, none of the analogues tested gave the first-order loss of activity expected for formation of an irreversibly inactivated PABS CoI.

The results of the inhibition study using AS CoI can be summarized as follows. First, it appears that a negatively charged moiety at C-1 and C-8 is required for binding, since phosphonate **9** is a strong inhibitor (competitive), whereas the diol **10** is not (Fig.I.4).

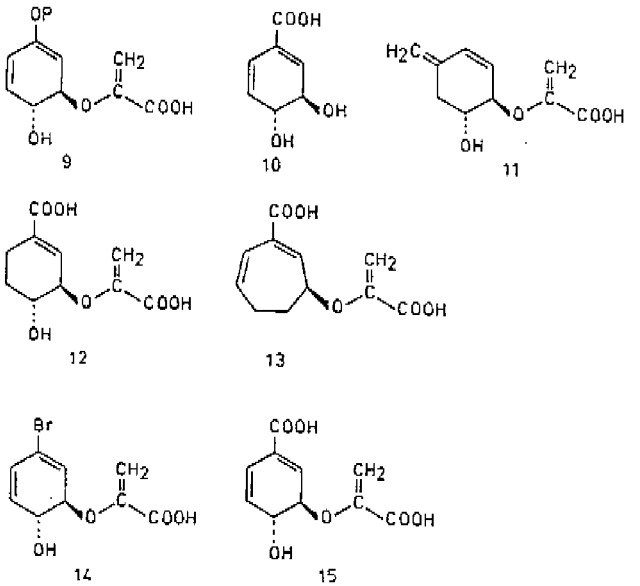


Fig.I.4

Chorismate analogues used by Walsh et al. (68) in a mechanistic study on *E.coli* PABS CoI.

Secondly, there is an apparent requirement for the planarity of the diene moiety around C-5-C-6-C-1-C-2 as evidenced by the lack of significant inhibition by the dihydrochorismate analogues **11** and **12**,

whereas the most potent reversible inhibition was observed with the cycloheptadienyl compound **13**, which lacks the C-4 hydroxyl group present in chorismate. Finally, compounds without the carboxyl at C-1, e.g. **14**, or with steric bulk at C-6, e.g. **11** are not bound at the active site but rather function as non-competitive inhibitors with AS CoI, presumably via binding to the tryptophan regulatory site (69).

Although the data for inhibition with PABS CoI are not as refined as that for AS CoI, it is apparent, that similar trends are followed. Again, the seven-membered ring **13** has the best Ki-value.

Since no evidence for the actual existence of proposed intermediates **3** (70,71,72,73) and **7** has been found yet by using the natural chorismate substrate, Walsh et al. (68) attempted the detection and isolation of an intermediate by using a series of alternate substrates for AS CoI and PABS CoI. Diastereoisomeric lactyl chorismate analogues (S)-**16** and (R)-**17** (Fig.I.5) were chosen, because it was expected that the poorer leaving group at C-3 might slow the aromatization step sufficiently to allow accumulation of the expected intermediate **18** or **19**, corresponding to **3** using AS CoI.

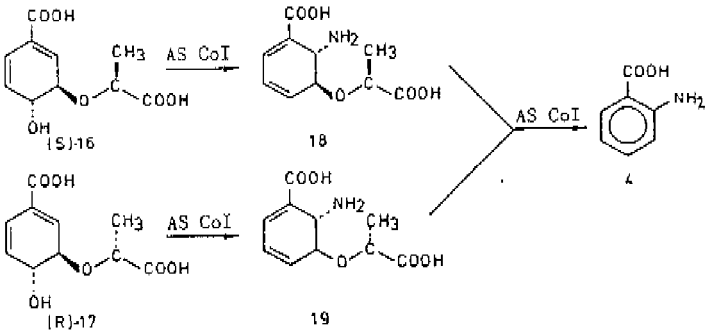


Fig.I.5
Alternate substrates for AS CoI and PABS CoI synthesized by Walsh et al. (65).

It was found, that the aromatization step now became the rate-limiting step during turnover. Also, AS and PABS active sites appeared to have opposite selectivities for the stereochemistry of the lactyl side

chain. AS preferred the S-isomer ($\frac{V/Km(Ch)}{V/Km(lactyl)}$ = 69)over the R-isomer ($\frac{V/Km(Ch)}{V/Km(lactyl)}$ = 872)whereas PABS showed opposite selectivity by V/K criteria (290 vs. 97 respectively).

The glycolate analogue **15** (Fig.I.4) is also turned over by both enzymes with surprisingly good V/K values. The observation that the Vmax value of **15** with PABS CoI is higher (140%) than that of chorismate, while the Vmax value of **15** with AS CoI is substantially lower (15%) than that of chorismate, suggests that aromatization is rate-limiting for AS but not for PABS, since glycolate is a poorer leaving group than pyruvate. Further support in this hypothesis is that **7** is processed to PABA at 10-20 times the rate of chorismate. Similarly, **3** is processed to anthranilate at only 1.2 times the rate of chorismate. Thus, for PABS CoI formation of **7** appears to be rate-limiting, while for AS CoI processing of **3** to anthranilate is rate-limiting. UV-Vis monitoring of accumulation of **18** or **19** during turnover of **16** or **17** respectively by AS CoI, was found to be consistent with the proposal of **18** or **19** as intermediates and therefore consistent with the proposal of **3** as an intermediate when chorismate would have been used. Unfortunately, possible accumulation of comparable intermediates during turnover of **16** or **17** by PABS CoI was not reported.

Tso & Zalkin (74) used a different approach to study enzyme mechanism of AS CoI. Residues essential for *Serratia marcescens* AS CoI function were studied by chemical modification reactions. Phenylglyoxal and 1,2 cyclohexanedione (75,76) modified arginine residues and inactivated AS CoI. From the inactivation data it can be concluded, that one arginine residue is essential for activity. Histidine residues in AS CoI were modified by ethoxyformic anhydride and by photooxidation (76,77). Upon modification of histidine residues the enzyme was also inactivated. Comparison of the number of carbethoxy groups incorporated between chorismate-protected and unprotected AS CoI indicated, that one histidine residue is required for activity. AS CoI was again inactivated using bromopyruvate (78,79). A differential labeling experiment indicated that loss of AS CoI activity was correlated with alkylation of one cysteine residue. A tryptic peptide containing the essential cysteine residue was isolated. The peptide has the amino acid sequence

le-Cys-Gln-Ala-Gly-Ser-Arg. A nearly homologous heptapeptide containing the essential cysteine residue is located in the primary structure of *E.coli* AS CoI at residues 376-382: Ile-Cys-Thr-Pro-Gly-Ser-Arg.

For each chemical modification chorismate protected against inactivation. Protection could result from direct shielding of residues essential for substrate binding or catalysis or indirectly from conformational changes. It is known, that chorismate binds to AS CoI in the absence of Mg^{2+}, and promotes a conformational change that is essential for the formation of an AS CoII-glutaminyl covalent intermediate and for hydrolysis of glutamine (80). Mg^{2+} is required in the catalytic step chorismate + "NH_3" to yield anthranilate + pyruvate. Data in (74) show, that Mg^{2+} enhances the capacity of chorismate to protect against inactivation by cyclohexanedione and photooxidation. It appears likely, that enhanced protection against modification of essential arginine and histidine residues results from increased steric shielding by the AS CoI*chorismate*Mg^{2+} complex over that obtained in the AS CoI*chorismate complex.

A plausible function of the essential arginine residue is in binding of the chorismate anion. There are numerous examples for the role of arginine residues in binding phosphate groups of substrates and coenzymes (81,82). Interaction of arginine with a carboxyl group has also been reported (83).

The essential histidine is a good candidate for a basic group perhaps required for abstracting the hydrogen on C-2 of chorismate or for protonation of the leaving hydroxyl or enolpyruvyl groups (64,84,85). It has been established, that the C-2 hydrogen of chorismate is not incorporated into the pyruvate formed in the reaction (85,86,87). One of several possible functions of the essential cysteine residue is that it participates in covalent catalysis by initial attack at C-2 of chorismate. According to this model, NH_3 would displace the enzyme thiolate to complete the formation of anthranilate. An analogous study of PABS CoI using chemical modification has not been possible yet due to the lack of sufficient amounts of relatively pure CoI. Therefore, it remains speculative to propose the analogous functions of essential arginine, histidine and cysteine residues.

A remarkable feature of the amino acid sequence of AS CoI as compared to that of PABS CoI is the complete absence of tryptophan in AS CoI, not only in *E.coli*, but also in *Salmonella typhimurium* (88). It has been suggested (46), that this may be an advantage to the organism, since the lack of tryptophan accounts for the continued synthesis of AS CoI during severe tryptophan starvation. A possible role for the continued synthesis of AS CoI would be to channel chorismate into the tryptophan pathway. Notably, also the AS CoII amino acid sequence is devoid of trp residues. PABS CoI and PABS CoII on the other hand contain seven and three trp residues respectively.

I.4.2. Component II.

A common feature of all PABS enzymes discussed in literature is that in vitro the amidotransferase activity is higher than the aminase activity by a factor 5 to 10. The subunit responsible for this raise in efficiency is CoII, the GAT subunit. From CoII it is known, that in all organisms studied sofar, a catalytically functional -SH group exists (13,80,89,90,91). In *S.typhimurium*, the glutamine analogue 6-diazo-5-norleucine, DON, inactivated AS due to binding to the essential cysteine. The rate of inactivation by low concentrations of DON was stimulated 25-fold by chorismate, suggesting ordered binding of first chorismate and then DON, or by analogy, glutamine (89,90).

In contrast to AS from *S.typhimurium* and *Pseudomonas putida* (80) binding of glutamine to the AS complex of *B.subtilis* (13) does not require chorismate and the rate of inactivation by DON is not influenced by chorismate. This suggests that subtle differences in the mechanism of glutamine binding exist among the enzyme complexes from various microorganisms.

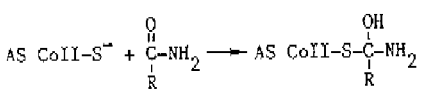
In *P.putida*, Cys 79 was shown to be the active site residue involved in the formation of the acyl-enzyme intermediate (38). Based on the observed homologies in the primary sequences of other amidotransferases examined, a 13-amino acid long sequence was proposed to be part of the catalytic domain. Active site Cys 84 in *Serratia marcescens* AS CoII was replaced with glycine using site-directed mutagenesis (92). The replacement abolished the amidotransferase activity but not the aminase activity in CoI. The NH_3-dependent

AS activity of the mutant enzyme supported tryptophan synthesis in media containing a high concentration of ammonium ions. Replacing Cys 84 with Gly eliminates the possibility that chemical modification of Cys 84 blocked amide transfer because of introduction of bulky modifying reagents rather than by specific chemical modification of an essential cysteine.

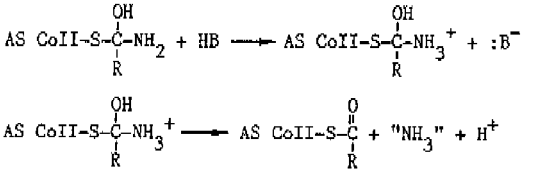
Bower & Zalkin (93) used pyridoxal-5-phosphate and NaCN to block DON-affinity labeling of the active site cysteine residue of *Serratia marcescens* AS CoII. Since pyridoxal-5-phosphate is useful for chemical modification of lysyl residues (94,95,96) their results were consistent with the formation of a lysyl-pyridoxal-phosphate Schiff base that is stabilized by NaCN. It was proposed, that a possible function for the lysyl residue is that it functions as a general acid-base to promote ionization of the cysteinyl residue. Alignment of protein chains from five organisms showed, that Lys 107 is the only lysyl residue conserved in all of them, whereas Lys 192 is conserved in four out of five. Lys 107 however is situated in a block of highly conserved residues, and therefore is the most likely candidate for the active site lysyl residue.

Amuro et al. (97) used site-directed mutagenesis to replace His 170 by tyrosine in *S.marcescens* AS CoII. The choice of His 170 was based on the fact that His 170 was the only conserved histidine when GAT subunits of four enzymes were compared: AS CoII, PABS CoII, GMP synthetase and carbamoyl-P-synthetase. Glutamine-dependent activity was undetectable in the Tyr 170 mutant, whereas NH_3-dependent activity was unchanged. DON-affinity labeling of AS CoII active site Cys 84 was used to distinguish whether His 170 has a role in formation or in breakdown of the covalent glutaminyl-Cys 84 intermediate. Reversion analysis was consistent with a proposed role of His 170 in catalysis as opposed to a structural role. A scheme for glutamine amide transfer was suggested by Amuro et al.(97)(Fig.I.6).

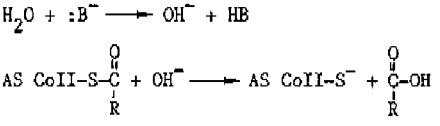
I. Formation of the glutamyl enzyme adduct.



II. Transfer of amide.



III. Hydrolysis of thioester.



AS CoII-SH represents AS CoII active site Cys 84, whereas in step I $:B^-$ was implicated as His 170. The identity and location of $:B^-$ in step III were not established. Perhaps the proposed active site Lys 107 (93) might be involved in this step.

I.5. Outline of this thesis.

In order to study *E.coli* p-aminobenzoate synthase Component I using site-directed mutagenesis, the gene coding for this component, pabB, had to be cloned first. In Chapter II, restriction mapping of about 23.7 kb of *E.coli* genomic DNA from the 40 min. region is described. Two genes were found to be located on this fragment, pabB and fadD, the latter coding for *E.coli* acylCoA synthetase. The pabB gene was subcloned into pUC19. The recombinant plasmid was subsequently used for transformation of an *E.coli* triple auxotrophic pabB mutant strain. Expression of PABS was detected by biological complementation of pabB deficiency, by crossfeeding towards other pabB mutant strains and finally by enzyme assay.

Chapter III describes the sequencing of pabB, cloned into M13mp18.

When this work was in progress, Goncharoff and Nichols published their results concerning the cloning and sequencing of pabB. Their data were found to be in complete agreement with ours.

Chapter IV deals with site-directed mutagenesis of pabB using a gapped heteroduplex technique, recently published by Kramer and Fritz (98).

In Chapter V, the results of the kinetic analysis of the pabB390-1 mutant enzyme, in which codon Trp 390 has been replaced with a stopcodon, are discussed.

Chapter VI describes the kinetic analysis of the pabB390-2 and pabB391 mutant enzymes, in which Trp 390 and Cys 391 have been replaced with tyrosine and serine, respectively. Based on the combined analyses of mutant-enzymes, a preliminary model for the reaction mechanism of PABS holo-enzyme is presented.

Finally, in Chapter VII a strategy for increasing PABS expression using a silent mutation construct is described.

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Chapter II.

Cloning of pabB and fadD.

II.1. Introduction.

Fulfilment of a particular metabolic requirement in *E.coli* mutant strains can often be achieved by a relatively low level of expression of the needed gene product. The ability of a cloned segment of DNA to complement an *E.coli* auxotrophic mutation in vivo therefore provides a simple way to detect the presence of a specific gene on one or more of these cloned segments.

Construction of a bank of cloned genomic DNA segments usually proceeds via disruption of the isolated genome in vitro, e.g. using restriction enzymes or mechanical sheering, followed by insertion of fragments into a suitable vector, either virus, plasmid or cosmid. A well-known source of *E.coli* genomic DNA fragments cloned into plasmid $pColE_I$ is the Clarke & Carbon gene bank (1,2,3). This bank has been screened for all kinds of *E.coli* genetic markers scattered among the entire genome, and a large number of gene assignments have already been made.

From these results, a gene-protein index was compiled by Neidhardt et al. (4). However, the presence of the pabB gene on one of the recombinant plasmids was not yet reported. Since the average size of DNA inserts was found to be about 12 kb (0.3-0.4 min. on the *E.coli* gene map), several genes can be present on a single insert. Therefore, attention was focused on $pColE_I$ recombinant plasmids reported to contain cloned genes located close to the pabB gene. One such gene, fadD, was reported to be in the 40 min. region of the *E.coli* gene map (5,6,7). The *E.coli* fadD gene codes for acylCoA synthetase (E.C.6.2.1.3) which takes part in fatty acid degradation, a process called β-oxidation, as shown in Fig.II.1. The distance between map locations of pabB and fadD was found not to be more than 0,17 min., as calculated from a co-transduction frequency of 91% (8). Also, Clark & Cronan (9) indicated that the fadD gene might be on four Clarke & Carbon plasmids, pLC4-21, pLC15-17, pLC15-32 and pLC30-32. This assumption apparently was based on assays of enzymatic activity of

acylCoA synthetase, although until now these results have not been published or confirmed otherwise.

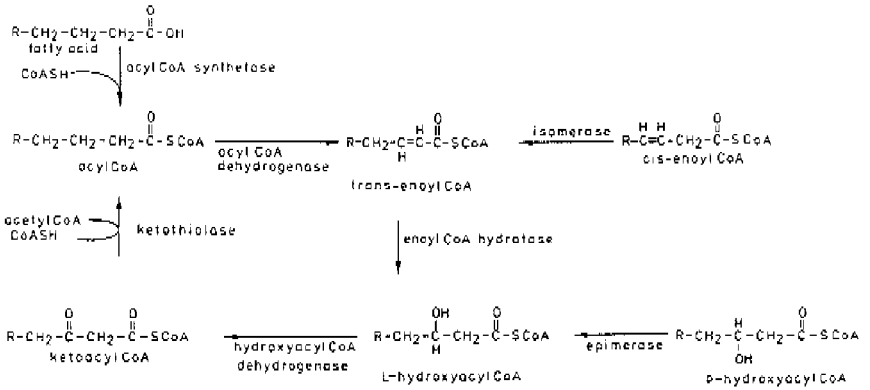

Fig.II.1 Fatty acid degradation in *E.coli* by β-oxidation.

Close examination of the gene-protein index published by Neidhardt et al. (4) revealed that exactly the same protein spots appeared after two-dimensional gel-electrophoresis of an *E.coli* extract, either containing plasmid pLC30-32 or pLC30-20. The presence of the *E.coli* recA marker on pLC30-20 was reported as well. As recA is located at 58 min. on the *E.coli* gene map (10), the distance from fadD is too large for both genes to be present on a single contiguous DNA fragment. The plasmids mentioned above were requested from the *E.coli* Genetic Stock Center and screened for the presence of pabB and/or fadD. The *E.coli* pabB gene is shown to be located on pLC30-32, whereas fadD is shown to be located on pLC30-32 as well as pLC15-17. Both genes were subcloned from the respective Clarke & Carbon plasmids and enzyme activities of the gene products determined after introduction into pabB- or fadD-mutated *E.coli* strains.*

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II.2. Materials and methods.

II.2.1. *E.coli* strains.

K27[1] CGSC 5478 F⁻/fadD88,mel-1,supF58 (5,11).
DC369[1] CGSC 6355 F⁻/zdj-225::Tn10,fadR16,butD12,mel-1,supF58 (12).
AB3292[1] CGSC 3292 F⁻/proA2,hisG4,pabA1,ilvC7,argE3,thi-1,lacY1, galK2,xyl-5,mtl-1,strA704,tfr-3,tsx-358,supE44 (13).
AB3295[1] CGSC 3295 F⁻/pabB3,his-4,ilvC7,argE3,thi-1,xyl-5,mtl-1, rpsl704,supE44 (13).
AB3303[1] CGSC 3303 F⁻/thi-1,pabB3,hisG4,argE3,lacY1,galK2,xyl-5, mtl-1,strA700 or strA704,tsx-29 or tsx-358,supE44 (13).
JA200[1] F⁺/deltrpE5,recA1,thr,leuB6,lacY, harbouring Clarke & Carbon plasmid pLC4-21, pLC15-17 pLC15-32, pLC30-20 or pLC30-32 (1,2,3,9,14).
PC1539[2] (=AN4) F⁻/pabB,argE,ilvC,pheA,tyrA,trp,lac,gal,tsx, (lam),phx,strA (15).
PC1550[2] CGSC 3294 F⁻/pabB,thi,ilvC,argE,proA,his,lacY,gal,xyl, mtl,tsx,phx,(lam),strA (13).
PC1551[2] F⁻/pabB,his,lacY,gal,xyl,mtl,tsx,phx,(lam), strA (13).
PC2247[2] F⁻/met,(lam),strA,phx, harbouring plasmid $pColE_I$.
W3110[3] F⁻/wildtype.

1 = obtained from B. Bachmann, *E.coli* Genetic Stock Center, Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut, U.S.A.
2 = obtained from Phabagen Collection, Department of Molecular Cell Biology, University of Utrecht, The Netherlands.
3 = obtained from P. Andreoli, Gist-Brocades, Delft, The Netherlands.

II.2.2. Media.

The rich medium used for routine subculture and growth was L-broth supplemented with 2 gr/l glucose (16,17). Minimal medium used was M9 (16) supplemented with 2 gr/l glucose, vitamins and individual amino

acids according to (18). Streptomycine (200 µg/ml), tetracycline (10 µg/ml), colicin E_I (own preparation, see below) and p-aminobenzoic acid (137 µg/l) were supplemented when required. In case of selection of the $fadD^+$ phenotype glucose was replaced with 5 mM oleate from a 1 M stock-solution in 10% Brij-58 neutralized with KOH and filter sterilized before use. Media were solidified with 15 gr/l agar when required.

II.2.3. Complementation assay.

Colonies of the Clarke & Carbon gene bank were used as donor strains in a conjugation experiment with PC1539, PC1550, PC1551, AB3292, AB3295, AB3303 and K27 as recipient cells essentially according to Miller (16). 500 µl samples were taken after maximally 25 min. and 120 min. of incubation, washed twice with 1 volume M9 minimal medium and 100 µl plated on pabB- or fadD-selective media. Colicin E_I immunity was tested by spotting 10 µl of a colicin E_I preparation on L-agar plates containing 100 µl of a suspension of exconjugants followed by incubation overnight at 37°C.

II.2.4. Colicin E_I preparation.

Colicin E_I was prepared from *E.coli* strain PC2247 essentially as in (19), with following modifications: resuspended cells were extracted three times in 0.1 M potassium phosphate buffer pH 7.0 containing 1 M NaCl, by vortexing 30 min. in an ice bath followed by centrifugation of cells. The pellet of the 40% - 60% saturated $(NH_4)_2SO_4$ fraction was suspended in 0.1 M potassium phosphate buffer pH 7.0 and sterilized by UV-irradiation at 254 nm. Contamination of remaining PC2247 cells was detected by plating 10 µl samples on L-agar plates. Wild-type *E.coli K12* strain W3110 was used as indicator-strain to determine the titer of the colicin E_I preparation by plating cells together with 10 µl spots of diluted colicin E_I stock solution. This final preparation could be diluted 10^2- to 10^3-fold to be used in selective media. Usually, the 40% - 60% $(NH_4)_2SO_4$ supernatant fraction contained enough colicin E_I to allow for 10-fold dilution, and was used in routine plating also.

II.2.5. DNA manipulations.

Since the original technique described by Clarke & Carbon in (2) did not give satisfactory results, hybrid pColE$_I$ plasmid DNA was prepared as described by Clewell & Helinski (20), without heat treatment of cells at 70°C before lysis.

Restriction endonuclease digestions and ligation of DNA restriction fragments with T_4 DNA-ligase were carried out in the buffers recommended by the suppliers. After incubation for 16 hours at 15°C, ligation mixtures were used directly for transformation into competent *E.coli* cells as described in (21).

II.2.6. p-Aminobenzoate synthase assay.

Cultures used for the preparation of cell extracts were grown in M9 supplemented medium and harvested during late log-phase of growth by centrifugation. Cells were washed once with 0.9 % saline and pellets were weighed. Disintegration of cells was performed by alumina grinding as in (22) by adding 0.5 gram Al_2O_3 for each gram (wet weight) of cells followed by grinding in a mortar and pestle during 3 min. at 4°C. To the resulting paste 4 ml of buffer 50 mM KPi/0.2 mM DTT/0.1 mM Na_2EDTA pH 7.6 were added for each gram of cells, followed by centrifugation at 30,000 g for 30 min. to remove cell debris. The supernatant was used for assay of PABS activity.

The standard reaction mixture for enzymatic synthesis of PABA contained 50 mM Tris/HCl pH 8.0, 10 mM $MgCl_2$, 2 mM DTT, 33 mM $(NH_4)_2SO_4$ or 50 mM L-glutamine, 0.2 mM chorismic acid and crude cell-free extract (containing 1 to 2 mg protein) in a final volume of 1 ml, as in (15). After incubation at 37°C the assay mixture was acidified with 0.1 ml 2 N HCl and PABA extracted into 3 ml ethylacetate. The concentration of PABA in the organic phase was determined fluorometrically (excitation 296 nm; emission 340 nm) on a Perkin Elmer 204 spectrofluorometer. A standard curve was made after extraction of 0.002 to 100 µM PABA from assay buffer into ethylacetate by the same procedure. Protein was measured according to Lowry et al. (23) with bovine serum albumin as standard.

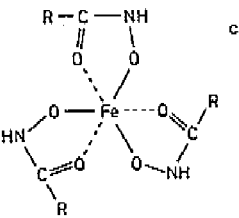
II.2.7. AcylCoA synthetase assays.

Cultures used for the preparation of cell extracts were grown in M9 supplemented medium and harvested after at least 2 days of incubation at 37°C, i.e. in stationary phase of growth. Cells were washed with 50 mM KPi buffer pH 7.5 before lysis. Cell-free extracts were prepared either by grinding as described above or in a French pressure cell (Aminco) at 15,000 psi. Cell debris was removed by centrifugation at 30,000 g for 30 min. The supernatant was used for assay of acylCoA synthetase activity by three different procedures.

(i) acylCoA synthetase assay according to Hestrin (24). This method is based on indirect assay in which the following reactions proceed:

I. Oleate + CoASH + ATP $\xrightarrow[\text{synthetase}]{\text{acylCoA}}$ Oleoyl-CoA + AMP + PPi

II. $R-\overset{O}{\overset{\|}{C}}-SCoA + NH_2OH \longrightarrow R-\overset{O}{\overset{\|}{C}}-\overset{H}{N}-OH + CoASH$

III. $R-\overset{O}{\overset{\|}{C}}-\overset{H}{N}-OH + Fe^{3+} \longrightarrow$  coloured complex

A 1.5 ml reaction mix contained: 0.40 ml 0.1 M NaF, 0.10 ml 7 % (v/v) Triton-X-100, 0.50 ml 0.08 M Oleic acid, 0.10 ml 0.2 M $MgCl_2$, 0.10 ml 0.4 M 2-mercaptoethanol, 0.20 ml 1.25 M Tris/HCl pH 8.5, 0.05 ml 1.6 mM CoASH stock solution, and 0.05 ml 0.2 M ATP. To this reaction mix cell-free extract was added, followed by incubation at 37°C during 1 hour. Then 1 ml hydroxylamine and 1 ml 1 M NaOH were added. About 1 min. after adding NaOH, concentrated HCl was added until pH 1.0 was obtained (about 1 ml). Adding 1 ml of Fe-reagens (0.2 M Fe in concentrated HCl) produced the coloured complex, the extinction of which was measured at 540 nm in a spectrofotometer.

(ii) acylCoA synthetase assay according to a protocol supplied by Sigma Chemical Company, based on the method of Spector et al. (25).

Like the method of Hestrin, this method is based on an indirect assay also, and following reactions proceed:

$$\text{I.}\quad \text{Oleate} + \text{ATP} + \text{CoASH} \xrightarrow[\text{synthetase}]{\text{acylCoA}} \text{Oleoyl-CoA} + \text{AMP} + \text{PPi}$$

$$\text{II.}\quad \text{ATP} + \text{AMP} \xrightarrow{\text{myokinase}} 2\ \text{ADP}$$

$$\text{III.}\quad 2\ \text{ADP} + 2\ \text{PEP} \xrightarrow[\text{kinase}]{\text{pyruvate}} 2\ \text{ATP} + 2\ \text{Pyruvate}$$

$$\text{IV.}\quad 2\ \text{Pyruvate} + 2\ \text{NADH} \xrightarrow[\text{dehydrogenase}]{\text{lactic}} 2\ \text{Lactate} + 2\ \text{NAD}^+$$

A 2.4 ml reaction mix contained: 2.00 ml assay buffer (0.2 M Tris/HCl pH 8.1/20 mM $MgCl_2$/1.8 mM Na_2EDTA/0.25% (v/v) Triton-X-100), 0.05 ml 14.5 mM ATP, 0.05 ml 42.8 mM Phosphoenolpyruvate, 0.05 ml 100 units/ml Myokinase solution, 0.05 ml Pyruvate kinase/Lactic dehydrogenase solution (1:5 dilution of stock containing 700 units/ml PK and 1000 units/ml LDH), 0.10 ml 49 mM CoASH and 0.10 ml 3.75 mM NADH. After equilibration to 25°C, 0.20 ml cell-free extract, containing acylCoA synthetase, was added and extinction at 340 nm monitored until reading remained constant (3-4 minutes). Then 0.20 ml 0.098 mM sodium oleate in 0.25% (v/v) Triton-X-100 was added, immediately mixed and the decrease in extinction at 340 nm recorded for approximately 10 minutes. The E_{340}/min. was obtained and unit/ml concentration determined (millimolar extinction coefficient of NADH=6.22).

(iii) acylCoA synthetase assay according to Bar-Tana et al. (26). This method is based on direct assay of 1-[^{14}C]-lauroyl-CoA formation from 1-[^{14}C]-labeled lauric acid as substrate. To 100 µl cell-free extract 22.5 µl 5 x assay buffer (12.5 mM ATP/40 mM $MgCl_2$/10 mM Na_2EDTA/100 mM NaF/0.5% (v/v) Triton-X-100/50 µM 1-[^{14}C]-lauric acid (59 Ci/µmol) in 0.2 M Tris/HCl pH 7.5) was added. Reaction was started by the addition of 2.5 µl 25 mM CoA in 0.2 mM Tris/HCl pH 7.5. Following incubation at 37°C during 10 min. the reaction was stopped by adding 625 µl isopropanol/n-heptane/1 M H_2SO_4 (40:10:1 (v/v)) and 125 µl 10% trichloroacetic acid. The mixture was extracted 4 times with n-heptane (625 µl portions). Radioactive 1-[^{14}C]-lauroylCoA in the water layer

was determined in a liquid scintillation counter. Protein was measured according to Lowry et al. (23) with bovine serum albumin as a standard.

II.3. Results and discussion.

II.3.1. F-mediated transfer.

Before using the *E.coli* strains listed above, all genetic markers relevant to this research were checked. No anomalies were detected in the expected phenotypes. Colonies of the Clarke & Carbon gene bank were screened for their ability to complement a pabB mutation or fadD mutation by F-mediated transfer to recipient cells as shown in Fig.II.2. Mating progeny were analyzed for the acquisition of colicin E_I immunity concomitant with pabB- or fadD-prototrophy. When prototrophy was established, colicin E_I immunity emerged simultaneously in almost all exconjugants, showing that the recombinant plasmid was responsible for the introduction of the pabB or the fadD marker, and that prototrophy was not a result of rare Hfr formation in the donor strains. To prevent the latter, time of conjugation was made short (25 min.). Also, after 120 min., samples were taken and exconjugants tested for the presence of the nearby his marker on 44 min. of the *E.coli* genome, in case AB3303 was used as a recipient. His prototrophs were not detected, so Hfr formation could be excluded. From the number of colonies restored to PABA prototrophy without obtaining colicin E_I immunity, the number of spontaneous revertants was established to be $1/2.10^{-8}$ (including AB3292, the pabA mutated strain).

From Fig.II.2 the following conclusions can be drawn: (i) contradictory to (9), not all Clarke & Carbon plasmids used here were able to complement the fadD phenotype, instead only pLC15-17 and pLC30-32 restored prototrophy in strain K27. (ii) pLC30-32 restored prototrophy in all pabB mutated strains, but not in the pabA mutated strain. Therefore, only pLC30-32 contains the pabB gene. (iii) pLC30-20 neither complements pabB nor fadD mutations, so the protein spots reported in (4) can not be ascribed to p-aminobenzoate synthase Component I or acylCoA synthetase.

Recipient Donor	AB3292 pabA	AB3295 pabB	AB3303 pabB	PC1539 phe,tyr trp,pabB	PC1550 pabB	PC1551 pabB	K27 fadD
JA200/pLC4-21	-	-	-	-	-	-	-
JA200/pLC15-17	-	-	-	-	-	-	+
JA200/pLC15-32	-	-	-	-	-	-	-
JA200/pLC30-20	-	-	-	-	-	-	-
JA200/pLC30-32	-	+	+	+	+	+	+

Fig.II.2 F-mediated transfer of Clarke & Carbon plasmids to recipient cells
+ = recipient cells growing on selective medium. PABA-independent growth was judged after 48 hours of incubation. Growth on oleate as carbon source was judged after 70 hours of incubation.

When PABA-independent growth was examined, extensive crossfeeding was noticed upon prolonged incubation, due to cells growing on PABA synthesized and excreted by pLC30-32 containing pabB mutant strains (Fig.II.3).

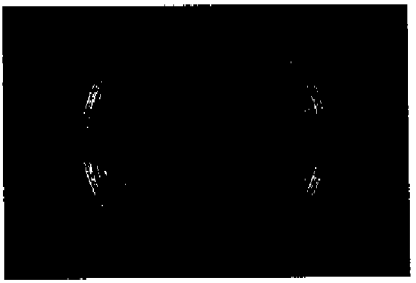


Fig.II.3
Crossfeeding by pLC30-32 containing PC1539 cells.

Since strain PC1539 is the only pabB mutant strain which is also mutated in phe, tyr and trp, i.e. it is a triple aromatic auxotroph, chorismate can no longer be channeled into the respective amino acid

pathways, and should be readily available for the folate pathway. However, upon introduction of pLC30-32, the growth rate of this strain did not increase as compared to other pabB mutated strains. In fact, AB3303/pLC30-32 had the highest growth rate, although still not matching that of wildtype cells growing on minimal medium. Possibly, irrespective of the amount available, only a small part of the chorismic acid pool is actually being used for vitamin synthesis.

The growth rate of fadD mutated strain K27 was even lower than pabB mutated strains upon restoring prototrophy, and selective plates could only be judged after at least 3 days of incubation. Most likely this is due to the fact that oleate used as carbon source has to be taken up by active transport using the fadL gene product (27,28); also, the fadD gene product itself, acylCoA synthetase, is proposed to be required for a group translocation step in transport (vectorial acylation) (Fig.II.4)(11,29,30) and expression of fadD is under control of the fadR gene product, a repressor (8,31). Induction of fadD expression is triggered by growth on exogenous fatty acids and expression is subject to catabolite-repression when glucose is present as carbon source.

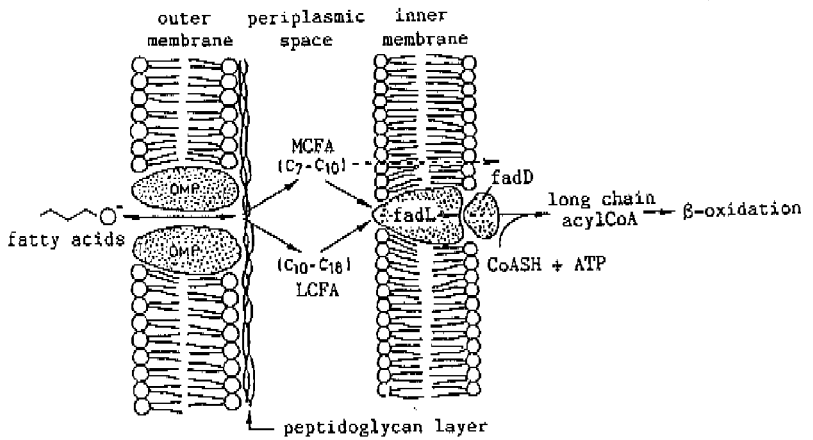


Fig.II.4 Long chain fatty acid transport in *E.coli*.
OMP = outer membrane protein; MCFA = medium chain fatty acids; LCFA = long chain fatty acids; fadL = long chain transport component; fadD = acylCoA synthetase.

Strain DC369, being mutated in fadR, produces acylCoA synthetase constitutively, due to the lack of repressor molecules. When compared to DC369, growth behaviour of K27/pLC30-32 was indistinguishable, whereas K27/pLC15-17 grew very poorly on selective medium. Since the fadR gene is intact in K27, this suggested a gene dose effect of fadD upon introduction of pLC30-32, which makes induction of fadD expression not necessary any more. Apparently, in K27/pLC15-17 the plasmid copy number is low, since induction has to proceed first, before growth can be resumed. This assumption was confirmed when preparative isolation of pLC15-17 from this strain failed to give the relatively high amount of plasmid isolated from K27/pLC30-32.

II.3.2. Restriction analysis of pLC30-32 and subcloning of pabB.

Plasmid pLC30-32 was isolated from JA200/pLC30-32 by gentle lysis using N-lauroylsarcosine, in order to avoid induction of relaxation to open circular plasmid form during preparation, as described in (20). Analysis of the plasmid DNA preparation on 0.8 % agarose gel showed a total length of 25 kb; pColE$_I$ itself is 6.3 kb in length, so the insert of chromosomal DNA measures 18.7 kb.

Strains PC1539 and AB3303 were used for transformation with purified pLC30-32. Again prototrophy was restored, the efficiency being about 1.10^4 cells transformed per µg plasmid. The somewhat lower transformation efficiency than the normally observed value of 1.10^6 cells per µg might be caused by the relatively large size of the plasmid or possible nicking to the open circular form during the transformation procedure.

Digestion of pLC30-32 with BamHI followed by ligation into BamHI digested pBR327 resulted into 39 colonies of PABA-independent PC1551 cells upon transformation and selection on minimal agar plates. When PstI was used in the same procedure, no colonies were detected. One of the PABA-independent, ampicillin resistant, tetracycline sensitive PC1551 colonies was picked and grown in rich medium. Recombinant pBR327 plasmid was then isolated and called pHK9. Comparison of BamHI, PstI, HindIII, or EcoRI digested pHK9 with λ-digested marker fragments on agarose gel showed the subcloned insert to be about 9 kb in length. Surprisingly, the BamHI digest of pHK9 did not result in the two

fragments expected (insert and vector). Instead, pHK9 was just linearized to one fragment of 12.3 kb, as was the case in the EcoRI and HindIII digests of pHK9. The PstI digest gave fragments of 5.3 kb, 5.1 kb, 1.1 kb and 0.8 kb. From these results it was concluded, that (i) one BamHI-site in pHK9 was lost during cloning, (ii) on the 9 kb pabB-containing insert there is no other BamHI, HindIII or EcoRI site (the latter two being unique in pBR327), (iii) the 9 kb insert must contain 3 PstI sites, since one is in pBR327, (iv) the 1.1 and 0.8 kb PstI fragments consist of insert DNA only, whereas the 5.1 and 5.3 kb fragments consist of partly vector, partly insert DNA, as pictured in Fig.II.5.

Double digestion of pHK9 with BamHI plus EcoRI or BamHI plus HindIII resulted in fragments of 11.9 and 0.4 kb. If the left hand BamHI site in Fig.II.5 was lost, fragments of 9.4 and 2.9 kb would have been found. This implies, that the right hand BamHI site is lost in pHK9.

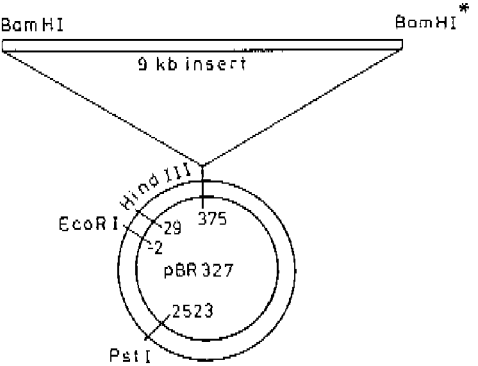


Fig.II.5
Recombinant plasmid pHK9 = plasmid pBR327 (3273 bp) containing a 9 kb insert from pLC30-32. Total length 12.3 kb. The insert contains 3 PstI sites, but no EcoRI or HindIII sites.
* = BamHI site lost (see text).

Plasmid pLC30-32 was digested with several enzymes also. The BamHI digest of pLC30-32 showed linearization of the plasmid, so it was concluded, that in fact not more than one BamHI site exists in pLC30-32. The 9 kb insert in pHK9 therefore is the result of ligation

of linearized pLC30-32 to the left hand pBR327 BamHI sticky-end, breakage of pLC30-32 (9 kb away from this site) and repair of the broken end to the right hand pBR327 BamHI sticky-end.

From this point on, only the results of pLC30-32 restriction analysis are described here, since pHK9 sites can be deduced from it and actual pHK9 digests were in complete agreement with deduced sites.

The Clarke & Carbon plasmids were originally constructed after digestion of $pColE_I$ with EcoRI, and ligation of chromosomal fragments by A-T-tailing. Therefore, the unique EcoRI site no longer exists in the recombinant plasmids. $pColE_I$ itself contains two PstI sites and one SmaI site. Results of digestion of pLC30-32 with BamHI, EcoRI, HindIII, PstI, SalI and SmaI, taken together with the known location of the SmaI site and PstI sites in $pColE_I$, allowed deduction of the complete map of pLC30-32, as shown in Fig.II.6.

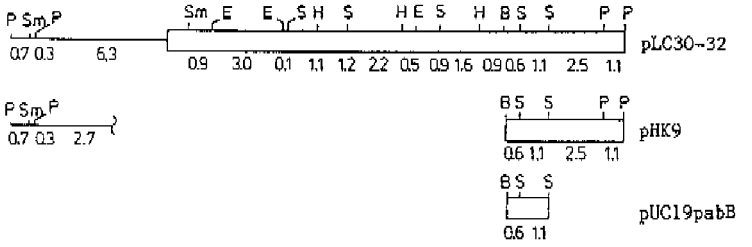


Fig.II.6 Restriction maps of pLC30-32, pHK9 and pUC19pabB. P = PstI; Sm = SmaI; E = EcoRI; S = SalI; H = HindIII and B = BamHI. Vectors pBR327 and pUC19 not shown.

From Fig.II.6 it follows, that the insert present in pHK9 contained 5.3 kb of *E.coli* chromosomal DNA and 3.7 kb of $pColE_I$ DNA. Because of this, search for the location of pabB was thereby limited to this 5.3 kb fragment. Since only two SalI sites and one PstI site are present, pHK9 was digested first with BamHI, then partially as well as completely with either SalI or PstI. Resulting fragments were ligated into suitably digested pUC19 and used for transformation into AB3303. The smallest fragment cloned restoring PABA prototrophy originated from a BamHI/SalI partial digest. Complete digestion with SalI after BamHI apparently did not leave the pabB gene intact. The length of the BamHI/SalI fragment measured 1.7 kb and was not digested any further,

since the expected length of the coding region was calculated to be about 1.4 kb, from the reported molecular weight of 48,000 D (15). Using pUC19 HindIII and EcoRI polylinker sites, the 1.7 kb pabB containing fragment was then transferred to M13mp10 and M13mp18 for sequencing and in vitro mutagenesis.

II.3.3. Restriction analysis of pLC4-21, pLC15-17 and pLC15-32.

In order to extend the restriction mapping of the *E.coli* 40 min. region as well as to deduce the location of the fadD gene, plasmids pLC4-21, pLC15-17 and pLC15-32 were isolated and digested with the same restriction enzymes as pLC30-32. Single and double digests resulted into the maps as shown in Fig.II.7.

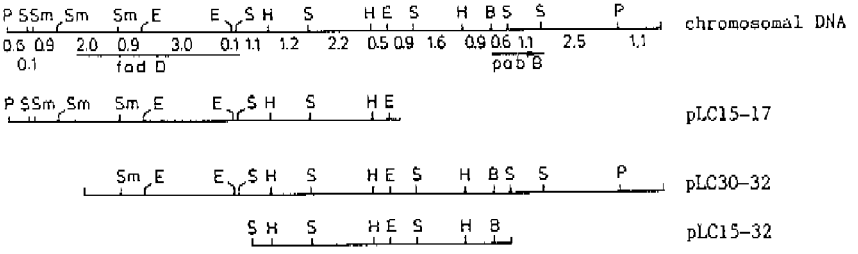


Fig.II.7.A
Restriction maps of pLC15-17, pLC30-32, pLC15-32 and the chromosomal DNA 40 min. region deduced. ⟶ = direction of transcription. P = PstI; S = SmaI; E = EcoRI; S = SalI; H = HindIII and B = BamHI. I Only insert DNA is shown.

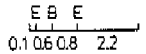


Fig.II.7.B
Restriction map of pLC4-21. E = EcoRI and B = BamHI. Only insert DNA is shown.

It can be seen, that pLC15-17, pLC30-32 and pLC15-32 contain an overlapping region of about 5 kb and that pLC4-21 does not overlap any of them at all.

Plasmids pLC15-17 and pLC15-32 both overlap with pLC30-32. From the results of F-mediated transfer into K27 it was known, that pLC15-17 and pLC30-32 contain the fadD gene, whereas pLC15-32 does not. This means that the fadD gene must be looked for on the overlapping region common to pLC15-17 and pLC30-32, subtracting the region shared with pLC15-32, which leaves a fragment of maximally 4.8 kb as indicated in Fig.II.7.

Plasmid pLC30-20 was not used for restriction analysis, since it was not mentioned in literature as a candidate for carrying the fadD gene, nor was the presence of either fadD or pabB detected by F-mediated transfer (Fig.II.2). The origin of the pLC30-20 insert remains unclear: the recA gene gives rise to a protein spot with well defined coordinates in 2-D electrophoresis, which could be detected upon expression of at least six other Clarke & Carbon plasmids; although pLC30-20 is reported to contain recA, the corresponding protein is not on the list of 9 proteins expressed. On the other hand, these 9 proteins exactly match those from pLC30-32, without pLC30-20 carrying fadD or pabB.

II.3.4. Subcloning fadD from pLC15-17.

From the complementation study and restriction analysis of Clarke & Carbon plasmids described in this chapter, the possible location of the *E.coli* fadD gene coding for acylCoA synthetase could already be reduced to a fragment of maximum 4.8 kb length (Fig.II.7). The molecular weight of the enzyme was found to be 47,000 D (33), so the length of the fadD gene will be about 1.4 kb. Attempts to delineate fadD more precisely on the 4.8 kb fragment were made by two different approaches, either by removing DNA fragments out of the pLC30-32 insert followed by religation (leaving the $pColE_I$ vector intact) or by subcloning fragments from the pLC15-17 insert into pUC19 as a new vector, as summarized in Fig.II.8.

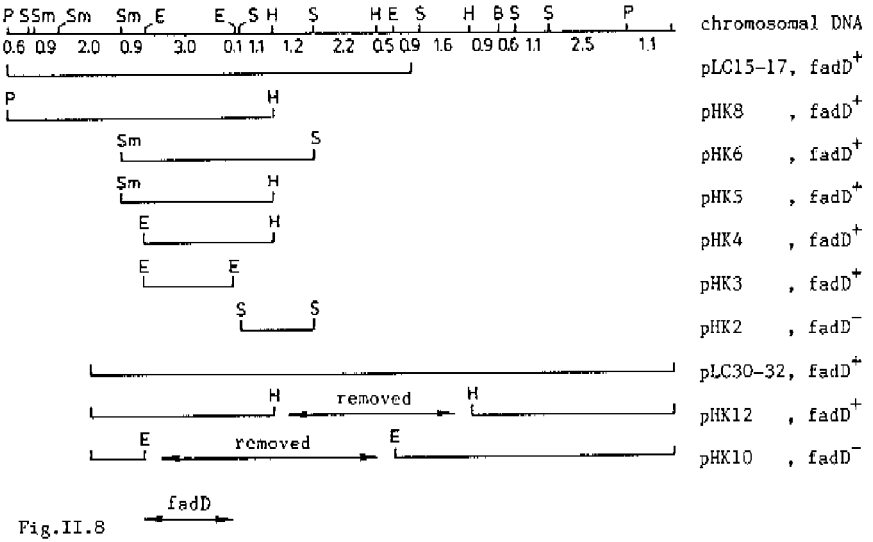


Fig.II.8

Deduction of localisation of fadD. Inserts shown at pHK2, pHK3, pHK4, pHK5, pHK6 and pHK8 were cloned into pUC19. Inserts shown at pHK10 and pHK12 were retained in pColE$_I$; fragments removed by HindIII or EcoRI digestion are indicated.

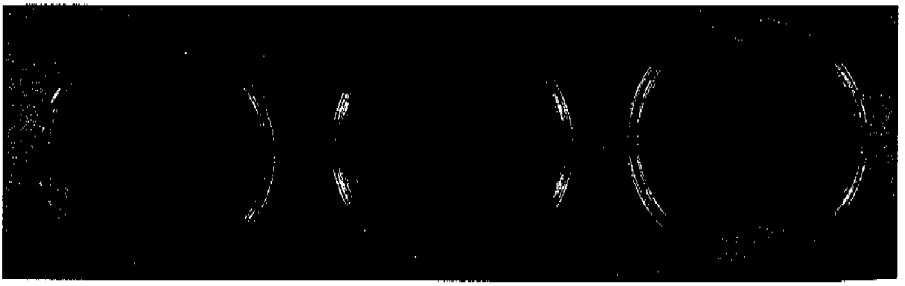
The copy number of pUC19 was expected to be higher than that of pLC15-17 in strain K27, and it was hoped for a more explicit detection of acylCoA synthetase activity either using biological complementation or using enzymatic assay.

Common to all recombinant plasmids tested was the fact that detection of acylCoA synthetase activity by biological complementation was nevertheless more difficult than the clear acylCoA synthetase activity detected in K27/pLC30-32 and DC369, despite the fact that the copy number of all plasmids was assumed not to be less than that of pLC30-32 in strain K27.

In Fig.II.9, results of growth on oleate-containing minimal medium is shown for strains DC369, K27/pHK5 and K27/pHK2.

As indicated in Fig.II.8, all plasmids complemented the fadD mutation except pHK2 and pHK10. Therefore, the fadD gene is present on the smallest common fragment cloned, i.e. the one in pHK3. Since this fragment still measures 3 kb, an attempt was made to further minimize its length. A colony of K27/pHK3 was picked from oleate plates and

cultured in Luria followed by preparative plasmid isolation. Upon analysis of the plasmid preparation on agarose gel it was found that the 3 kb insert had spontaneously deleted to a fragment of 1.7 kb, since pHK3 was only linearized by EcoRI digestion.



DC369	K27/pHK5fadD$^+$	K27/pHK2
fadR$^-$	fadR$^+$	fadR$^+$
fadD$^+$	fadD$^-$	fadD$^-$
constitutive	inducable	mutated

Fig.II.9
Growth on oleate containing minimal medium.

Because at that time there were no other restriction sites known in the 3 kb EcoRI fragment in pLC15-17, other restriction endonucleases were used to digest pHK3 in order to find one that digests the 1.7 kb insert. HpaI was found to linearize pHK3 without cutting into pUC19, and therefore the 1.7 kb insert contains one HpaI site. pLC15-17 was analysed for the location of HpaI sites, of which three were present. Fig.II.10 shows the updated restriction map of pLC15-17 and deleted pHK3. The HpaI site common to both plasmids is 0.1 kb away from one of the EcoRI sites and therefore the 1.7 kb insert in pHK3 could be positioned on the map of pLC15-17 as indicated.

The results of locating fadD by restriction analysis and complementation described here are consistent with the less accurate method of calculating distances from co-transduction frequencies, summarized in Fig.II.11 (8,33,34,35).

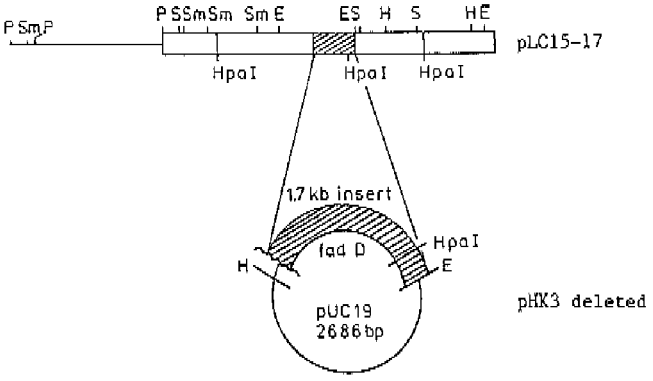


Fig.II.10 Updated restriction map of pLC15-17 and deleted pHK3. P = PstI; Sm = SmaI; S = SalI; E = EcoRI and H = HindIII.

From Fig.II.11, the distance between pabB and fadD can be estimated to be about 0.06-0.17 min., whereas restriction analysis showed the distance between pabB and fadD to be 8.5 kb, i.e. 0.21 min. No further subcloning was performed from this point on, but pHK3 was used for enzymatic assays described in section II.3.6.

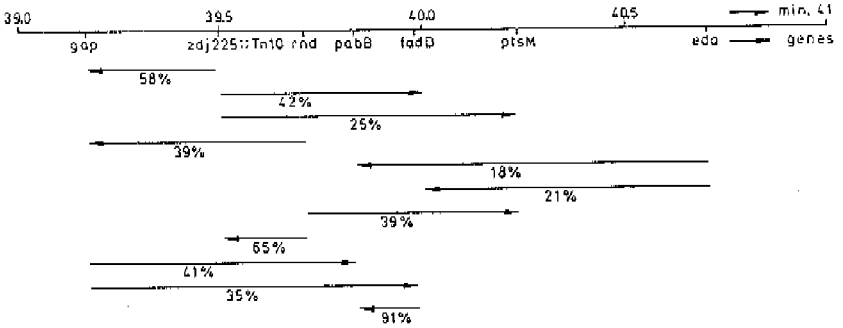


Fig.II.11 Co-transduction frequencies of *E.coli* genetic markers surrounding the 40 min. region. Frequency = $(1-{}^{d}/_{2})^{3}$, d = distance in minutes.

II.3.5. Enzymatic assay of p-aminobenzoate synthase.

p-Aminobenzoate synthase activity was determined in cell-free extracts prepared by a grinding technique. The efficiency of this technique was checked by comparing the protein concentration of cell-free extracts with that obtained in (15), in which a French pressure cell or Sorvall Ribi cell disintegrator was used. On average, 14 to 15 mg/ml was extracted as compared to 13 to 16 mg/ml in (15). Also, enzyme activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assayed as a reference. GAPDH activity was found to be comparable with GAPDH activities obtained by different disintegration techniques reported in literature.

In Table II.1, results of PABS assays are summarized. First, it can be concluded, that glutamine-dependent PABS activity in pabA mutated strain AB3292 and in pabB mutated strain PC1539 are below detection limit, as expected. Secondly, PABS activity in PC1539/pLC30-32 and PC1539/pHK9 are both in the range of 10-12.5 pmol/min./mg. This means that if transcription and translation are of the same efficiency in both strains, then copy numbers of both recombinants are comparable.

	stored °/n -20°C	PC1539 extract ($pabA^+$) added	Vmax pmol/min./mg.
AB3292 ($pabA^-$,$pabB^+$)	+	-	< 0.8
PC1539 ($pabA^+$,$pabB^-$)	+	-	< 0.8
PC1539/pLC30-32	+	-	11.5
PC1539/pHK9	+	-	10 and 12.5
PC1539/pHK9	+	+	35
PC1539/pHK9	-	-	58.4
PC1539/pUC19pabB	-	+	111
AN1 ($pabA^+$,$pabB^+$, * phe,tyr,trp)	-	-	125 - 150
AN3 ($pabA^-$,$pabB^+$) *	-	+	37.5

Table II.1 Amidotransferase assay of PABS in a number of *E.coli* strains (glutamine-dependent PABA production). * = reported in (15).

Thirdly, it was noticed, that immediate assay upon preparation of cell-free extract gave a raise in PABS activity by a factor of 5, mounting up to 58.4 pmol/min./mg. Therefore, storage overnight at -20°C reduces the activity of the holo-enzyme to about 20%, possibly due to instability of the pabA gene product, CoII, which is essential for the glutamine-dependent activity and was noticed to be more unstable than CoI (15). Fourthly, adding fresh extract of PC1539 cells, containing CoII only, raises PABS activity by a factor of 3 (going from 10-12.5 to 35 pmol/min./mg in PC1539/pHK9) when stored CoI extract is used, and raises PABS activity by a factor of 2 (going from 58.4 to 111 pmol/min./mg in the extracts of PC1539/pHK9 and PC1539/pUC19pabB) when freshly prepared CoI extract is used. In the triple aromatic auxotroph, AN_1, which is wild-type for both pabA and pabB, a value of 125-150 pmol/min./mg is reported in cell-free extracts. On the other hand, when reconstructing PABS holo-enzyme from extracts of AN_3 (pabA mutant) and PC1539 (pabB mutant) a value of 37.5 pmol/min./mg was reported (15). The value of 58.4 pmol/min./mg found with PC1539/pHK9 therefore indicates a possible gene dose effect due to cloning of the gene coding for CoI. This is confirmed by the raise in PABS activity to 111 pmol/min./mg when freshly prepared extract of CoII is added, since apparently not all CoI subunits were aggregated to holo-enzyme due to a shortage of CoII (present only once on the PC1539 genome).

The results of a glutamine-dependent assay in which time of incubation was varied is shown in Fig.II.12.

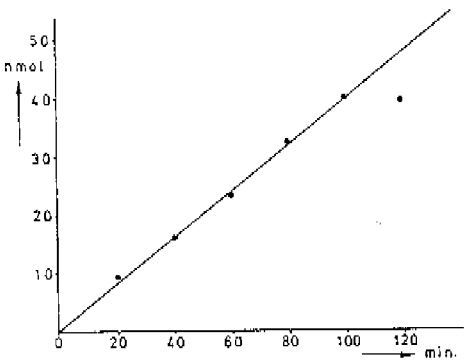


Fig.II.12
Glutamine-dependent assay of PABS in cell-free extract of PC1539/pHK9. No component II added. Extract used directly.

It was concluded, that the holo-enzyme is stable up to 100 min. of incubation before non-linearity appears.

II.3.6. Enzymatic assay of acylCoA synthetase.

Attempts to detect the presence of acylCoA synthetase activity in cell-free extracts by the method of Hestrin et al. (24) were not successful, since omitting extract, ATP or CoASH still produced a coloured reaction complex. Therefore all other components in the reaction mix were omitted one by one. This experiment showed β-mercaptoethanol to be capable of producing the brownish colour by itself without acylCoA synthetase activity. β-Mercaptoethanol was replaced with DTE and extracts were assayed again. Although the expected coloured complex was formed using either ethylacetate (without enzyme) or purified acylCoA synthetase supplied by Sigma chemical Co. (isolated from *Pseudomonas* species), the method of Hestrin showed to be unreliable. The pH-dependence of the complex E_{540} was such, that even the E_{540} of the reaction using only ethylacetate was hardly reproducible. Assays based on cell-free extracts were not reproducible at all (even an extract of strain K27 alone could raise the colour when pH was slightly changed).

Also the second method tested failed to give useful results. The protocol supplied by Sigma Chemical Co., based on the method of Spector et al. (25) gave satisfactory results when purified acylCoA synthetase was used, but when the indirect NADH oxidation was monitored in the presence of cell-free extracts, results were obscured by the abundancy of other enzymes using NADH as co-enzyme (e.g. GAPDH). Differential spectra were made comparing extracts of K27 and K27/pLC30-32, but expression of acylCoA synthetase was too low to ascribe part of the overall NADH oxidation as being specific to acylCoA synthetase.

The third method tested was based on radioactive substrate, which is removed from the assay mix after the enzymatic reaction, leaving the radioactive product available for detection by liquid scintillation counting. Kameda & Nunn applied this method successfully when they isolated acylCoA synthetase from *E.coli* strain RS3010, which is mutated in fadR and expresses acylCoA synthetase constitutively,

like DC369. They showed that the enzyme has highest affinity for fatty acids with chain lengths of 6 to 18 carbon atoms, and maximum activity was observed with lauric acid. Therefore, we set out to detect acylCoA synthetase in strain K27 transformed with recombinant plasmids, not using 1-[C^{14}]-oleic acid as in complementation studies, but using 1-[C^{14}]-lauric acid as a substrate. Using lauric acid in selective media gave the same results in complementation studies as oleic acid.

As in the method of Spector et al., the commercial acylCoA synthetase preparation purified from *Pseudomonas* species gave the expected amount of 1-[C^{14}]-lauroyl-CoA formation, dependent on the number of enzyme units present in the assay. However, no significant amount of 1-[C^{14}]-lauroylCoA was formed when reaction was based on cell-free extracts of DC369 or K27/pLC30-32, although the exact protocol as published in (32) was followed. The reason for this is not clear, although it might be speculated, that being a membrane associated enzyme, acylCoA synthetase activity might be lost during lysis of cells by grinding (mechanical shearing) or by too high pressure in the French pressure cell. On the other hand, pressure might just as well be too low to set the enzyme free, since e.g. cells of K27 appear different from RS3010 (sphaere vs. rod-shape) when viewed under the microscope, so a different pressure needed to lyse cells can be expected.

Since DC369 expresses acylCoA synthetase constitutively like RS3010 and grows well on selective media, too low expression of the enzyme can not be the reason for the failure of its detection in this assay. Optimalisation of preparing the cell-free extracts by French pressure cell is therefore currently being undertaken.

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Chapter III.

Sequencing of *E.coli* pabB.

III.1 Introduction.

The introduction of the chain termination method for DNA sequencing developed by Sanger et al. (1) has provided a distinct advantage over techniques like the method of Maxam & Gilbert (2), due to its speed and simple performance. Moreover, the use of M13 phage vector (3) is particularly well-suited for sequence analysis, since a single strand template can easily be obtained and a universal primer (4) can be used for all sequencing reactions of DNA fragments cloned into the polylinker region (5,6,7,8).

In recent years, the elaborate synthesis of oligonucleotide primers by the phosphoramidite method (9) has become more convenient with the introduction of automated DNA synthesis. As a consequence, dideoxy sequencing is facilitated, since readily available primers obviate the necessity of cloning several overlapping subfragments (while using the universal primer), in order to establish the sequence of a gene of interest. Instead, the complete gene cloned into M13 phage can serve as a template for sequencing successive regions of at least 200 nucleotides, starting with the universal primer, followed by the use of new primers complementary to the sequence just established.

After cloning into M13mp10, the complete *E.coli* pabB gene was sequenced this way, using a total of 15 oligonucleotide primers.

III.2 Materials and methods.

III.2.1 Preparation of template ss DNA (adapted from (10)).

A fresh overnight culture of strain JM101A ($F^-proA^+B^+$,$lacI^qZ^-$,delM15, traD36/thi,dellac-pro,phx,recA,supE) was diluted 1:100 with "Antibiotic Medium 3" (17.5 g/l Antibiotic Medium 3, premixed, Difco); 2.5 ml of the dilution was transferred to a sterile 15 ml culture tube and infected with 5 µl of M13mp10pabB phage stock (originally prepared from the supernatant of a culture of JM101A cells transformed with

M13mp10pabB RF DNA). After incubation overnight at 37°C 1.5 ml was transferred to an Eppendorf tube, centrifuged during 5 minutes and 1 ml was withdrawn from the supernatant. To this 1 ml 160 µl of 25% (w/v) Polyethyleneglycol 6000 (PEG 6000)/3 M NaCl were added, mixed and left at room temperature during 15 minutes. The mixture was then centrifuged for 30 min. and supernatant removed. To the phage pellet 100 µl 0.01 M Tris/HCl pH 7.5/0.001 M Na_2EDTA (TE) was added and extracted with 50 µl of TE-saturated phenol. After 10 min. at room temperature the mixture was centrifuged for 5 min. and the aqueous phase transferred to a new tube. The aqueous phase was re-extracted with 50 µl TE-saturated phenol/chloroform (1:1(v/v)), followed by extraction with 100 µl chloroform. To 90 µl of the aqueous phase 10 µl 3 M NaAc pH 5.6 and 300 µl ethanol (-20°C) were added and incubated at -20°C for at least one hour. The precipitated ss DNA was then centrifuged 15 min. and the supernatant was removed. The pellet was washed with 1 ml 70% ethanol (-20°C), centrifuged again for 5 min., and supernatant was removed. After drying in a vacuum desiccator the pellet was dissolved in 10 µl TE-buffer and stored at -20°C until use. The typical yield of this procedure was about 2-3 µg of virion DNA.

III.2.2 Oligonucleotide synthesis.

Oligonucleotide primers were synthesized by the phosphoramidite method (9,11), on an Applied Biosystems 381A DNA synthesizer, using β-cyanoethyl-protected phosphoramidites instead of methyl-protected phosphoramidites. The latter require thiophenol for deprotection (12) followed by treatment with concentrated ammonia to cleave the product from the column, whereas β-cyanoethyl-protected oligonucleotides can be deprotected and cleaved from the support with concentrated ammonia only. After deprotection and cleavage at 55°C during 8 hours or overnight incubation, oligonucleotides were precipitated with 0.1 volume of 3 M NaAc pH 5.6 and 3 volumes of ethanol (-20°C). Precipitation was overnight at -20°C. Oligonucleotides were pelleted by centrifugation (15 min. at room temperature), washed with 70% ethanol (-20°C) and dissolved in 100 µl TE. After extraction with 100 µl TE-saturated phenol and extraction with 200 µl diethylether, the concentration of this oligonucleotide stock-solution was measured by

U.V.-spectrofotometry. On average, a 0,2 µmol scale synthesis yielded 0.6-0.8 mg purified 18-mer. In case the oligonucleotide was used for sequencing, the 5'OH terminal end was not phosphorylated. In case it was used for in vitro mutagenesis phosphorylation was performed as described in Chapter IV.

III.2.3 Sequence analysis.

Sequencing of ss DNA was performed essentially as in (1). The sequence established was analysed using a computer program written by Roger Staden, Analyseq. V8.1, which was taken from the CAOS/CAMM (Computer Assisted Organic Synthesis/Computer Assisted Molecular Modelling) facilities of the Dutch National Center for Computer Assisted Chemistry, Catholic University Nijmegen.

III.3 Results and discussion.

Sequencing of the *E.coli* pabB gene cloned into M13mp10 was started using a 17-mer sequencing primer as described in (13): 5'-GTAAAACGACGGCCAGT-3', which is complementary to the M13 phage region immediately downstream its polylinker sequence. After reading about 150 nucleotides, a new primer was synthesized and sequencing was proceeded. At the time this protocol was repeated with a third sequencing primer, Goncharoff & Nichols (14) published the complete *E.coli* pabB sequence. Comparison with the pabB 3' region of M13mp10pabB, from which more than 500 nucleotides had been sequenced, showed no ambiguities. Nevertheless, sequencing of the rest of the pabB gene was continued. In Table III.1, the location and functions of all primers used is shown.

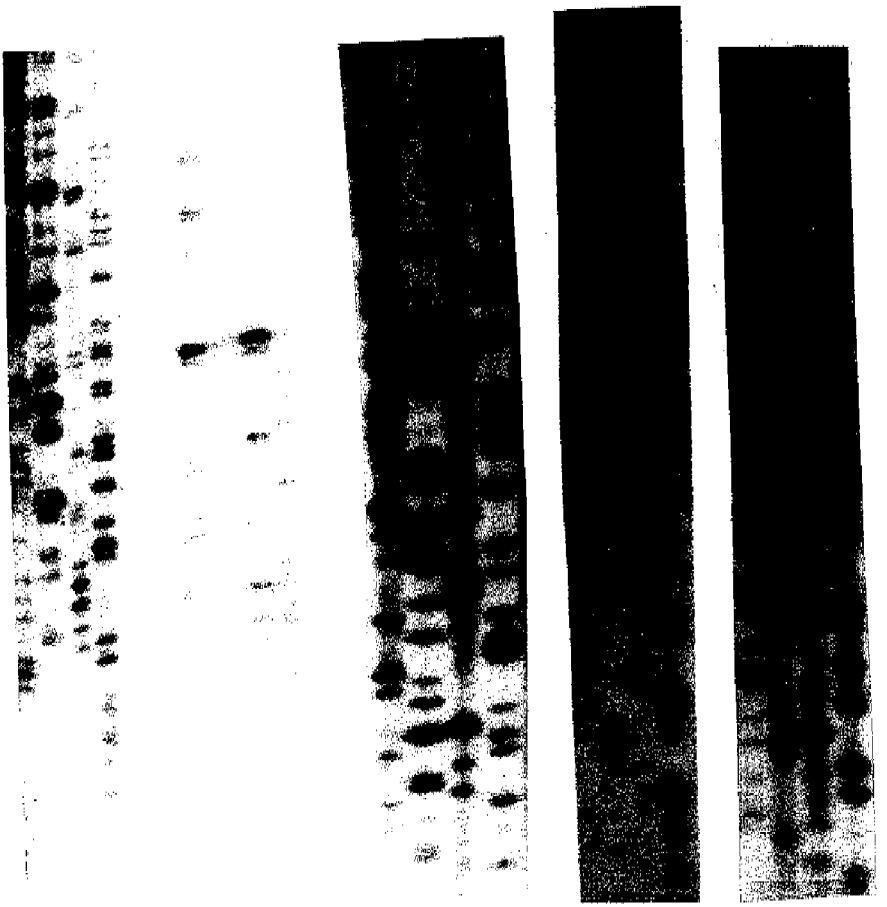
The results of Goncharoff & Nichols offered the opportunity to design sequencing primers that could serve dual purposes. Not only were they designed to function as sequencing primers, but also to function in the in vitro mutagenesis of *E.coli* pabB. Each primer was constructed such, that its position was close to a mutagenesis target site. In this way, candidate M13mp18pabB phages containing a newly formed mutation could be screened directly by sequencing with a primer which on its turn can be used as a mutagenic probe.

Primer	Sequence/Location (5'→3')	Purpose
Commercial 17-mer	^{1524}GTAAAACGACGGCCAGT^{1508}	sequencing until primer 1403
1403	^{1420}GCAAAAGTTGAAAGCCCG^{1403}	sequencing mutation EcoRI 1369 sequencing mutation Lys 449→stop sequencing until primer 1203
EcoRI 1369	^{1381}GGCTACCGA*ATTCCACGT^{1364}	i.v.m. to EcoRI site 1369
Lys 449	^{1353}CACTTCCTA*CAGCATACG^{1336}	i.v.m. Lys 449 (AAG)→stop (TAC)
1203	^{1217}CTGGTATCCATGTTG^{1203}	sequencing until primer 1203 sequencing mutation Cys 391→Ser sequencing mutation Trp 390→Tyr sequencing mutation Trp 390→stop sequencing until primer His 339
Cys 391	^{1179}GCTGCCCCT*CCAGCCATT^{1162}	i.v.m. Cys 391 (TGC)→Ser (AGC)
Trp 390-1	^{1177}TGCCGCACT*AGGCATTGC^{1160}	i.v.m. Trp 390 (TGG)→stop (TAC)
Trp 390-2	^{1177}TGCCGCAA*T*AGGCATTGC^{1160}	sequencing mutation His 339→Asn i.v.m. Trp 390 (TGG)→Tyr (TAT)
His 339	^{1023}GACCAGATT*ATGCACCGC^{1006}	i.v.m. His 339 (CAT)→Asn (AAT)
Pro278/Arg279	^{843}CGGCAGC*CGC*GGTAGCGT^{826}	sequencing mutation Pro278/Arg279 i.v.m. Pro278/Arg279, silent; creates SacII restriction site
Leu214/Ala215	^{651}ACGTTGC*GCA*AGATTCAC^{634}	sequencing mutation Leu214/Ala215 i.v.m. Leu214/Ala215, silent; creates NsiI restriction site
Trp 166	^{505}TTTGCAGA*T*A*ACGCCCGAC^{488}	sequencing mutation Trp 166→Tyr/Phe i.v.m. Trp 166 (TGG)→Tyr (TAT) and Trp 166 (TGG)→Phe (TTT)
His 147	^{447}ACGCTGGTT*GTCCACAAT^{430}	sequencing mutation His 147→Asn i.v.m. His 147 (CAC)→Asn (AAC)
Arg93/Pro94	^{289}TATCCGTC*GCC*CCAATCGT^{272}	sequencing mutation Arg93/Pro94 i.v.m. Arg93/Pro94, silent; creates XmaIII restriction site
Ile48	^{152}GCGACCACC*ATATCAAAC^{135}	sequencing mutation Ile48 i.v.m. Ile48, silent; creates EcoRV restriction site
Ala18	^{65}AAATAAAATTCC*GCCGCG^{48}	sequencing mutation Ala18 i.v.m. Ala18, silent; creates SacII restriction site
Ala7	^{30}ACTAATCACC*GCGCGACA^{13}	sequencing mutation Ala7 i.v.m. Ala7, silent; creates SacII restriction site
XhoI-15	$^{-5}$CGTGACTGC*AG*TGGCTAT^{-22}	sequencing mutation XhoI-15 i.v.m. to XhoI site -15 sequencing until BamHI site -134

Table III.1 Sequencing and in vitro mutagenesis (i.v.m.) primers
* = mismatch compared to the sequence of pabB established.

In Fig.III.1, the results of sequencing, using a number of oligonucleotide primers from Table III.1, are shown.

Fig.III.1 Autoradiographs of sequencing gels, after using the following primers (see also Table III.1): 1 = 17-mer; 2 = 1403; 3 = Lys 449; 4 = 1203; 5 = His 339; 6 = Trp 166; 7 = His 147; 8 = Ile 48; 9 = Ala 18; 10 = Ala 7. From left to right, A, G, C and T lanes are shown.



6 7 8 9 10

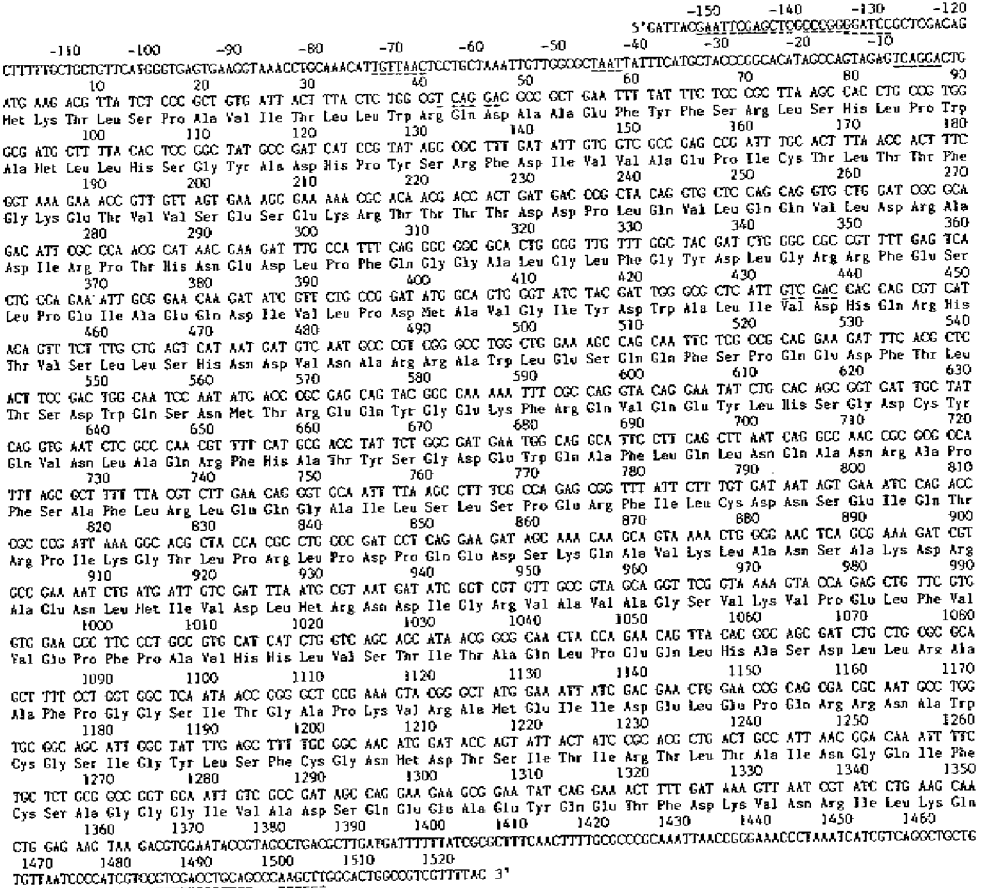
Fig.III.2 shows the nucleotide sequence determined, together with the amino acid sequence predicted from it.

As stated in (14), the amino acid sequence is based on the longest open reading frame of 1377 bp, from which the first 18 bases are assumed not to be translated. This region contains a putative Shine-Delgarno ribosome binding site (15) 5'-TcAGGA-3', as underlined in Fig.III.2. Three nucleotides downstream there is an ATG startcodon. The encoded methionine is considered to be the first amino acid in the primary sequence of PABS CoI, because from this point on, translation produces a protein of 453 amino acid residues with a calculated molecular weight of 51,217 D. This agrees well with the molecular weight of 48,000 D determined by chromatography on Sephadex G-100 by Huang & Gibson (16). Not far downstream what is believed to be the real ribosome binding site (RBS), there is another putative RBS of exactly the same sequence, 5'-TcAGGA-3', as shown in Fig.III.2. However, there is no startcodon close to this region; only 46 nucleotides downstream, the next in-frame ATG codon is encountered (starting translation from this ATG codon would produce a protein with a calculated molecular weight of 47,567, which would also agree well, even better, with the empirically determined one). Whether this alternative RBS is functional is unknown.

In Fig.III.2, the SalI site, which destroyed the ability of pabB to complement pabB-mutated strains, is indicated. It is clear, that digestion at this site would remove 144 amino acid coding information from the N-terminal end, thereby destroying PABS CoI enzyme activity.

Although Goncharoff & Nichols reported, that they did not find other open reading frames exceeding a total length of 300 bp, it can be shown that there is one open reading frame of 306 bp, starting at 938 and ending at 1244 (-1 frame compared to the PABS CoI coding frame). Since there is no putative promoter, no ribosome binding site nor startcodon, this open reading frame is not translated. Another open reading frame of 291 bp is present, starting at 173 and ending at 464 (+1 frame compared to the PABS CoI coding frame). There is an ATG codon present, but a RBS is missing. Therefore, the 78 amino acid (MW 8,580 D) coding information can not be expressed.

FigIII.2 Nucleotide sequence of *E.coli* pabB and the amino acid sequence deduced. Sequences underlined: -152→-147:EcoRI site; -146→-141:SstI site; -139→-134:SmaI site; -134→-129:BamHI site; -73→-67:RNA-polymerase binding site;-46→-43: Pribnow-box;-9→-4: Ribosome binding site; 42→47:alternative ribosome binding site (see text);438→433: SalI site;1484→1489: SalI site;1490→1495: PstI site;1499→1504: HindIII site.



Directly upstream the ATG startcodon putative regulatory sequences can be found (14): from nucleotide positions -73 to -67 there is a possible RNA-polymerase binding site 5'-TGTTAAC-3' (17), from positions -46 to -43 there is a Pribnow-like box 5'-TAAT-3' (18), suggesting initiation of transcription at position -36 (19). No attenuator-like sequences in the 5' region are found (as in trpE).

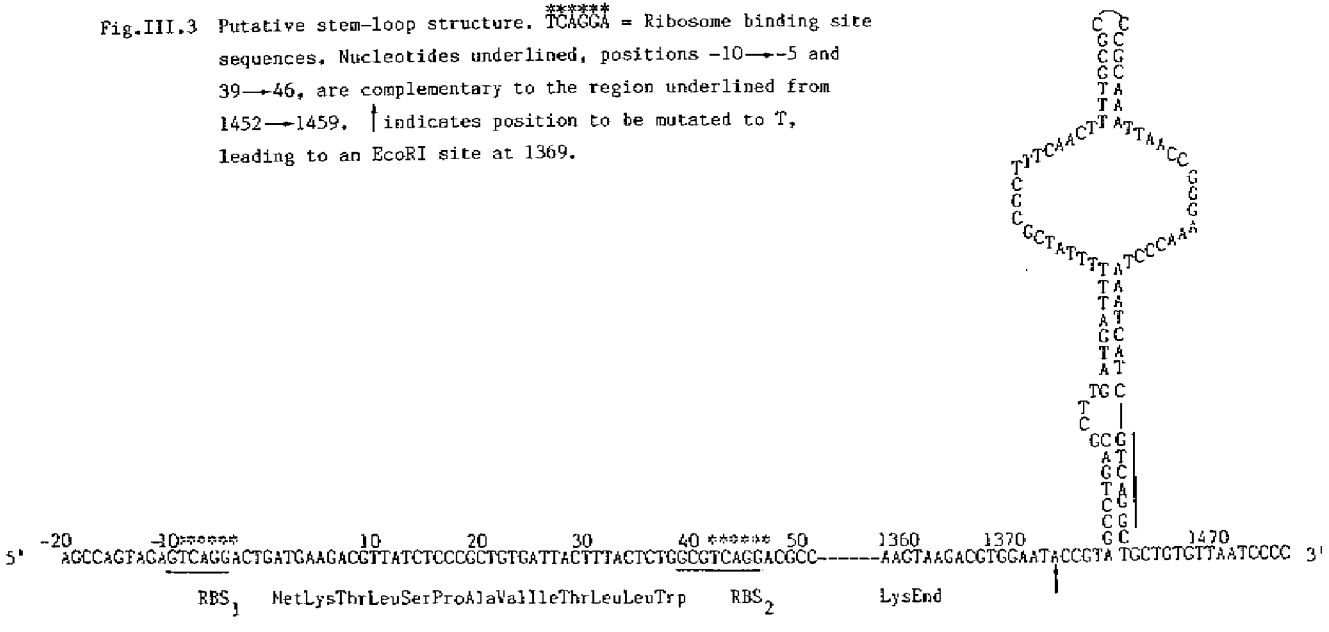
From Fig.III.2, it can be concluded that there is no obvious rho-independent transcription termination sequence present. However, when the pabB sequence was analysed using CAOS/CAMM facilities (Analyseq. V8.1 Roger Staden) 3 inverted repeats emerged, all of them downstream the TAA stop codon. When taken together, a putative stem-loop structure can be constructed, as shown in Fig.III.3.

It can be speculated, that this stem-loop structure has some function in arresting RNA polymerase, or in transcription termination, although it does not resemble the normally found G-C rich region followed by a track of T-bases (at which an mRNA transcript ends). Instead, tracks of 6 T-bases and 4 T-bases (positions 1395-1401 and 1415-1418) are present within the putative loop.

Another speculation about the function of this stem-loop structure is that it might influence translation in some way. In this respect, it is remarkable to notice, that the stem-sequence is complementary to the ribosome binding site that is presumably functional during expression. The stem-sequence is complementary to another RBS-like region also, since both RBS sequences are identical. Theoretically, RBS_1 (functional) can hybridize to nucleotides 1380-1385, but the number of basepairs (6) is less than the number of basepairs (8) in the structure as shown in Fig.III.3. RBS_2 (non-functional) can hybridize to nucleotides 1380-1387, i.e. 8 basepairs. Therefore, there could be competition between RBS_2 and sequence 1454-1461, for hybridisation with sequence 1378-1387. Of course, this is only possible, when the mRNA transcript has not ended before sequence 1454-1461. Switching of hybridisation between these two competing sequences might be functional in allowing translation to proceed or not. It is not apparent however, what the trigger for breakage/reunion of the stem-loop structure is.

The first indication, that the stem-loop structure as indicated in Fig.III.3 can exist in vivo, came from a sequencing experiment.

Fig.III.3 Putative stem-loop structure. ****** = Ribosome binding site sequences. Nucleotides underlined, positions -10→-5 and 39→46, are complementary to the region underlined from 1452→1459. ↑ indicates position to be mutated to T, leading to an EcoRI site at 1369.



M13mp10pahB ss DNA was used as template with Klenow DNA-polymerase and a 17-mer sequencing primer (Table III.1). When the reaction temperature was lowered to 0-4°C, a normal pattern was observed until position 1463, where sequencing was aborted, as shown in Fig.III.4.

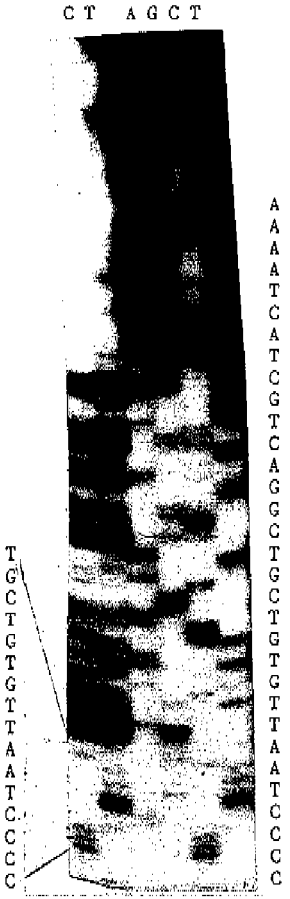


Fig.III.4 Sequencing of the region 1476 1360, using 17-mer sequencing primer. Left: 0-4°C, right: room temperature.

In order to establish the existence of the proposed stem-loop structure and its function, it was decided to construct a mutagenic oligonucleotide, that will introduce an A into a T base mutation at position 1373. An EcoRI restriction site will be the result, at position 1369-1374. The mutagenic oligonucleotide, called EcoRI 1369,

5' ^{1381}GGCTACGGA*ATTCCACGT^{1364} is now being used for in vitro mutagenesis by the gapped heteroduplex technique (see Chapter IV). Once the desired EcoRI 1369 mutant is isolated, the complete pabB gene can be taken out of the cloning vector (M13mp18) by a single digestion with EcoRI, since a second EcoRI site is present in the 5' region, the one which belongs to the phage polylinker. The resulting pabB gene will have all of its 5' regulatory sequences, whereas the putative 3' regulatory sequence is removed. The pabB gene can subsequently be studied with respect to its expression. Moreover, the pabA gene can now be coupled in tandem, in order to achieve coordinate expression of both genes from the same mRNA.

Alignment of the pabB coding sequence with that of *E.coli* trpE shows similarity of 40% at the nucleotide level and 26% at the amino acid level, if gaps are inserted as pictured by Goncharoff & Nichols. These results suggested, that pabB and trpE arose from a common ancestor, as expected (20,21). A remarkable feature of the 5' (40 amino acid-) coding region of *E.coli* trpE is the fact that it can not be aligned with *E.coli* pabB. Recently, it was shown by Matsui et al. (22), that this region, including Leu^{35}-Leu-Glu-Ser^{38}, is essential for the allosteric regulation of AS, and that Ser^{38} may be the binding site for tryptophan in feedback inhibition. If this is true, than non-alignment of this region is not surprising, since pabB is not inhibited by tryptophan.

Also remarkable in the alignment of both genes is that about 70% of all conservative amino acids are found in the C-terminal half of CoI.

Another feature of AS CoI is that it is completely free of Trp residues (AS CoII also), whereas PABS CoI contains seven and PABS CoII contains three Trp residues. It has been proposed (23), that AS is tryptophan-free so that chorismate can be channeled into the tryptophan biosynthetic pathway under severe tryptophan starvation conditions.

Alignment of the heptapeptide Ile^{317}-Cys-Thr-Pro-Gly-Ser-Arg^{323} of AS CoI with PABS CoI reveals that the essential Cys 318 of AS CoI (24) is not conserved in PABS CoI. Instead, Ala 318 has replaced this cysteine residue. McLeish et al. (25) have suggested the presence of an essential sulfhydryl group in PABS CoII, but not in PABS CoI, based on chemical modification studies.

As far as the AS CoI-essential Arg and His residues are concerned (24), they have not been located in the AS CoI amino acid sequence yet. Thus, it is unknown, if they are conserved in PABS CoI. However, if all His residues in both Components I are considered, only 2 (out of 12 present in PABS CoI) are conserved: His 147 and His 339. Of course this does not necessarily mean that one of them is essential in either enzyme, since enzyme mechanisms will be different. Also, results of McLeish et al. (25) have provided evidence against the presence of an essential His residue in PABS CoI, since PABS CoI was not inhibited after chemical modification using diethylpyrocarbonate (26) or bromopyruvate. On the other hand, the proposed function of His, as a candidate for abstracting the hydrogen on C-2 of chorismate or for protonation of the leaving hydroxyl or enolpyruvyl groups, might be similar in both enzymes.

Although the AS CoI-essential Arg residue could be conserved in PABS CoI if the proposed function in binding the chorismate anion is correct, locating its position will be more difficult, since 11 out of 28 Arg residues are conserved in PABS CoI.

Comparison of AS CoI and PABS CoI amino acid sequences as described here was basic for the development of strategies for in vitro mutagenesis experiments, described in the following Chapter.

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Chapter IV.

In vitro mutagenesis of *E.coli* pabB.

IV.1 Introduction.

IV.1.1 General.

In the last decade, oligodeoxyribonucleotide-directed in vitro mutagenesis has become one of the most powerful tools in molecular genetics, since it provides a means to alter a defined site in a region of cloned DNA (1-11). Therefore, this technique is of direct importance in modification of naturally occurring proteins and in the rational design and synthesis of new proteins with unprecedented functions.

The principle of the method is to use heteroduplex DNA, formed by annealing a vector, containing the target gene as a single strand, with a complementary synthetic oligonucleotide, containing the desired codon change. After extension and ligation of the oligonucleotide, the newly formed duplex DNA is subsequently transformed into cells in which the vector can replicate. The progeny recombinant DNA contains either the wild-type gene or the gene with the mutation specified by the synthetic oligonucleotide.

The method stems from the combination of a number of observations like (i) marker rescue of mutations in ϕX174 by restriction fragments (12,13), (ii) the stability of DNA duplexes containing mismatches (14-17) and (iii) the ability of *E.coli* DNA-polymerase to extend oligonucleotide primers hybridized to single strand templates (18-19). The general concept of using an oligonucleotide as an ideal mutagen was discussed years before the experiment was feasible (20), and it took almost twenty years before Hutchison et al. (21) and Gillam & Smith (22,23) first created a number of mutations in single strand phage ϕX174. Similar experiments using ϕX174 (24) were reported, until Wasylyk et al. (25) demonstrated the first example of oligonucleotide-directed mutagenesis of a cloned fragment in a vector derived from a single strand phage, fd. Since then, many useful new strategies for site-specific in vitro mutagenesis of DNA have been developed, in

order to make the basic procedure as technically straightforward as possible and to improve the efficiency with which the heteroduplex DNA produces progeny from the mutant-containing strand (26–35).

IV.1.2 In vitro mutagenesis techniques.

The basic biochemical procedure, which was originally used, anneals the mutagenic oligodeoxyribonucleotide to a single strand circular genome, as primer for DNA-polymerase in the presence of DNA-ligase (11,22,23).

A serious drawback of using a single strand template for in vitro mutagenesis is the problem of background progeny of wild-type molecules resulting from inefficient conversion of template to covalently closed circular (CCC) DNA (36).

An elegant way to reduce this problem is to carry out oligonucleotide mutagenesis on a gapped duplex target obtained by annealing a second, wild-type primer (two-primer technique) or linearized denatured vector RF DNA (gapped duplex technique) to recombinant phage ss DNA.

In addition to the higher efficiency of mutagenesis using the gapped duplex approach as compared to the single strand approach, it has become possible to take advantage of the presence of the gapped strand in subsequent selection procedures by locating one or more selectable markers on one of either strands. As a consequence, efficiency of mutagenesis is raised to as high as 80–95%.

The cassette mutagenesis technique involves the replacement of a short segment of a gene clone with a synthetic, mutant oligonucleotide duplex (36–44). The obvious advantage of this technique is the high yield of mutants, but a disadvantage is the dependence on conveniently located restriction sites to remove a wild-type segment. If these restriction sites are missing, they have to be introduced by the methods discussed above. If the desired restriction site will be in a protein coding region, only mutations are allowed that create the restriction site without changing the amino acid sequence upon expression of the gene. In other words, the mutation should be "silent" with respect to the amino acid that the triplet involved is coding for.

IV.1.3 Selection and enrichment of mutants.

A powerful selection and enrichment strategy, which was used for in vitro mutagenesis of pabB described in this thesis, is based on the presence of nonsense (amber) mutations in the template M13mp10 vector sequence (32-35,46-48). The gene to be mutated is cloned in the amber-containing template, and single strand DNA is isolated. Wild-type M13mp10 revertant RF DNA is then digested with the same restriction enzymes used to clone the gene in the amber template. Linear, wild-type M13mp10 is melted at elevated temperature, the mutagenic oligonucleotide and single strand recombinant template added, and slowly cooled down to room temperature. The gapped heteroduplex containing the annealed mutagenic oligonucleotide is then converted to CCC DNA by DNA-polymerase and DNA-ligase. Some time after the polymerisation reaction has started, single strand DNA binding protein (gene 32 protein of T_4 phage) is added to overcome possible arrest of DNA-polymerase by secondary structure (7,49). However, enzymatic attachment in vitro is not absolutely necessary. A mix-heat-transfect protocol is available (34), although efficiency of mutagenesis is of course reduced. After transfection of the mixture into a recipient host cell,which is amber-suppressing but defective in DNA-repair (mutS or mutL), both wild-type and mutant progeny are propagated. The phage-containing supernatant of this culture is taken for infection of a non-suppressing host cell. As a consequence, only wild-type phage, originating from the gapped strand containing the desired mutation, will segregate.

IV.2 Materials and methods.

IV.2.1 *E.coli* strains, plasmids and phages.

BMH71-18	F'lacI^q, ZdelM15, proA$^+$B$^+$/del(lac-proAB), thi, supE.
BMH71-18mutS	F'lacI^q, ZdelM15, proA$^+$B$^+$/del(lac-proAB), thi, supE, mutS::Tn10.
MK30-3	F'lacI^q, ZdelM15, proA$^+$B$^+$/del(lac-proAB), recA, galE, strA.
JM101A	F'lacI^q, ZdelM15, proA$^+$B$^+$, traD36/del(lac-proAB),

supE, thi, recA.

PC1539 F^-/pabB, argE, ilvC, pheA, tyrA, trp, lac, gal, tsx, (lam), phx, strA.

Phages M13mp10 and M13mp18 were from Pharmacia.

Plasmid pUC19 was obtained from R.N.H. Konings, Department of Molecular Biology, Catholic University Nijmegen.

Strains BMH71-18, BMH71-18mutS and MK30-3 were obtained from H.-J. Fritz, Max-Planck Institute fur Biochemie, Abteilung Zellbiologie, Munich, F.R.G.

Strains PC1539 and JM101A were obtained from Phabagen Collection, Department of Molecular Cell Biology, University of Utrecht, the Netherlands.

IV.2.2 Media.

The rich medium used for routine subculture and growth was L-broth, supplemented with 2 g/l glucose (50). Streptomycine sulphate (200 µg/ml), tetracycline (10 µg/ml) and ampicillin (25 µg/ml) were supplemented when required. Selection of colourless plaques of M13mp18pabB, wild-type as well as mutated, was performed on L-broth agar plates without glucose, using 3 ml topagar (6 g/l agar in L-broth) containing 10 µl 100 mM IPTG and 50 µl X-Gal (2% in dimethylformamide). Media used during in vitro mutagenesis were Antibiotic Medium 3 (17.5 g/l); EHA plates contained 13 g/l Trypton, 8 g/l NaCl, 10 g/l agar, 2 g/l sodium citrate.$2H_2O$ and 7 ml/l 20% glucose, added after autoclaving; topagar contained 10 g/l Trypton, 5 g/l NaCl and 6.5 g/l agar.

IV.2.3 DNA preparations.

Plasmid DNA and M13mp RF DNA were isolated according to Clewell & Helinski (51),followed by banding in CsCl gradients using ultracentrifugation. M13mp ss DNA was isolated as described in Chapter III. Mutagenic oligonucleotides were synthesized as described in Chapter III, 5'-phosphorylated with T_4-polynucleotide kinase according to Richardson (52) and purified by preparative acrylamide gel-electrophoresis and elution on Biotrap (Schleicher & Schuell). All

oligonucleotides were tested for specific annealing by using them as sequencing primers on M13mp10pabB as described in Chapter III.

IV.2.4 In vitro mutagenesis.

In vitro mutagenesis of the pabB gene was performed essentially according to the gapped heteroduplex technique of Kramer & Fritz (35,46), with following modifications: both the M13mp18 RF DNA digested with EcoRI and HindIII, and the M13mp10pabB virion DNA isolated by PEG-precipitation, were subjected to electrophoresis on 1% agarose gel and purified by electro-elution on Biotrap. Removal of the complete polylinker sequence from M13mp18 RF was necessary to make M13mp10 and M13mp18 compatible in this respect. For example, M13mp10pabB does not contain the KpnI site present in M13mp18. Linear M13mp18 RF DNA (0.5 µg) and M13mp10pabB ss DNA (1.3 µg) were mixed in 40 µl 187.5 mM KCl/12.5 mM Tris/HCl pH 7.5. To 8 µl of this mixture 2 µl of 5'-phosphorylated mutagenic oligonucleotide (2 pmol/µl) were added. Construction of gapped duplex DNA and annealing of the primer were performed in a single step by heating 2 min. to 100°C and cooling to room temperature during 45 min. Next, 4 µl 500 mM Tris/HCl pH 8.0 /600 mM ammoniumacetate/50 mM dithiothreitol/50 mM $MgCl_2$, 4 µl 2 mM NAD, 4 µl of 4x2.5 mM dNTP's, 15 µl water, 1 µl *E.coli* DNA-ligase 5 units/µl) and T_4 DNA polymerase (1 unit/µl) were added. The mixture was incubated during 15 min. at room temperature; 2 µl of T_4 gene 32 protein were added and incubation continued for another 90 min. at room temperature. The reaction was stopped by adding 2.5 µl 200 mM Na_2EDTA pH 8.0 and heating to 65°C for 10 min. The mixture was extracted with 40 µl phenol (equilibrated with TE-buffer)/chloroform (1:1 v/v), three times with 200 µl diethylether, and immediately used for transfection of *E.coli* BMH71-18mutS competent cells according to Cohen et al. (53) and cultured overnight at 37°C. Phage containing supernatant of this propagation step was used to infect *E.coli* MK30-3 cells. Dilutions 10^6 to 10^9 were taken for segregation in MK30-3 by plating 100 µl samples on L-agar plates. Single plaques were picked, grown in 10 ml cultures and ss DNA was isolated after overnight incubation at 37°C. Mutants were detected directly by single-track sequencing of the isolated ss DNAs. One single plaque was chosen,

grown on larger scale and ss DNA isolated preparatively. From this ss DNA, the region of interest containing the desired mutation was sequenced using all four dideoxy NTPs and compared with wild-type.

IV.2.5 Subcloning mutated pabB.

From a plaque of M13mp18 recombinant phage, containing a mutated pabB gene, *E.coli* JM101A cells were infected, cultured overnight at 37°C and cells collected by centrifugation. RF DNA was isolated as described before. Following EcoRI and HindIII digestion, the mutated pabB gene was excised out of the RF, separated from vector DNA by 1% agarose gel-electrophoresis and purification by Biotrap elution. Then the pabB gene was cloned into pUC19 (digested with EcoRI and HindIII) using T_4 DNA-ligase. JM101A competent cells were prepared and transformed with pUC19pabB ligation mix (54). Selection of colourless, ampicillin-resistant colonies was performed on L-broth agar plates containing IPTG and X-Gal. A single colony was picked and grown in L-broth (containing ampicillin). Plasmid DNA was isolated as described.

IV.2.6 Restriction analysis of Trp 390 mutants.

In case of mutants pUC19pabB390-1 and pUC19pabB390-2 plasmid DNA was digested with BstNI after 5'-^{32}P-phosphorylation in the buffer recommended by the supplier, Anglian Biotechnology. Subsequently, fragments were analysed on 8% polyacrylamide gel followed by overnight autoradiography.

IV.3 Results and discussion.

IV.3.1 Target sites of *E.coli* pabB chosen for site directed mutagenesis.

From the *E.coli* pabB nucleotide sequence shown in Chapter III, Fig.III.2, and from comparison of PABS CoI and AS CoI amino acid sequences as shown in Fig.IV.1, a number of potential target sites for in vitro mutagenesis emerged.

Fig.IV.1 Comparison of *E.coli* PABS CoI and AS CoI amino acid sequences. ▭ = conservative residues. ○ = residues chosen for mutagenesis.

The mutagenic oligonucleotides that were designed are shown in Table III.1, and can be divided into three groups, depending on purpose. First of all, 7 mutagenic oligonucleotides were constructed to achieve codon changes in the pabB coding sequence such that either an amino acid will be replaced after in vitro mutagenesis, or a stopcodon is introduced, the latter leading to premature translation termination, and therefore to a C-terminal deleted PABS CoI. One of these oligonucleotides was constructed as a mixed primer, i.e. during synthesis of the oligonucleotide two phosphoramidites were delivered simultaneously to the growing strand such that during mutagenesis two different mutations are introduced using only one (mixed) primer. Sequencing possible mutants afterwards will determine which mutation has actually been introduced in a particular mutant phage.

The second group of oligonucleotides contains 8 primers that were designed tomake the pabB gene suitable for future experiments using the cassette mutagenesis technique. This technique is completely dependent on the availability of conveniently located restriction sites within a sequence of interest. Since the *E.coli* pabB sequence is rather limited in this respect, these primers were designed to introduce a new restriction site, not present in the M13 vector, and present only once or not at all in the pabB sequence. A summary of sites available in the wild-type pabB gene, and new sites to be introduced is shown in Table IV.1.

Site	Palindrome	Restriction endonuclease
-152	GAATTC	EcoRI
-146	GAGCTC	SstI
-139	CCCGGG	SmaI
-134	GGATCC	BamHI
- 15	CTCGAG*	XhoI
17	CCGCGG*	SacII
50	CCGCGG*	SacII
72	CTTAAG	AflII
139	GATATC*	EcoRV
277	CGGCCG	XmaIII
382	GATATC	EcoRV
433	GTCGAC	SalI
586	AAATTT	-
642	TGCGCA*	MstI
647	AACGTT	-
712	CGCGCG	-
713	GCGCGC	BssHII
724	AGCGCT	HaeII
832	CCGCGG*	SacII
937	GATATC	EcoRV
1074	GCGCGC	BssHII
1369	GAATTC*	EcoRI
1484	GTCGAC	SalI
1490	CTGCAG	PstI
1499	AAGCTT	HindIII

Table IV.1
Summary of sites available in wild-type pabB and new sites to be introduced by site-directed mutagenesis (*).

While creating a new restiction site, care was taken not to disturb, or interrupt, the pabB coding sequence by introducing a missense or nonsense mutation, respectively. In other words, as far as coding capacity is concerned, only those palindromes were chosen, which are silent towards the amino acid sequence expressed.

The third group of oligonucleotides consists of 4 primers specifically designed to study proposed regulatory regions of the *E.coli* pabB gene. These primers were designed to create restriction sites either in the 5' region or in the 3' region. Since they are silent with respect to the coding sequence, they can be regarded also to belong to the second group of primers described before.

As far as the first group of mutagenic oligonucleotides is concerned, the following amino acid replacements were conceived:
1. Trp 390 to stopcodon.
The motives to change Trp 390 codon into a stopcodon were as follows. First of all, it would be interesting to determine how many amino acid residues can be removed, starting from the C-terminal end of PABS CoI, before enzyme activity as a whole, or a specific function in particular (like subunit binding or the capacity to use NH_3 as an amino donor) would be severely affected. In this way, amino acid residues essential for catalysis could possibly be delineated. For example, in case of Trp 390 to stopcodon change, 3 out of 6 cysteine residues would be removed. At the time this mutation was achieved, no data concerning the presence of an essential -SH group in PABS CoI were available. Therefore, specific deletion could give a first indication.

Secondly, stability of the truncated PABS CoI with respect to oxidation, as compared to the wild-type PABS CoI, would be worthwhile to study, because the high susceptibility of wild-type CoI towards denaturation in general has arrested its purification to homogeneity until now.

When designing this mutation, it was felt, that the chance of retaining enzyme activity was small, since creating a deletion of 63 amino acids is rather drastic and likely to affect some function in enzyme catalysis. Nevertheless, the mutagenesis was performed because the amber codon which was introduced could still be translated as tyrosine in an *E.coli* pabB amber suppressing strain, as an alternative

way to mutate Trp 390 to tyrosine using site directed mutagenesis, as discussed hereafter.

The Trp 390 to stopcodon mutation destroyed a BstNI restriction endonuclease recognition site (5'-^{1166}CCTGG^{1170}-3' ⟶ 5'-^{1166}CCTAG^{1170}-3'), which facilitated screening of potential mutants.

2. Trp 390 to tyrosine.

Trp 390 was chosen to be changed into tyrosine for the following reasons. In PABS CoI there are seven Trp residues, whereas AS CoI contains none. It has been proposed (55), that this may cause chorismate to be channeled into the tryptophan biosynthetic pathway. Consequently, PABS CoI might only be produced after a certain tryptophan level has been reached in cells. In order to test growth behaviour of cells under tryptophan starvation conditions with a completely tryptophan-free PABS CoI, it is necessary to replace all seven Trp residues one by one. Of course, enzyme activity should be retained on wild-type level as much as possible. In general, best candidates to replace Trp residues are the other two aromatic amino acids, tyrosine and phenylalanine, since they are almost isosteric and the 3-D structure of PABS CoI should not be distorted too much. However, if the Trp residue in question does have some function in catalysis rather than a structural function, it probably will not be possible to substitute it for other residues. Trp 390 was chosen to be replaced with tyrosine rather than phenylalanine, since AS CoI contains a tyrosine residue at site 390 in its amino acid sequence, as can be seen from the alignment with PABS CoI in Fig.IV.1. It is interesting to note that Trp 390 in PABS CoI is surrounded by a large number of conservative amino acids when compared to AS CoI. Replacement with tyrosine in AS CoI apparently was allowed during evolution without destroying substrate binding and the use of NH_3 as an amino donor, features common to both enzymes. Residue 390 was chosen as the first of Trp residues to be mutated, since it is closest to the C-terminal end, which is the most conservative half of PABS CoI. Additionally, as in the Trp 390 to stopcodon mutation, a BstNI site is destroyed (5'-^{1166}CCTGG^{1170}-3' ⟶ 5'-^{1166}CCTAT^{1170}-3'), which facilitated screening of potential mutants. Also, no new primer had to be synthesized in order to sequence potential mutants, because the

same sequencing primer as in the Trp 390 to stopcodon change could be used.

3. Cys 391 to serine.

The essential cysteine residue in AS CoI, Cys 318, is not present in PABS CoI since it reads alanine at site 318. Therefore, it was reasoned, that PABS CoI does not contain cysteines, essential for catalysis, at all, or one of the six non-conservative Cys residues is a possible candidate. Cys 318 in AS CoI is proposed to attack at C-2 of chorismate, directing amination towards C-2. In PABS CoI, this could be different, since the amino-group reacts with C-4 of chorismate. Whether this function is still performed by an essential, dislocated, cysteine in PABS CoI, remains to be determined. As an alternative to search for essential Cys residues in PABS CoI by creating deletions as in case of Trp 390 to stopcodon mutation, all six of them could be mutated themselves, for example to serine. Changing -SH to -OH isosterically should leave the 3-D structure of the enzyme intact (presuming the -SH group does not take part in disulfide bridging for structural purposes) and specifically probe for cysteines essential to catalysis rather than structure.

Cys 391 was chosen to start with, since it is in the most conservative half of the enzyme, and the same sequencing primer as in the mutations discussed above could be used. Again, the Cys 391 to serine mutated PABS CoI could also be studied with respect to inactivation through oxidation.

4. Lys 449 to stopcodon.

As discussed above, creating C-terminal deletions in a protein coding sequence can be used as a tool to probe for catalysis-essential amino acid residues. Since the deletion resulting from the Trp 390 to stopcodon mutation was likely to have large effects on enzyme activity, it was considered also to use smaller deletions. An example of such a deletion, removing only five amino acids from the PABS CoI C-terminal end, is the mutation of Lys 449 codon to a stopcodon. Lys 449 was chosen since it is the first residue downstream the last conservative amino acid Leu 448. Therefore, this deletion would remove a small number of residues from the C-terminal end, leaving all conservative residues intact. If enzyme activity and other functions would be retained in the deleted CoI, then the next step would be to

remove conservative residues by stepwise deletions in the order of 1-5 amino acids.

5. Trp 166 to tyrosine or phenylalanine.

This mutation was chosen for the same reasons as the Trp 390 to tyrosine mutation, with this exception, that either tyrosine or phenylalanine is introduced (using a mixed probe). Comparison with the AS CoI amino acid sequence suggests that replacement with glutamine would be allowed, but substitution of Trp 166 with a more isosteric residue was preferred, in order to effect the 3D structure as less as possible.

6. His 147 to asparagine.

7. His 339 to asparagine.

From AS CoI it is known, that one essential histidine residue is present (56). The proposed function of this histidine, the location of which is unknown in AS CoI, is to function as a basic group required to abstract the hydrogen on C-2 of chorismate or to protonate the leaving hydroxyl or enolpyruvyl group. From the alignment of PABS CoI with AS CoI in Fig.IV.1, it can be concluded, that only two histidines are conservative, His 147 and His 339. If in both enzymes histidine performs the same function, then either of the two conservative His residues is a good candidate to be the catalytically essential one. However, if histidine in AS CoI abstracts hydrogen from C-2 of chorismate, then the chance of finding the same histidine residue in PABS CoI abstracting hydrogen from C-4 of chorismate is unlikely. If histidine is required for protonation of leaving groups in AS CoI, then a histidine residue in PABS CoI could perform this function also. Substitution of His residues with asparagine was chosen since asparagine is almost isosteric with histidine.

IV.3.2 Results of in vitro mutagenesis.

From the oligonucleotides available as shown in Table III.1, in vitro mutagenesis was started with the ones that belong to the first group, as already discussed, leading to amino acid substitutions in PABS CoI. First, they were all tested for specific priming by using them in a dideoxy sequencing experiment. As a result, no alternate hybridisation targets in either gene or vector sequence emerged from the

autoradiographs after gel-electrophoresis, as predicted by previous computer sequence analysis. This is illustrated in Fig.III.1, where several mutagenic oligonucleotides were used to read parts of the pabB sequence (Trp 390-2 and Cys 391 not shown).

According to literature, efficiency of mutagenesis using the gapped heteroduplex technique of Kramer & Fritz depends on the length of the mutagenic oligonucleotide (and the number of mismatches included), the gap-size of the heteroduplex target, the presence of internucleotide phosphorothioate linkages, the route followed before transfection, and heat-shock temperature, as summarized in Table IV.2. During in vitro mutagenesis of pabB, route B' was followed, with a gap-size of 1650 nucleotides and 18-mer mutagenic oligonucleotides. Although an efficiency of at least 65% was expected according to Table IV.2, first attempts gave only low phage titers after segregation through *E.coli* MK30-3. After modification of the Kramer & Fritz protocol (46) by performing the gapped duplex construction as well as the mutagenic primer annealing in a single step, high segregation titers were found, as shown in Table IV.3. Possibly, incubating twice at 100°C, first during gapped heteroduplex construction, then during oligonucleotide annealing, destroyed some of the total DNA that serves as starting material for enzymatic gap-filling, leading to less CCC DNA and therefore low titers upon transfection.

From the plaques that appeared after infection of MK30-3, 10 to 20 were selected and grown on MK30-3 again. Phage-containing supernatants of Lys 449, Trp 166, His 147 and His 339 experiments were stored until further use. ss DNA of Trp 390-1, Trp 390-2 and Cys 391 potential mutants were analyzed by single-track sequencing. As a result, 8 out of 9 plaques contained the Trp 390 to stopcodon mutation, 5 out of 19 contained the Trp 390 to tyrosine mutation, and 11 out of 20 contained the Cys 391 to serine mutation. Although more plaques would have to be screened to draw definite conclusions from these mutagenesis efficiencies, the results suggest, that a double mismatch (in the case of Trp 390-2) lowers efficiency as compared to a single mismatch. Also, efficiencies of the Trp 390-1 (89%) and Cys 391 (55%) compare well with the mean value of 65% (Table IV.2) reported in experiments of Kramer & Fritz applying the same route (B').

Oligomer-length[1] (nucleotides)	Gap-size (nucleot.)	Internucleotidic phosphorothioate	Route[2]	Average mutant efficiency (%)
16	117	-	B	73
16	120	-	B	63
16	1640	-	B	53
16	120	-	B'	78
16	1640	-	B'	65
16	117	-	A	22
16	120	-	A	16
24	120	-	A	39
32	120	-	A	31
40	120	-	A	46
48	120	-	A	39
16	1640	-	A	5
24	1640	-	A	14
32	1640	-	A	12
40	1640	-	A	23
48	1640	-	A	23
22	120	+	A	29
28	120	+	A	55
22	1640	+	A	12
28	1640	+	A	31
28	1640	+	A' (30°C)	41
28	1640	+	A' (37°C)	40
28	1640	+	A' (45°C)	53

Table IV.2

[1]Oligomers contained one mismatch.

[2]Route A = Mix-heat-transfect + heatshock 30°C

Route A' = Mix-heat-transfect + heatshock as indicated

Route B = Gap-filling by *E.coli* DNA-polymerase + T_4 DNA-ligase heatshock 30°C

Route B' = Gap-filling by T_4 DNA-polymerase + *E.coli* DNA-ligase + T_4 gene product 32, heatshock 30°C.

Compiled from refs. 32,34,35 and 46.

Oligomer-length (nucleotides)	Number of mismatches	Gap-size (nucleot.)	Route	Segregation titer	Mutant efficiency
Cys 391 18	1	1650	B'	$33.5 . 10^{10}$/ml	11/20 (55%)
Trp 390-1 18	1	1650	B'	$0.9 . 10^{10}$/ml	8/9 (89%)
Trp 390-2 18	2	1650	B'	$0.5 . 10^{10}$/ml	5/19 (26%)
Lys 449 18	1	1650	B'	$2.2 . 10^{10}$/ml	-
Trp 166 18	2 (mixed)	1650	B'	$2.5 . 10^{10}$/ml	-
His 147 18	1	1650	B'	$2.6 . 10^{10}$/ml	-
His 339 18	1	1650	B'	$3.3 . 10^{10}$/ml	-

Table IV.3

No internucleotide phosphorothioates present.

Route = see Table IV.2

The high efficiency of Trp 390-1 mutagenesis could be caused by using an 18-mer mutagenic oligonucleotide versus 16-mers in Table IV.2. As a consequence, in the Cys 391 mutagenesis, a value above 65% would be expected also. A possible explanation for the somewhat lower value of 55% could be the fact that the Cys 391 mutagenic oligonucleotide contains 3'-T-T nucleotides, whereas Trp 390-1 and the 16-mers used by Kramer & Fritz have a 3'-G-C end. Tight hybridisation at the very 3' end could help to increase marker yield as a reflection of more efficient priming by DNA-polymerase during the in vitro gap-filling reaction.

One of the plaques was chosen, which showed mutation after single track sequencing of ss DNA. After re-infection of MK30-3 ss DNA was purified. Fig.IV.2 shows the autoradiographs of sequencing the region surrounding codons 390 and 391 of pabB, in case of wild-type, Trp 390 to stopcodon, Trp 390 to tyrosine and Cys 391 to serine mutation.

The mutated pabB genes were digested out of M13mp18 using EcoRI and HindIII restriction enzymes, and subcloned into pUC19 by standard techniques. Single colourless colonies of JM101A, after selection on ampicillin-containing X-Gal indicator plates, were picked and grown on large scale for plasmid preparation. After purification of recombinant plasmids by CsCl-gradient centrifugation, the presence of a mutated pabB gene was verified by detecting the loss of a BstNI site in case

of codon 390 mutation, as shown in Fig.IV.3.

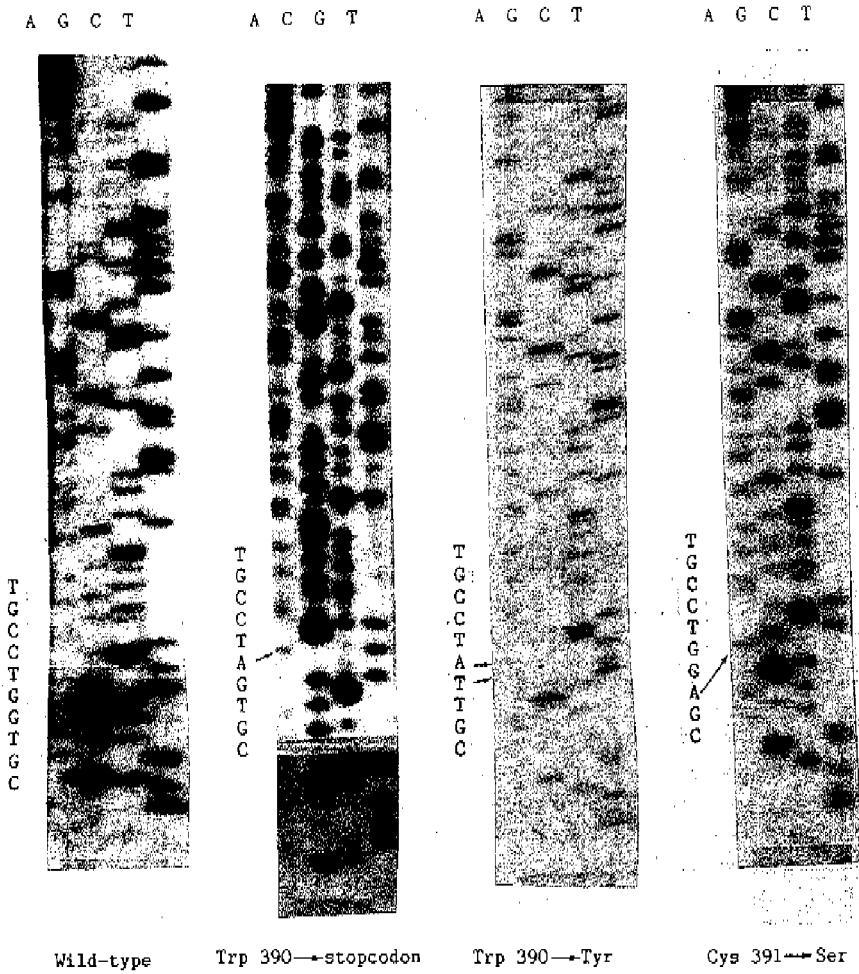

Fig.IV.2 Autoradiographs of sequencing mutated pabB genes.

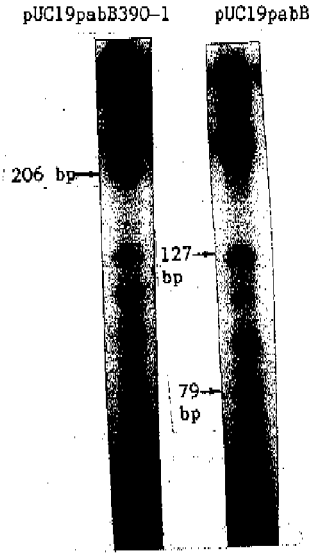

Fig.IV.3
Autoradiographs of BstNI digests of pUC19pabB390-1 and pUC19pabB. Restriction fragments of 79 bp and 127 bp are not separated by BstNI in pUC19pabB390-1; instead, a fragment of 206 bp appears.

Recombinant pUC19 plasmids were then transformed into pabB mutated strain PC1539, to study growth behaviour of transformed cells, and kinetic parameters of mutated CoI, as described in the next chapter.

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Chapter V.

Kinetic analysis of PABS CoI with 63 amino acids deleted from its C-terminal end by site-directed mutagenesis.

V.1 Introduction.

The understanding of the functional roles of amino acids in proteins, enzymes in particular, has strongly increased from two major developments in protein biochemistry: knowledge of the 3-D structure from X-ray crystallography, and the ability to produce large quantities of sometimes otherwise inaccessible enzymes by genetic engineering. During the past five years these methods have been combined with a third, new discipline, called "protein engineering", which makes use of a variety of site-directed mutagenesis techniques.

Protein engineering has become industrial prospect because more detailed understanding of the role of amino acid side chains in protein structure and function is possible, when many point mutations in the coding sequence of a protein are available. Indeed, application of this principle has already resulted in the generation of a large number of mutant proteins for structure-function studies and in the production of new proteins with useful biochemical properties (1-20). At this moment, about 40 proteins have been subjected to protein engineering, as reviewed in (21-23), and the number is increasing rapidly. Impressive applications have been the engineering of the enzymes tyrosyl-tRNA synthetase (24-27) and subtilisine (28-30).

In contrast to anthranilate synthase (31,32), site-directed mutagenesis of p-aminobenzoate synthase has not been reported in literature yet. Here, the results of enzymatic analysis of a PABS enzyme will be described, of which 63 amino acids were deleted from the C-terminal end of Component I by site-directed mutagenesis.*

* Submitted for publication in Journal of Bacteriology.

V.2 Materials and methods.

V.2.1 Construction of PC1539/pUC19pabB390-1.

In vitro mutagenesis of the *E.coli* pabB gene using mutagenic oligonucleotide Trp 390-1 was described in Chapter IV. The resulting plasmid pUC19pabB390-1 was used for transformation into *E.coli* pabB mutated strain PC1539 according to (33). PABA-independent growth of PC1539/pUC19pabB390-1 was tested on minimal medium plates as described in Chapter II.

V.2.2 Preparation of cell-free extracts.

Cultures used for the preparation of cell-free extracts were grown in M9 supplemented medium and harvested during late log-phase or stationary phase of growth by centrifugation. Cells were washed once with 0.9% saline and pellets were weighed. Disintegration of cells was performed by alumina grinding as in (34) by adding 0.5 gram Al_2O_3 for each gram (wet weight) of cells followed by grinding in a mortar and pestle during 3 min. at 4°C. To the resulting paste 4 ml buffer 50 mM KPi/0.2 mM DTT/0.1 mM Na_2EDTA pH 7.6 were added for each gram of cells, followed by centrifugation at 30,000 g for 30 min. to remove cell debris. Cell-free extracts were used for PABA synthesis directly.

V.2.3 Enzyme assay.

Assay of p-aminobenzoate synthase was described in Chapter II. For the determination of Km of chorismic acid, substrate concentration was varied from 0.002 to 0.4 mM. For the determination of L-glutamine-dependent PABA synthesis, a cell-free extract of PC1539 cells grown in L-broth containing 2 to 4 mg protein, was included in the 1 ml assay mixture. Km's of glutamine or NH_3 were determined by variation of concentrations from 0.1 to 25 mM or 0.1 to 33 mM, respectively.

Protein was measured according to Lowry et al. (35) with bovine serum albumin as a standard.

V.3 Results.

In order to determine the activity of PABS CoI in vitro and in vivo, pUC19pabB was transformed into *E.coli* pabB mutant strain PC1539. This particular strain was chosen from all pabB mutant strains (see Chapter II), since it is the only one being triple aromatic auxotroph. Thus, chorismate can not be consumed in other conversions in cell metabolism, and therefore it is available for PABA production only.

After the introduction of a stopcodon in the PABS CoI coding sequence, recombinant plasmid pUC19pabB390-1 was transferred to PC1539 also, and transformants were detected by selection on ampicillin containing L-agar plates. Both colonies from PC1539/pUC19pabB and PC1539/pUC19pabB390-1 were then tested for PABS enzyme activity by plating on selective minimal medium. Surprisingly, PC1539/pUC19pabB390-1 not only grew on selective medium, but was also shown to have a generation time identical to PC1539/pUC19pabB, when growth curves were established (not shown). This means that PC1539/pUC19pabB390-1 must still be able to synthesize PABA in sufficient amounts despite the mutation.

The possibility, that the nonsense mutation introduced into the pabB gene might be translated in PC1539 cells by means of suppressor activity, was then considered. If, for example, the stopcodon would be translated via $tRNA^{trp}$, then a wild-type CoI would be the result, explaining the unchanged growth behaviour of PC1539 cells. Another possibility would be translation via $tRNA^{tyr}$, which would result in a Trp to Tyr amino acid change, instead of a deleted CoI. At the time the Trp 390 to stopcodon change was achieved, the deliberate Trp 390 to tyrosine change by site-directed mutagenesis was not available yet, so kinetic parameters could not be compared directly with a hypothetical Trp to Tyr codon change by cellular suppressing activity. As turned out later (see Chapter VI), kinetic data were completely different. In order to rule out the influence of suppressor activity in PC1539 cells completely, PC1539 cells were made F^+ first, by mating with a wild-type F^+ strain, F^+-exconjugants being detected by infecting single colonies with wild-type M13mp18 virus. Next, PC1539 F^+ cells were plated together with M13mp10 (amber) virus (multiplicity of infection = 1) on L-agar plates. As compared to a control infection

with wild-type M13mp18 virus, no plaques appeared in case of M13mp10, showing that PC1539 is unable to suppress the amber mutations in M13mp10. Therefore, PC1539 is suppressor-negative.

E.coli PC1539 cells containing either the wild-type PABS or the deleted PABS were grown to late log-phase, harvested and cell-free extracts were prepared simultaneously. Progress curves of PABA production during incubation at 37°C in the glutamine assay were established. As shown in Fig.II.12 (no extra pabA-extract added) and Fig.V.1A (extra pabA-extract added), a linear relationship up to 100 min. was found in the case of wild-type PABS. Fig.V.1B shows the progression curve of PABA production in case of the deleted PABS.

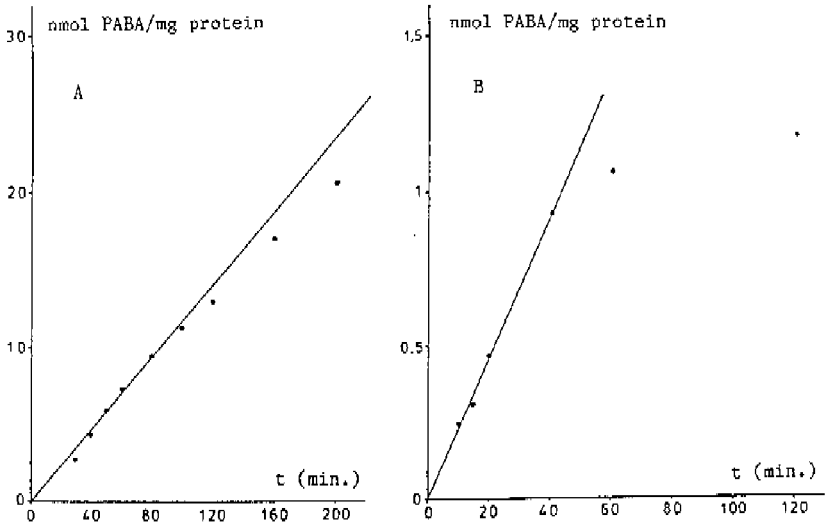

Fig.V.1 Progress curves of PABA production in the glutamine-dependent assay. Extra Component II added. A = wild-type PABS. B = deleted PABS.

It is clear, that non-linearity started at about 40 min. in the glutamine-assay of deleted PABS. No explanation for the appearance of non-linearity in both cases can be given yet, although instability of

wild-type components I and II has been noted before (36). From the progression of PABA production in Fig.V.1B it can be concluded, that the deleted CoI must be more easily inactivated than wild-type CoI. Likely, the loss of 63 C-terminal amino acids has rendered CoI more susceptible to denaturation or proteolysis. Although Walsh et al. (37) reported, that addition of protease inhibitors either before or after cell-lysis did not result in any changes in specific activity, indicating that proteolytic degradation is not a problem, experiments to determine proteolytic activity in PC1539 cells are currently being undertaken (Walsh et al. used a different *E.coli* strain, BN116, the characteristics of which were not described).

It was decided to establish kinetic parameters directly from crude extracts, because attempts to purify wild-type PABS to homogeneity were not successful yet (36,37,38). Moreover, the deleted PABS is more easily inactivated, and enzyme activity of deleted PABS has decreased significantly below wild-type PABS (Fig.V.1B). Two precautions were taken in the kinetic measurements: enzyme activities were assayed immediately after isolation of crude extracts and time of incubation did not exceed 40 min. Furthermore, the wild-type and deleted CoI extracts were prepared simultaneously and were kinetically characterised in parallel experiments in order to rule out any difference in assay conditions. In a number of independent experiments, the kinetic parameters of wild-type CoI and deleted CoI were determined, and found to be as shown in Fig.V.2 and Table V.1.

In case glutamine was used as a substrate, a concentrated extract of PC1539 cells grown on rich medium was added as a source of CoII in order to compensate for an expected overproduction of CoI in transformed cells due to the cloning in pUC19.

As the total amount of protein extracted during the preparation of cell-free extracts varied only slightly in several experiments, and because the concentration of wild-type and deleted PABS enzyme produced in vivo is likely to be identical (same host cell and cloning vector, same quantity of CoII, no change in the regulatory region of the gene coding for CoI), it is justified to conclude that a change in Vmax is due to a change in k_{cat} and not to a change of enzyme concentration.

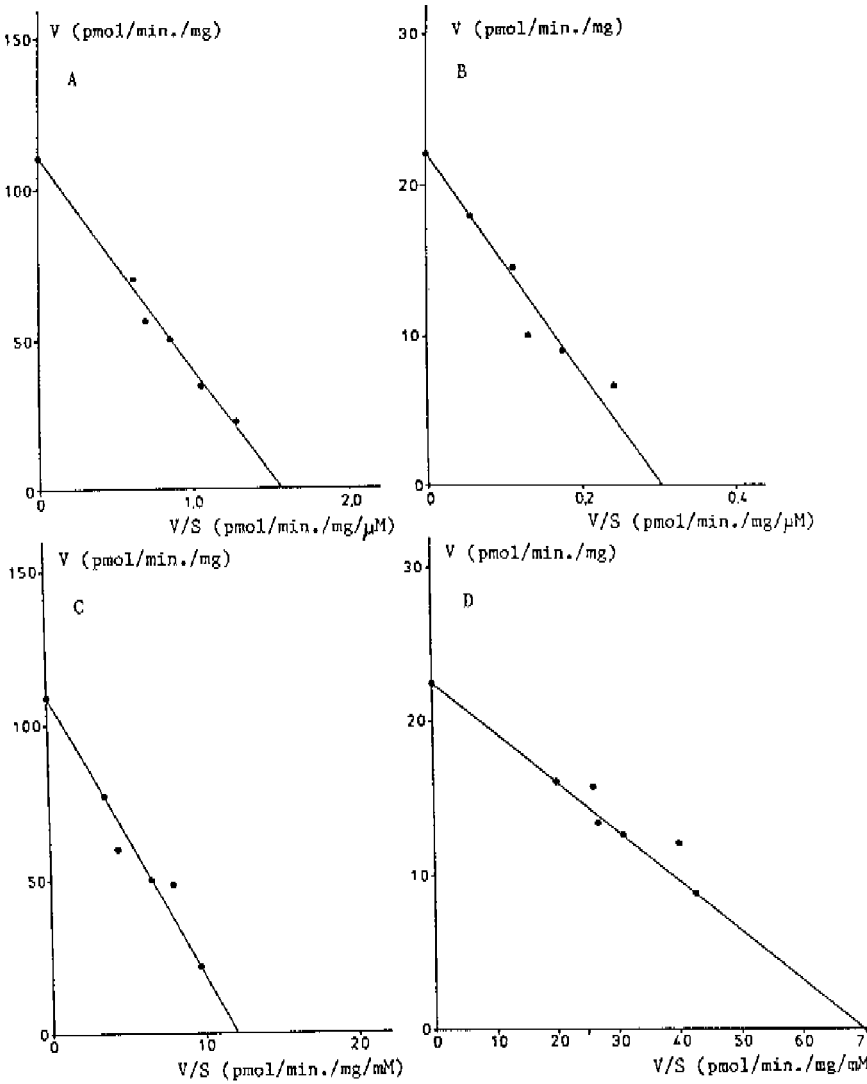

Fig.V.2 Eadie-Hofstee plots of V versus V/S of the glutamine assay of wild-type PABS (A,C) and deleted PABS (B,D). Chorismate was varied in A and B, while glutamine was present at saturating concentration of 50mM. Glutamine was varied in C and D, while chorismate was present at saturating concentration of 200 μM.

Cell-free extract	NH_4^+-dependent assay				Gln-dependent assay		
	Component II added	Vmax(NH_3) pmol/min./mg	Km(Ch) μM	Km(NH_3) mM	Vmax(Gln) pmol/min./mg	Km(Ch) μM	Km(Gln) mM
PC1539/pUC19pabB	-	214	50	10			
PC1539/pUC19pabB	+	134	55	-	111	72	9.2
PC1539/pUC19pabB390-1	-	0*	-	-			
PC1539/pUC19pabB390-1	+	0*	-	-	22	74	0.3

Table V.1

Kinetic parameters of wild-type CoI and deleted CoI and of the respective reconstituted PABS enzymes. Results of the glutamine-dependent assay are also shown in Fig.V.2. * = Slight amount of PABA observed did not exceed the blank value measured in the absence of added NH_3 or Gln and corresponded to a Gln concentration of 0.13 mM in the crude extract.

A number of points emerge from the results presented. First of all, PC1539 cells are still able to produce sufficient amounts of PABA for growth, using the mutated pabB gene cloned into pUC19. Secondly, NH_4^+-dependent PABA production was found to be absent. Thirdly, Vmax(Gln) was found to be 22 pmol/min./mg, i.e. fivefold lower than the wild-type value of 111 pmol/min./mg. Fourthly, it can be concluded, that both in vitro and in vivo the reconstitution of PABS from components I and II is still possible, despite the deletion of 63 amino acids. Finally, it is remarkable, that Km for chorismate has not changed at all, whereas Km for glutamine has been depressed by more than one order of magnitude.

V.4 Discussion.

From the data presented it can be concluded, that the introduction of a stopcodon in the nucleotide sequence of pabB has indeed produced a modified CoI upon expression: (i) the pabB390-1 CoI is more easily inactivated than the wild-type CoI as judged from the progression curves in Fig.V.1; (ii) PABA production in the NH_4^+-dependent assay is virtually absent, whereas PABA production in the glutamine-dependent

assay is still operative, though at a fivefold lower level; (iii) Km(Gln) has changed considerably, in contrast with Km(Ch); (iv) preliminary experiments on the purification of CoI exhibit a different behaviour of the pabB390-1 gene product as compared to the wild-type: it does not precipitate at 50% $(NH_4)_2SO_4$ saturation, whereas almost all of wild-type CoI does. This phenomenon indicates a clear change of overall properties such as is to be expected for a rather large deletion.

Changing codon Trp 390 in CoI of *E.coli* PABS into a stopcodon has no effect on the growth behaviour of cells, although the kinetic parameters are strongly affected (Table V.1). Since the regulatory part of the mutant gene was not changed, it can be assumed that the observed decrease in Vmax(Gln) is due to a decrease in k_{cat} of CoI.

It might be argued, that the deletion causes a conformational change responsible for loss of activity of CoI, and that on attachment of CoII the proper conformation is restored. Such an explanation, however, does not apply, because the NH_4^+-dependent activity does not return on addition of CoII (Table V.1).

From the fact, that pabA mutant strain AB3292 (39,40) does not grow in the absence of PABA, because it lacks CoII, whereas CoI is fully intact, it can be concluded, that in vivo only the glutamine-dependent activity is responsible for growth. Likewise, in PC1539/pUC19pabB390-1 it is the glutamine-dependent activity that is responsible for growth. Therefore, no matter if the NH_4^+-dependent reaction has been abolished completely, as long as the glutamine-dependent reaction is possible, cells are able to grow. From the results presented in Table V.1, it can thus be concluded, that 22 pmol/min./mg, as measured in cell-free extract, apparently is sufficient for growth of wild-type *E.coli* cells.

The reduced Vmax(Gln) in the mutant is counteracted by the decreased Km(Gln) value. In Fig.V.3, V versus Gln-concentration plots are shown for the wild-type and the truncated enzyme. The curves cross at Gln-concentration = 2 mM. This signifies, that in vivo the PABA production does not suffer from the mutation when the physiological concentration of glutamine is about 2 mM and is even larger when Gln-concentration $<<$ 2 mM. Viewed in this light, the calculated Gln-concentration of 0.13 mM fits well with the fact that cells

containing the mutated pabB grew just as well as wild-type cells.

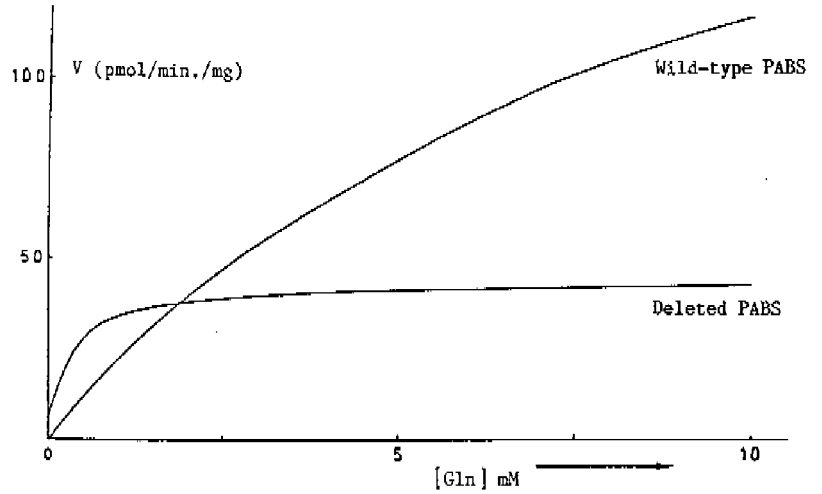

Fig.V.3
V versus glutamine-concentration plots of wild-type and deleted PABS at saturating chorismate concentration of 200 μM. Curves cross at glutamine-concentration of 2 mM.

From these results, it is concluded, that the 63 amino acid deletion of PABS CoI has inactivated the NH_4^+-dependent reaction completely, whereas the glutamine-dependent reaction is still possible. This implies, that the deleted PABS holo-enzyme is able to bind glutamine, the binding-site of which resides on the intact CoII, and that the ammonia abstracted from glutamine is still being channeled to the deleted CoI, where it can react with chorismate to produce PABA. From the unchanged Km(Ch) it is assumed that the chorismate binding-site has not been affected by the deletion. On the other hand, since the NH_4^+-dependent reaction can not take place, uptake of NH_4^+-ions from the medium directly is not possible anymore. In other words, the ammonium binding-site has been affected by removing the 63 C-terminal amino acids of CoI. In addition, this means, that the ammonia from glutamine is not delivered (by CoII) to the original NH_4^+ binding-site in CoI.

The decreased Km(Gln) in the glutamine-dependent assay of the deleted PABS holo-enzyme will be discussed in Chapter VI, in which a model concerning a possible reaction scheme for the conversion of chorismate plus glutamine to PABA is presented.

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Chapter VI.

Kinetic analysis of PABS CoI having Cys 391 replaced with serine or Trp 390 replaced with tyrosine by site-directed mutagenesis.

VI.1 Introduction.

Chemical modification studies of AS CoI by Tso & Zalkin (1) have shown the presence of one arginine residue, one histidine residue, and one cysteine residue (377) to be essential for catalysis. In the comparison of AS CoI and PABS CoI amino acid sequences (Fig.IV.1)(2), cysteine 377 is aligned with residue 318 in *E.coli* PABS CoI. If a cysteine residue essential for catalysis exists in PABS CoI, it must be dislocated, since it reads alanine at position 318. After codon 391 mutation in the *E.coli* pabB gene, such that cysteine is replaced with serine upon expression, it will be shown in this chapter that the mutated PABS is still functional, both in the NH_4^+-dependent conversion, as well as in the glutamine-dependent conversion.

From the observation, that *E.coli* AS CoI is completely free of tryptophan residues, it was concluded (3), that its biological significance might be channeling of chorismate into the tryptophan biosynthetic pathway. In order to investigate *E.coli* cells under tryptophan starvation conditions with a tryptophan-free PABS, all tryptophan residues have to be replaced by site-directed mutagenesis, without destroying enzyme activity.

It will be shown here, that Trp 390 can be replaced with tyrosine successfully with respect to retaining enzyme activity, of PABS CoI as well as of the holoenzyme.

Finally, a possible reaction scheme for the conversion of chorismate plus glutamine to PABA will be presented, based on kinetic parameters of mutant enzymes mentioned above, and the deleted PABS CoI described in the previous chapter.

VI.2 Materials and methods.

VI.2.1 Construction of PC1539/pUC19pabB390-2 and PC1539/pUC19pabB391.

In vitro mutagenesis of the *E.coli* pabB gene using mutagenic oligonucleotide Trp 390-2 or Cys 391 was described in Chapter IV. The resulting plasmids pUC19pabB390-2 and pUC19pabB391 were used for transformation into *E.coli* pabB mutated strain PC1539 according to (4). PABA-independent growth of transformed cells was tested on minimal medium plates as described in Chapter II.

VI.2.2 Kinetic analysis.

Preparation of cell-free extracts, enzyme assay and protein determination were as described in Chapter V.

VI.3 Results and discussion.

As in the case of pUC19pabB390-1, recombinant plasmids pUC19pabB390-2 and pUC19pabB391 were also transformed into *E.coli* mutant strain PC1539, since this is the strain best suited for studying PABS enzyme activity, being triple aromatic auxotroph. After picking PC1539 cells, transformed with recombinant plasmids carrying a mutated pabB gene, from selective L-agar plates containing ampicillin, colonies of PC1539/pUC19pabB390-2 and PC1539/pUC19pabB391 were tested for PABS enzyme activity by plating on selective minimal medium. Both were able to grow without the addition of PABA to the medium, and growth behaviour was indistinguishable from wild-type PC1539/pUC19pabB. Since PC1539 was shown to be suppressor-negative (Chapter V), this means that PC1539 cells containing a PABS CoI in which Trp 390 and Cys 391 have been replaced with tyrosine and serine respectively, are still able to produce PABA in sufficient amounts despite these mutations.

From the progression curves of PABA production of the wild-type PABS and deleted PABS as shown in the previous chapter (Fig.V.1) it was known, that the deleted CoI is more easily inactivated during incubation at 37°C than the wild-type CoI. So, before kinetic parameters of the Trp 390 to tyrosine mutant and Cys 391 to serine

mutant were established, inactivation behaviour relative to wild-type CoI was tested. This was done by preincubation of cell-free extract at 37°C in assay mixture without the substrates chorismate and ammonium. After preincubation, remaining activity was tested in the NH_4^+-dependent assay without CoII, the results of which are shown in Fig.VI.1.

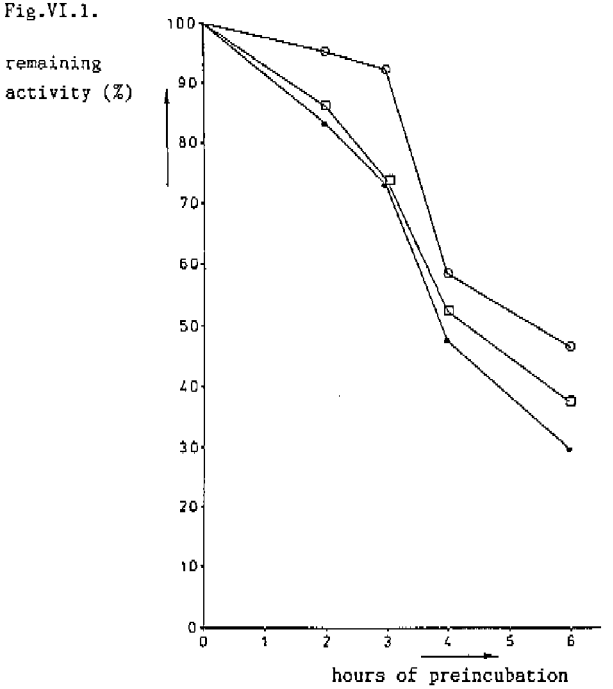


Fig.VI.1 Inactivation of mutated Components I after preincubation in assay buffer at 37°C. Remaining activity (%) relative to the activity determined at t = 0 was measured during 30 min. of NH_4^+-dependent assay. ○ = Trp 390 to tyrosine mutated CoI; □ = Cys 391 to serine mutated CoI; ● = Wild-type CoI.

From Fig.VI.1 it can be concluded, that the mutations of residues 390 or 391 do not lead to a more pronounced inactivation behaviour of CoI, as compared to the deleted CoI. In fact, both mutated components I were found to be at least of the same stability as wild-type CoI; the Trp 390 to tyrosine mutated CoI remained almost fully active (92% of

initial activity) after three hours of preincubation, whereas the wild-type and the Cys 391 to serine mutated CoI had already lost about 27% of their activity.

Because both PC1539/pUC19pabB390-2 and PC1539/pUC19pabB391 were able to grow on minimal medium without PABA, it can be concluded, that in vivo the glutamine-dependent conversion is still possible, since it was established before (Chapter V) that in vivo only the glutamine-dependent activity is responsible for growth. Therefore, reconstitution of the respective mutated components I with wild-type CoII can take place in both cases. In contrast with the deleted CoI, the NH_4^+-dependent conversion is possible also.

Kinetic parameters of the mutated components I in both assays were again determined from crude extracts directly, taking the same precautions as described in case of the deleted CoI (immediate assay and parallel experiments). Results of these experiments are summarized in Table VI.1.

PABS	Vmax(NH_3) pmol/min./mg	Km(Ch) μM	Km(NH_3) mM	Vmax(Gln) pmol/min./mg	Km(Ch) μM	Km(Gln) mM
Wild-type	214	50	10	111	72	9.2
Deleted	0	0	0	22	74	0.3
Trp 390→Tyr	208	18	15	103	10	5.6
Cys 391→Ser	390	31	-	103	-	5.7

Table VI.1 Kinetic parameters of wild-type and mutated PABS Components I, and of the respective holoenzymes.

As far as the Trp 390 to tyrosine mutated PABS is concerned, both Vmax(NH_3) and Vmax(Gln) are virtually unchanged as compared to wild-type PABS. Km(Ch) has decreased substantially, both in the

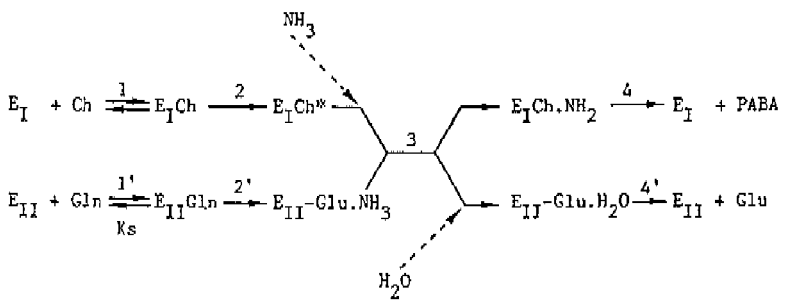
dependent assay as well as in the glutamine-dependent assay. Also, Km(Gln) has decreased, but Km(NH_3) has increased.

As far as the Cys 391 to serine mutated PABS is concerned, enzyme activity in the NH_4^+-dependent assay has been raised by a factor of 1.8, whereas Vmax(Gln) is virtually unchanged. In this mutant also, Km(Gln) and Km(Ch) both have decreased.

From the results presented, it can be concluded, that neither Cys 391, nor Trp 390, is essential for catalysis, since both the NH_4^+-dependent conversion as well as the glutamine-dependent conversion can proceed. The finding, that these residues are not essential for catalysis is in agreement with the results of enzyme assays performed with the deleted PABS. In the deleted PABS these residues were not present any more, but the glutamine-dependent reaction was still functional. Moreover, the results presented here agree with the results of McLeish et al.(5), which indicated the presence of an essential cysteine in CoII only (needed for binding glutamine), but not in CoI.

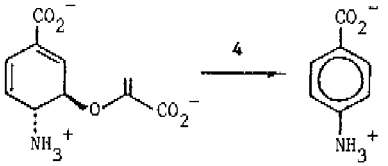
In both mutants discussed in this chapter, the NH_4^+-dependent reaction has not suffered severely from the amino acid substitutions; in fact Vmax(NH_3) is even higher than wild-type Vmax(NH_3) in the Cys 391 to serine mutant. Therefore, uptake of NH_3 from the medium is not inactivated.

A minimum number of reaction steps that are necessarily involved in the conversion of chorismate into PABA can be represented by the following scheme:



In the E_I-chorismate complex, chorismate is converted in step 2 into some as yet unknown intermediate that is capable of reacting with NH_3

that either comes from E_{II} or directly from the medium. Uptake of NH_3 from the medium is inactivated in CoI having 63 amino acids deleted from its C-terminal end. In step 3, the intermediate **7** (Fig.I.2) is produced. Synthetic amino enol pyruvate **7** was shown to be a chemically and kinetically competent intermediate (6,7,8).



7 PABA

Compound **7** is converted to the final product in the aromatization reaction 4, which has been shown not to be rate limiting (6).

Since the NH_4^+-dependent conversion can be inactivated, by preventing the utilization of NH_3 from the medium, without simultaneous inactivation of the glutamine-dependent conversion, it is concluded, that ammonia originating from glutamine arrives from a site different from the site responsible for uptake of ammonia from the medium.

In the hydrolysis of glutamine by E_{II} it is reasonable to assume the occurrence of an acylenzyme intermediate as has been observed in numerous serine and thiol proteases (9). This was also found in the case of AS CoII (10–17).

Intrinsically reaction 4', the deacylation step, is likely not to be rate limiting, comparable with the hydrolysis of amides by proteases. For reaction 4' to proceed, however, it is necessary to replace NH_3 in the E_{II}-glu.NH_3 complex by H_2O (see also the scheme for glutamine amide transfer by AS CoII as suggested by Amuro et al. (18), Fig.I.6). Reaction 4' will therefore be prohibited as long as reaction 3 has not occurred; it will be resumed only as far as reaction 3 proceeds. Of the two steps, reaction 3 is therefore the rate limiting one and reaction 4' the kinetically irrelevant one.

This scheme provides a partial explanation for the decrease of Km(Gln) in the mutant enzymes. For an enzyme reaction with an acylenzyme intermediate Km equals (9):

$$Km = \frac{k_3}{k_3 + k_2} \cdot Ks \qquad \text{and} \qquad Vmax = \frac{e.k_2.k_3}{k_2 + k_3}$$

For the present case:

$$Km(Gln) = \frac{k_3}{k_3 + k_2'} \cdot Ks(Gln) \quad (1) \quad \text{and} \quad Vmax = \frac{e.k_2'.k_3}{k_2' + k_3} \quad (2)$$

A more systematic derivation of these equations is presented in the Appendix.

Since E_{II} was not mutated, Ks(Gln) and k_2' may be unchanged. Furthermore, e is considered to be approximately equal. On the other hand, in the deleted E_I k_3 has been reduced. It follows from equations 1 and 2 that Km(Gln) should be reduced to the same extent as Vmax, i.e. fivefold in case of the deleted PABS. Actually, the decrease of Km(Gln) is thirtyfold. This suggests, that k_3 has decreased thirtyfold, partly compensated by a sixfold increase in e. On the other hand it is imaginable, that CoII, on combining with CoI, acquires a somewhat different conformation that exhibits other k_2' and/or Ks values. It will require purification of the wild-type and the truncated enzymes to settle this question.

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Chapter VII.

Cloning strategy for increasing PABS expression.

VII.1 Introduction.

In microorganisms, the multibranched shikimic acid pathway contains an important common intermediate, chorismic acid. An intriguiging feature of chorismate-converting enzymes is the way in which a large difference in the amount of end-products like o-aminobenzoate (anthranilate, AA) and p-aminobenzoate (PABA) is maintained. Although the physiological importance of this phenomenon is evident (PABA being synthesized as a precursor for a vitamin, folic acid, and AA being synthesized as a precursor for an aromatic amino acid, Trp), it is not yet clear, in which way chorismate is mainly channeled into the aromatic amino acid pathway in vivo.

Since enzyme activity of PABS in cell-free extracts is measured in the pmoles/min./mg range, whereas AS activity is measured in the nanomoles/min./mg range, the different amounts of PABA and AA produced in vivo might be due to different efficiencies of underlying catalytic mechanisms. This is only true, if the same amounts of PABS and AS are present in the cells. Unfortunately, attempts to purify PABS to homogeneity by different research groups (1-4) have not been successful so far. Therefore, it can not be determined, if AS is converting chorismate with higher efficiency than PABS, as long as purified PABS is not available.

Apart from the mechanism of enzyme catalysis, several other ways to regulate specific conversion of chorismate to vitamin or amino acid exist. For example, AS is subject to feed-back inhibition by tryptophan, and the region of the AS amino acid sequence probably responsible for it, has been determined (5). In case of PABS, no feed-back inhibition has been reported.

Also, the complete absence of tryptophan residues in the AS amino acid sequence might play a role in chorismate channeling (6), as mentioned.

So far, only intrinsic features of the enzymes themselves, which could influence the amount of product synthesized, have been

discussed. Of course, regulation of enzyme expression on transcription or translation level can be another way to maintain a high cellular AA to PABA synthesis ratio. In this respect, the Trp operon is a classical example of regulation on both levels. AS is encoded by trpE, the first gene on polycistronic mRNA; fine-tuning of tryptophan synthesis in cells is achieved by controlling transcription and translation of this mRNA, not only by means of a repressor protein, but also by means of attenuation, the mechanism of which has been elucidated by Yanofsky et al. (7).

As far as PABS is concerned, the gene coding for CoI, pabB, is not on the same mRNA as the gene coding for CoII, pabA. The way in which coordinate expression is achieved is not yet known. Also, for the pabB-mRNA, no operator or repressor protein has been found.

The strategy for optimizing PABS CoI expression described in this chapter was developed in the first place to investigate the way in which cells regulate PABS enzyme production in vivo, and secondly to facilitate purification of PABS CoI in vitro.

VII.2 Materials and methods.

VII.2.1 Materials.

Plasmid pKK223-3 was obtained from Pharmacia.

VII.2.2 Oligonucleotide synthesis.

Synthesis of mutagenic oligonucleotide Ala-7 5' ^{30}AGTAATCACC*GCGGGAGA^{13} 3' was performed as described in Chapter III.

VII.3 Results and discussion.

It was found by the group of Gil et al. (8-12), that the pabS gene of *Streptomyces griseus*, which complements both pabA and pabB mutations in *E.coli*, is subject to in vivo repression by phosphate, only in *S.griseus*, but not in *E.coli*. The pabS gene is expressed in *E.coli* only after deletion of about 1 kb of the original cloned fragment (4.5 kb). Transcriptional readthrough from the pBR322 Tet promoter is

responsible for expression. The 1 kb deleted portion was found to contain the *S.griseus* pabS promoter as well as a phosphate control sequence (PCS), located upstream of the pabS gene.

Another pab gene, pabP (13) has been cloned, from *Streptomyces acrimycini*, the expression of which is very low as compared to the expression of pabS.

From these results, it was suggested, that in *Streptomyces* there seem to be specific genes, like pabS, involved in secondary metabolism (candicidin biosynthesis). These genes can be expressed at high level by an efficient promoter and regulation is dependent on cellular phosphate concentration. The pabP gene on the other hand, is suggested to function in primary metabolism, producing PABA for vitamin synthesis only. A PCS is missing in case of the pabP gene. Although promoter sequences of both genes have not been published yet, the low expression of the pabP gene might be due to a difference in promoter strength as compared to the pabS gene.

Plasmid pUC19pabB was requested from us by P.Liras (research group of Gil et al.), in order to test the cloned insert for the presence of a PCS sequence. It was found, that expression of the cloned fragment containing the pabB gene was not under control of cellular phosphate (personal communication). This can either mean, that a PCS has not been cloned (150 nucleotides upstream the pabB gene are present, see Chapter III, whereas a 1 kb fragment upstream the pabS gene contains the *S.griseus* PCS), or that a PCS does not exist in *E.coli*. The latter is most likely, for the following reasons: (i) the *E.coli* strain from which the pabB has been cloned, does not synthesize antibiotics which would require PABA as a precursor, (ii) expression of the *E.coli* pabB gene is comparable to expression of the pabP gene and to the pabS gene from which the PCS and promoter have been deleted.

From the results presented above it is concluded, that expression of *E.coli* PABS could possibly be increased in two different ways: first, by removal of the complete original 5' region of pabB, including the putative weak promoter and the ribosome binding site (RBS), followed by insertion of the remaining coding sequence into an expression vector containing a strong promoter and an efficient RBS. Secondly, by cloning the gene coding for ColI, pabA, in tandem with pabB, to achieve coordinate, high expression of both genes. In order

to do so, transcriptional stop signals of the pabB gene as well as the pabA promoter must be removed, such that one contiguous mRNA can be transcribed with high efficiency from the strong promoter, inserted upstream pabB. The expression vector chosen was pKK223-3, as shown in Fig.VII.1.

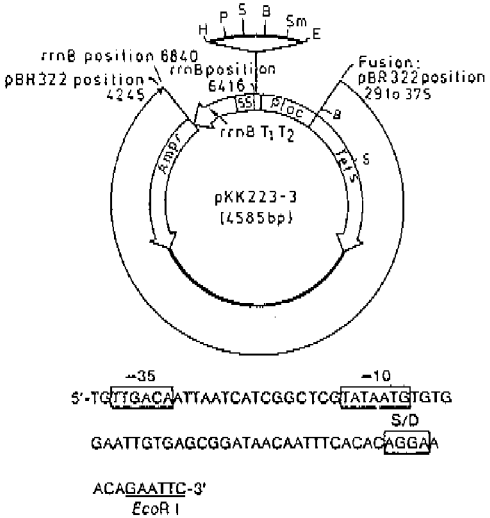

Fig.VII.1 Expression vector pKK223-3. H = HindIII; P = PstI; S = SalI; B = BamHI; Sm = SmaI; E = EcoRI. P_{tac} and S/D sequences shown separately. rrnB = ribosomal RNA transcription terminators.

In the following section, a strategy for optimizing PABS CoI expression is described. Results of cloning the pabB gene into the expression vector pKK223-3, using this strategy, are under way.

As stated in Chapter IV, the *E.coli* pabB gene contains only a limited number of restriction sites that could be used for cassette mutagenesis. Especially in the 5' region, restriction sites that could be used to remove regulatory sequences, are missing. Therefore, it was decided to use the 5' BamHI site at position -134 (Fig.III.2) and to create a second, new restriction site downstream the putative ribosome binding site. Since there are only 3 nucleotides between the RBS and the ATG start codon, this implies that the restriction site has to be introduced in the pabB coding sequence. Thus, it should be silent with

respect to the coding sequence. The first silent mutation, that can be created with a single base substitution, and that is unique in pabB, is positioned within the Ala-7 codon, and the restriction site created will be a SacII site.

Mutagenic oligonucleotide Ala-7: 5' ^{30}AGTAATCACC*GCGGGAGA^{13} 3' was synthesized to be used for in vitro mutagenesis by the gapped heteroduplex technique, as described in Chapter IV. Sequencing of possible Ala-7 mutated M13mp18pabB ss DNAs is now in progress.

Because the expression vector does not contain a SacII site in its polylinker, and because the first 7 codons will be removed after digestion also, a double-strand adaptor sequence will be necessary to clone the remaining pabB coding sequence into pKK223-3. Two single strand oligonucleotides were synthesized, adaptor-19 and adaptor-25, which were 5' phosphorylated and annealed as shown in Fig.VII.2.

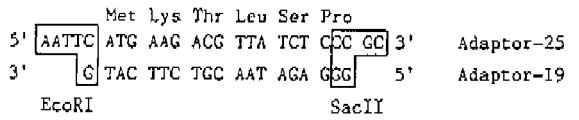

Fig.VII.2 Double strand adaptor produced by annealing single strand 19-mer and 25-mer oligonucleotides.

The Ala-7 mutated pabB can be cloned in pKK223-3 using the annealed adaptor and restriction sites as indicated in Fig.VII.3. Since pKK223-3 contains the strong trp-lac (P_{tac}) promoter (14), a higher transcription rate is to be expected. Moreover, expression of PABS can be induced by the addition of IPTG, which derepresses the tac promoter. Alternatively, a $lacI^q$ genotype may be introduced into the triple aromatic auxotroph strain PC1539. Adjacent to the pabB coding region (3' end) a DNA segment containing the strong rrnB ribosomal RNA transcription terminators (15,16) will be present, such that only pabB will be over-expressed. Translation of the pabB containing mRNA can be expected to increase also, since a vector with similar configuration, i.e. using the lacUV-5 RBS, has been used to over-express the lambda cI gene giving yields of 18 to 26% in cellular extracts following IPTG induction (17); pKK223-3 has also been used to express dehydroquinate

synthase (18).

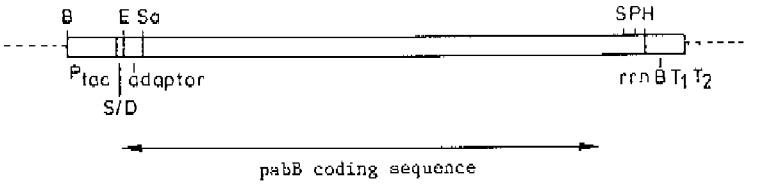

Fig.VII.3 Construct of pKK223-3/pabB, after ligation of HindIII + EcoRI digested pKK223-3, the double strand adaptor as shown in Fig.VII.2, and SacII + HindIII digested Ala-7 mutated pabB. B = BamHI; E = EcoRI; Sa = SacII; S = SalI and P = PstI. P_{tac} = trp-lac promoter, S/D = Shine-Delgarno sequence and rrnB T_1T_2 = ribosomal RNA transcription terminators.

The strategy outlined here will be tested first with the pabB gene alone. If expression is satisfactory, i.e. the NH_4^+-dependent assay in vitro should reach the nmoles/min./mg range, it can be considered to introduce the pabA gene in tandem with pabB (after removal of pabB transcription termination signals), just upstream the rrnB sequences.

VII.4 References.

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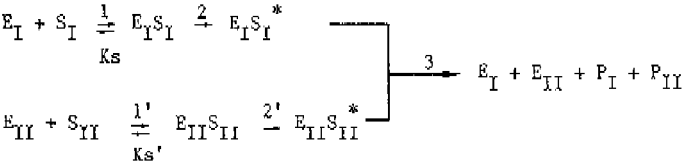
Appendix.

Kinetics of two parallel running, interconnected, enzyme reactions.

One enzyme molecule carries two active sites that catalyze different reactions. It is irrelevant whether these sites are contained in one protein chain or in two different subunits. The case is examined in which each active site catalyzes one reaction step before the step they have in common:



The reaction rate, v, is equal to

$$v = \frac{dP_I}{dt} = \frac{dP_{II}}{dt} = k_3[E_IS_I^*][E_{II}S_{II}^*]/e \qquad (1)$$

in which e denotes the total enzyme concentration; the rate is proportional to $E_IS_I^*$ concentration times the occupancy of the second site, $[E_{II}S_{II}^*]e$. It is a common procedure to measure the effect of the concentration of one substrate on the reaction rate when the enzyme is saturated with the other substrate, i.e. $S_I >> Ks$. The following conservation equations then apply:

$$e = [E_IS_I] + [E_IS_I^*] \qquad (2)$$

$$e = [E_{II}] + [E_{II}S_{II}] + [E_{II}S_{II}^*] \qquad (3)$$

Applying the steady state assumption to all intermediates provides:

$$\frac{d[E_IS_I^*]}{dt} = 0 = k_2[E_IS_I] - k_3[E_IS_I^*][E_{II}S_{II}^*]/e \qquad (4)$$

$$\frac{d[E_{II}S_{II}^*]}{dt} = 0 = k_2'[E_{II}S_{II}] - k_3[E_IS_I^*][E_{II}S_{II}^*]/e \qquad (5)$$

$$\frac{d[E_{II}S_{II}]}{dt} = 0 = k_1'[E_{II}][S_{II}] - (k_{-1}' + k_2')[E_{II}S_{II}] \qquad (6)$$

Combination of equations (2) and (4) yields:

$$[E_IS_I^*] = e/(1 + \frac{k_3}{ek_2}[E_{II}S_{II}^*]) \qquad (7)$$

Combination of equations (3) and (6) yields:

$$E_{II}S_{II} = \frac{e - [E_{II}S_{II}^*]}{1 + (k_{-1}' + k_2')/k_1'[S_{II}]} = \frac{e - [E_{II}S_{II}^*]}{1 + Ks'/[S_{II}]} \qquad (8)$$

Making the usual assumption $k_{-1}' >> k_2'$.
Insertion of equations (7) and (8) into equation (5) yields:

$$k_2' \frac{e - [E_{II}S_{II}^*]}{1 + Ks'/[S_{II}]} = \frac{k_3[E_{II}S_{II}^*]}{1 + [E_{II}S_{II}^*]k_3/k_2e} \qquad (9)$$

Although it is possible to solve $[E_{II}S_{II}^*]$ from equation (9) and insert it into equations (7) and (1) the equations generally become unwieldly and do not exhibit simple Michaelis-Menten type of substrate concentration dependence. Such a solution is possible only when $k_2 >> k_3$. Equations (7) and (9) then simplify into

$$[E_IS_I^*] = e \qquad (7a)$$

and $$[E_{II}S_{II}^*] = e/\left[1 + \frac{k_3}{k_2}(1 + Ks'/[S_{II}])\right] \qquad (9a)$$

Insertion of equations (7a) and (9a) into equation (1) finally yields:

$$v = \frac{ek_{cat}}{1 + Km'/[S_{II}]} \qquad (10)$$

in which

$$k_{cat} = \frac{k_2'k_3}{k_2' + k_3} \qquad (10a) \qquad \text{and} \qquad Km' = \frac{k_3}{k_2' + k_3} Ks' \qquad (10b)$$

It is evident from equation (10) that when k_3 decreases, km' decreases to the same extent.

Summary.

This thesis describes the cloning, sequencing and in vitro mutagenesis of the *E.coli* pabB gene coding for p-aminobenzoate synthase Component I. The goal of this study was to gain insight in the catalytic mechanism of the enzyme by kinetic analysis of engineered Components I upon expression of mutated pabB.

In Chapter I, literature available on the genetic organisation and the reaction mechanism of the closely related enzymes p-aminobenzoate synthase (PABS) and anthranilate synthase (AS) has been reviewed. Both enzymes utilize chorismic acid as a substrate, converting it to p-aminobenzoate or o-aminobenzoate respectively. PABS and AS both consist of two subunits, Component I and Component II. In vitro, Component I is responsible for aminase activity, whereas Component II is the glutamine amidotransferase component.

In *E.coli*, the gene coding for PABS Component I (CoI), pabB, is located on 40 min. on the genome. Chapter II describes restriction mapping of 23.7 kb from this region, using Clarke & Carbon plasmids pLC15-17, pLC15-32 and pLC30-32. Apart from the pabB gene, the *E.coli* gene coding for acylCoA synthetase (E.C.6.2.1.3), fadD, was cloned from this region also.

Chapter III describes the sequencing of pabB, cloned into phage M13mp18. Putative regulatory sequences are discussed, and the amino acid sequence deduced from the coding sequence has been aligned with the amino acid sequence of AS CoI. From this comparison, a number of residues were chosen to be mutated.

Chapter IV deals with site-directed mutagenesis of pabB, using a gapped heteroduplex technique. Codon Trp 390 was mutated to a stopcodon, deleting 63 amino acids from the C-terminal end of PABS CoI upon expression. Alternatively, residue Trp 390 was replaced with tyrosine, and residue Cys 391 was replaced with serine.

In Chapter V, the results of kinetic analysis of the deleted PABS CoI are presented. It is shown, that the deleted CoI is still functional in vivo, although the NH_4^+-dependent conversion of chorismate to p-aminobenzoate is inactivated. The glutamine-dependent conversion is the only one used in *E.coli* metabolism. Therefore, reconstitution of deleted CoI and CoII is still possible.

Since the deleted CoI is unable to use NH_4^+-ions from the medium directly, it is concluded, that the ammonia from glutamine, which is channeled from CoII

to CoI, is not delivered to an initial NH_4^+-binding site in CoI.

Chapter VI describes the kinetic analysis of mutant-enzymes, in which Trp 390 and Cys 391 have been replaced with tyrosine and serine, respectively. Based on the combined analyses of mutant-enzymes, a preliminary model for the reaction mechanism of the PABS holo-enzyme has been presented.

Finally, in Chapter VII a strategy for increasing PABS expression using a silent mutation construct has been described.

Samenvatting.

Dit proefschrift beschrijft het kloneren, de sequentie-bepaling en in vitro mutagenese van het *E.coli* pabB gen coderend voor p-aminobenzoate synthase Component I. Doel van dit onderzoek was inzicht te verkrijgen in het katalytisch mechanisme van het enzym door kinetische analyse van de veranderde Component I na het tot expressie brengen van het gemuteerde pabB gen.

Hoofdstuk I geeft een samenvatting van de beschikbare literatuur betreffende de genetische organisatie en het reactiemechanisme van de nauw verwante enzymen p-aminobenzoaat synthase (PABS) en anthranilaat synthase (AS). Beide enzymen gebruiken chorismaat als substraat, waarbij respectievelijk p-aminobenzoaat of o-aminobenzoaat wordt gevormd. PABS en AS zijn samengesteld uit twee subunits, Component I en Component II. Component I bezit in vitro aminase activiteit, terwijl Component II verantwoordelijk is voor de amidotransferase activiteit.

Het gen coderend voor PABS Component I (CoI), pabB, werd gelokaliseerd op 40 min. van het *E.coli* genoom. Hoofdstuk II beschrijft de restrictie-mapping van 23.7 kb van deze regio, waarbij gebruik werd gemaakt van Clarke & Carbon plasmiden pLC15-17, pLC15-32 en pLC30-32. Behalve het pabB gen werd ook het *E.coli* gen coderend voor acylCoA synthetase (E.C.6.2.1.3), fadD, gekloneerd uit dit gebied.

Hoofdstuk III beschrijft de sequentie-bepaling van pabB, gekloneerd in M13mp18. Mogelijk regulatoire sequenties worden besproken, en de aminozuurvolgorde afgeleid uit de coderende sequentie wordt vergeleken met de aminozuurvolgorde van AS CoI. Op basis van deze vergelijking werden een aantal residuen gekozen voor mutagenese.

Hoofdstuk IV behandelt de plaats-specifieke mutagenese van pabB, waarbij gebruik gemaakt werd van de zgn. "gapped heteroduplex" techniek. Codon Trp 390 werd gemuteerd tot stopcodon, waardoor een deletie van 63 aminozuren ontstond, wanneer PABS CoI tot expressie werd gebracht. Residu Trp 390 werd daarnaast vervangen door tyrosine, en residu Cys 391 door serine.

Hoofdstuk V toont de resultaten van de kinetische analyse van de gedeleteerde CoI. Het blijkt, dat de gedeleteerde CoI nog functioneert in vivo, hoewel de NH_4^+-afhankelijke omzetting van chorismaat naar p-aminobenzoaat is geïnactiveerd. Reconstitutie van de gedeleteerde CoI met CoII bleek nog wel mogelijk; vandaar de conclusie dat alleen de glutamine-afhankelijke conversie functioneert in het metabolisme van *E.coli*. Het feit, dat de gede-

leteerde CoI niet in staat is om NH_4^+-ionen op te nemen uit het medium betekent, dat ammonia afkomstig van glutamine, en via CoII overgebracht naar CoI, niet arriveert op een eerste NH_4^+-bindingsplaats in CoI.

Hoofdstuk VI beschrijft de kinetische analyse van mutant-enzymen, waarin Trp 390 en Cys 391 respectievelijk werden vervangen door tyrosine en serine. Gebaseerd op de gecombineerde analyse van mutant-enzymen is een model opgesteld voor het reactiemechanisme van het PABS holo-enzym.

In Hoofdstuk VII tenslotte, wordt een strategie beschreven voor verhoging van de expressie van PABS, gebruikmakend van een zgn. silent mutatie.

Curriculum vitae.

De auteur van dit proefschrift werd geboren op 16 april 1956 te Berghem. Na het behalen van het Gymnasium β diploma aan het Titus Brandsma Lyceum te Oss in 1974, werd in datzelfde jaar begonnen met de studie Biologie aan de Katholieke Universiteit Nijmegen. Het doctoraalexamen (B4) met als Hoofdvak Moleculaire Biologie (Prof. Dr. J.G.G.Schoenmakers) en bijvakken Microbiologie (Prof. Dr. Ir. G.D. Vogels) en Biochemie (Prof. Dr. H.J. Hoenders) werd behaald in maart 1981. Tijdens de doctoraalstudie werd het C-diploma, Deskundigheid Stralingshygiëne, behaald. Van 1 april 1981 tot 1 juli 1988 was hij werkzaam bij de vakgroep Organische Chemie van de Technische Universiteit Eindhoven. In april 1982 werd het diploma Microbiële veiligheid bij rec. DNA werkzaamheden behaald. In de periode juni 1984 tot 1 juli 1988 werd het onderzoek, beschreven in dit proefschrift, verricht onder leiding van Prof. Dr. H.M. Buck. Per 1 juli is de auteur van dit proefschrift in dienst getreden van de Technische Universiteit Eindhoven als universitair docent bij de vakgroep Organische Chemie.

Dankwoord.

Op deze plaats wil ik allen, die een bijdrage hebben geleverd aan de totstandkoming van dit proefschrift dank zeggen.

Dit geldt in het bijzonder voor Prof. Dr. H.M. Buck, Prof. Dr. E.M. Meijer en Prof. Dr. L.A.AE. Sluijterman, vanwege hun aandeel in het onderzoek dat ten grondslag ligt aan dit proefschrift.

Prof. Dr. R.N.H. Konings en Prof. Dr. J.G.G. Schoenmakers ben ik dank verschuldigd voor hun gastvrijheid, die mij in de gelegenheid stelde een aantal experimenten uit te voeren op de afdeling Moleculaire Biologie van de Katholieke Universiteit Nijmegen.

Een woord van dank gaat uit naar de medewerkers van de Sectie Bio-Organische Chemie, DSM, Geleen, voor het beschikbaar stellen van apparatuur en chemicaliën, en Dhr. T. Brandsma van het Philips Natuurkundig Laboratorium, Eindhoven, voor het gebruik van zijn fluorescentiespectrofotometer.

Alle studenten van de Hogeschool Interstudie, Oss, en de Hogeschool West-Brabant, die in het kader van hun stageperiode aan het onderzoek hebben deelgenomen, wil ik dank zeggen voor hun bijdrage. Ook de studenten van de Technische Universiteit Eindhoven, die in het kader van dit promotie-onderzoek hun afstudeerwerk hebben verricht, ben ik dank verschuldigd.

Henk Eding wil ik mijn dank betuigen voor het consciëntieus verzorgen van alle tekeningen en de lay-out.

Tenslotte wil ik alle medewerkers van de SBD, Athene T.U.E., en de vak-groep Organische Chemie, m.n. mijn kamergenote Drs. Jana van de Goor, hartelijk danken voor hun bijdrage in de vorm van wetenschappelijke discussies, alsook voor het creëren van een prettige werksfeer.

Stellingen.

1. De bewering van L. Clarke & J. Carbon, dat de door hen geconstrueerde *E.coli* genenbank voldoende recombinant pColE$_I$ plasmiden bevat om representatief te zijn voor het gehele *E.coli* genoom, wordt in de praktijk niet bevestigd gezien het ontbreken van o.a. het gap gen coderend voor glyceraldehyde-3-fosfaat dehydrogenase en het fadL gen coderend voor long chain fatty acid transport protein.

   L. Clarke & J. Carbon (1976) Cell 9, 91-99.
   C.L. Ginsburgh; P.N. Black & W.D. Nunn (1984) J. Biol. Chem. 259, 8437-8443.

2. De identificatie van *E.coli* genen met bijbehorende proteïne spots na 2-D gel-electrophorese, op recombinant plasmiden pLC4-21, pLC15-32 en pLC30-20, zoals weergegeven door F.C. Neidhardt et al., is onjuist.

   F.C. Neidhardt; V. Vaughn; T.A. Phillips & P.L. Bloch (1983) Microbiol. Rev. 47. 231-284.
   D.P. Clark & J.E. Cronan, Jr. (1981) Meth. Enzymol. 72, 693-707.

3. Bij het toepassen van oligodeoxyribonucleotiden als selectieve anti-sense inhibitors van gen-expressie verdient het de voorkeur gebruik te maken van fosfaat-gemethyleerde analoga i.p.v. fosforothioaat analoga.

   C.A. Stein; C. Subasinghe; K. Shinozuka & J.S. Cohen (1988) Nucl. Acids Res. 16, 3209-3221.
   M.H.P. van Genderen; L.H. Koole & H.M. Buck (1988) Proc. Kon. Ned. Acad. van Wetensch. B 91, 179-183.

4. De door C.C. DiRusso gegeven locatie van het *E.coli* fadD gen coderend voor acylCoA synthetase, op 24 min. van het genoom, is in tegenspraak met de eerder gepubliceerde locatie van fadD op 40 min. van het genoom door P.N. Black et al., en komt evenmin overeen met de resultaten vermeld in dit proefschrift.

   C.C. DiRusso (1988) Nucl. Acids Res. 16. 7995-8009.
   P.N. Black; B. Said; C.R. Ghosn; J.V. Beach & W.D. Nunn (1987) J. Biol. Chem. 262. 1412-1419.
   Hoofdstuk II van dit proefschrift.

5. Gezien de gedetailleerde kennis betreffende het reactiemechanisme en de 3-D structuur van Horse Liver Alcohol Dehydrogenase, die al geruime tijd beschikbaar is, wordt het opmerkelijk, dat het gen coderend voor dit enzym nog niet is gekloneerd ten behoeve van *in vitro* mutagenese experimenten.

H. Eklund; C.-I. Bränden & H. Jörnvall (1976) J. Mol. Biol. 102, 61-73.
H. Eklund & C.-I. Bränden (1987) in Biological Macromolecules and Assemblies, Chapter 2, 73-142.

6. De ongebruikelijke weergave van specifieke enzymactiviteit, door C.-Y.P. Teng et al. en C.T. Walsh et al., in units per mg, of in pmol per min. per unit, in combinatie met een unit-definitie als de hoeveelheid enzym benodigd om in 30 min. 1 nmol product te vormen, maakt de interpretatie van experimentele gegevens onnodig verwarrend.

C.-Y.P. Teng; B. Ganem; S.Z. Doktor; B.P. Nichols; R.K. Bhatnagar & L.C. Vining (1985) J. Am. Chem. Soc. 107, 5008-5009.
C.T. Walsh; M.D. Erion; A.E. Walts; J.J. Delany III & G.A. Berchtold (1987) Biochemistry 26, 4734-4745.

7. De zorgvuldigheid, waarmee referees het manuscript van *C.T. Walsh et al., (1987) Biochemistry 26, 4734-4745* hebben beoordeeld, kan onvoldoende worden genoemd, gezien b.v. consequent wordt gesproken over p-aminobenzoaat synthase, terwijl p-aminobenzoaat synthase Col wordt bedoeld, intermediair 4-amino-4-deoxychorismaat regelmatig wordt verwisseld met analogon 5,6-epoxy-5,6-dihydrochorismaat, en de genetische markers van de gebruikte *E.coli* niet zijn vermeld.

8. De interpretatie van experimentele data, verkregen uit kinetische analyse van NAD^+ analoga, door J.-P. Samama et al., is veelal kwalitatief van aard, zonder relaties te leggen tussen geometrie en functie van het analogon in het enzym Lactaat Dehydrogenase.

J.-P. Samama; N. Marchal-Rosenheimer; J.-F. Biellmann & M.G. Rossmann (1981) Eur. J. Biochem. 120, 563-569.

9. De uitspraak van J. Gerlt, dat " The term site-directed mutagenesis may have connotations that presumably stem from the common view that any field or area of investigation that resides on the properties of DNA is too complex to be viewed as chemistry, and therefore must be biology" geeft aan, dat biologie en chemie nog teveel als aparte disciplines worden gezien, waardoor het perspectief van multidisciplinair onderzoek wordt onderschat.

J. Gerlt (1987) Chem. Rev. 87, 1079-1105.

H.J.M. Kocken *Eindhoven, 27-1-1989*