β -KETOACYL-ACP SYNTHASE I OF ESCHERICHIA COLI: NUCLEOTIDE SEQUENCE OF THE *fabB* GENE AND IDENTIFICATION OF THE CERULENIN BINDING RESIDUE

by

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The *fabB* gene of *E. coli* encoding β -ketoacyl-ACP synthase I has been isolated by complementation and sequenced. The enzyme has been purified and its NH₂-terminal residues sequenced. Identification of the active site was accomplished by tagging with ³H-cerulenin and radio sequencing of the region. Comparison of the deduced primary structures of the *fabB* gene product with the *FAS2* gene product of *Saccharomyces cerevisiae* revealed the probable active site in chalcone synthases of higher plants.

1. INTRODUCTION

During each successive cycle of elongation in the synthesis of *E. coli*'s long chain fatty acids, an enzyme condenses a two carbon unit to a primer or the growing chain. The ten carbon stage is a branch point at which a double bond may be kept in the chain (53, 60). The resulting saturated 16 carbon (C₁₆) and unsaturated 16 and 18 carbon (C_{16:1}, C_{18:1}) chains are the predominant fatty acyl moieties of the membrane phospholipids. Two groups of mutants defective in condensation reactions are known (16, 17,

60). Those with mutations in the *fabB* gene are thought to be impeded in the elongation step at the branch point leading to C_{16:1} and C_{18:1}, whereas those with mutations in the *fabF* gene are hampered in elongation of C_{16:1} to C_{18:1}. The two genes encode β -ketoacyl-ACP synthases I and II, respectively. Recently a third condensing enzyme has been identified, namely acetoacetyl-ACP synthase (27) which is responsible for the first condensation reaction yielding a four carbon chain.

In this paper we describe the isolation and

Abbreviations: ACP = acyl carrier protein; bp = base pair(s); EDTA = ethylene diamine tetraacetic acid; kb = kilobase (pairs); nt = nucleotide(s); ORF = open reading frame; PAGE = polyacrylamide gel electrophoresis; RNase = ribonuclease; SDS = sodium dodecyl sulfate; Tris = tris-(hydroxymethyl)-aminomethane.

sequencing of the *fabB* gene as well as the purification and sequencing of the NH₂-terminal and active site of β -ketoacyl-ACP synthase I. This has been done as a step toward understanding the attributes of the three condensing enzymes resulting in the specificities noted above. Furthermore, the information obtained provides tools for exploring the condensing enzyme multigene family and its enzymes in higher plants.

2. MATERIALS AND METHODS

2.1. Miscellaneous items

Restriction endonucleases, T4 ligase, DNA polymerase I, T4 DNA polynucleotide kinase, RNase A, RNase T1 and calf intestine alkaline phosphatase were from Boehringer Mannheim. AMV reverse transcriptase and human placental ribonuclease inhibitor were from Amersham. Exonuclease III and S1 nuclease were from Promega Biotech. T7 DNA polymerase and the Sequenase kit were purchased from United States Biochemical Corporation. α -³²P-dATP (> 600 Ci · mmol⁻¹), γ -³²P-ATP (> 7000 Ci · mmol⁻¹) and α -³⁵S-dATP (1000-1500 Ci · mmol⁻¹) were obtained from New England Nuclear. Cerulenin and trypsin were from Sigma. Q-Sepharose FF, Mono Q, PEP-RPC and Superose-12 came from Pharmacia. Proteins were assayed using the BCA reagent from Pierce.

2.2. Bacterial strains, plasmids and phages

Escherichia coli B was a gift from Dr. J. PREISS (University of California, Davis). The host for λ EMBL4 was *E. coli* LE 392 (45). *E. coli* DM 86 carrying the temperature sensitive *fabB15* allele (*fabB^{ts}*) (42) was obtained from Drs. J. CRONAN and M. NARASIMHAN. *E. coli* DH5 α :F', *endA1*, *hsdR17* (*r_k*, *m_k*⁺), *supE44*, *thi-1*, λ , *recA1*, *gyrA96*, *relA1*, $\delta 80dlacZ$ $\Delta M15$ was used as a host for the pUC13 plasmid (43). λ EMBL4 clones 9D2, 12F12, 2G11, 2C9, 6F7 and 3A11 (32) were a gift from Dr. Y. KOHARA.

2.3. Preparation of DNA

The DNA from *E. coli* B was extracted as described by MIURA (44). Large scale prepara-

tions of plasmid DNA from *E. coli* were obtained by the alkaline lysis method (39), and purified by CsCl equilibrium centrifugation. Mini-preparations of plasmid DNA were made by the rapid screening method of BIRNBOIM and DOLY (4).

2.4. Isolation of the *fabB* gene

Chromosomal DNA from *E. coli* was partially digested with Sau3A and size fractionated using a 5-25% NaCl gradient (28). Fractions containing DNA fragments of 1.5-8.5 kb size range were pooled, concentrated by precipitation and ligated to BamHI cleaved, dephosphatased pUC13 vector. This mixture was used to transform *E. coli* DM 86 according to the standard method of HANAHAN (21). After amplification in LB (39) plus ampicillin 50 μ g · ml⁻¹, the transformed cells were plated on minimal medium (68) containing ampicillin (50 μ g · ml⁻¹), thiamine (0.005%), glucose (0.2%) and methionine (0.006%). Transformants were screened for complementation of the *fabB^{ts}* allele by incubating the plates 48 h at 42 °C. These selective conditions were used since the DM 86 strain grows at temperatures > 38 °C only when an appropriate unsaturated fatty acid is added to the medium. In subsequent transformation experiments the cells were selected for ampicillin resistance on LB plates for 12 h at 37 °C and then screened for complementation on minimal plates at 42 °C.

2.5. Nucleotide sequence analysis

The isolated pUC13 clone containing the *fabB* gene (pfabB1) was terminally deleted from one end of the insert by exonuclease III and S1 nuclease digestion (24). An appropriate set of deleted plasmid subclones was selected and sequenced by the dideoxy chain termination method (52) using denatured plasmid DNAs as templates (22). Sequencing reactions were carried out with reagents from the Sequenase kit including a modified T7 DNA polymerase (63) and universal pUC primers. The nucleotide sequence of the complementary strand was determined similarly, except that pfabB1 was used as template and seven specific 15mer oligodeoxyribonucleotides synthesized on an

Applied Biosystem 380A DNA Synthesizer served as primers. Analysis of sequence data was performed according to QUEEN and KORN (48).

2.6. Mapping the 5' end of *fabB* mRNA

Total RNA from *E. coli* B was extracted as described by GILMAN and CHAMBERLIN (18). A 40mer oligodeoxyribonucleotide complementary to nucleotides 42-81 in the *fabB* gene was end-labelled (39), and 1 pmol was hybridized to 100 μ g total RNA in 30 μ l buffer [40 mM-PIPES (pH 6.4), 1 mM-EDTA, 0.4 M-NaCl, 80% formamide] by incubation for 10 min at 65 °C followed by 12 h at 30 °C. The mRNA with the hybridized primer was recovered by ethanol precipitation and resuspended in 50 μ l of the following buffer: 50 mM-Tris-HCl (pH 8.3), 140 mM-KCl, 10 mM-MgCl₂, 10 mM-dithiothreitol, 4 mM-sodium pyrophosphate, 0.5 mM-dATP, 0.5 mM-dCTP, 0.5 mM-dGTP, 0.5 mM-dTTP containing 50 U human placental ribonuclease inhibitor. Primer extension was done at 42 °C for 90 min by using 40 U AMV reverse transcriptase. EDTA was added to 20 mM to stop the reaction. The RNA was digested for 30 min at 37 °C by adding 2.5 U RNase A and 50 U RNase T1. This was followed by phenol extraction and ethanol precipitation. The DNA was dissolved in 6 μ l TE [10 mM-Tris-HCl (pH 7.5) 1 mM-EDTA]. Four μ l loading buffer (95% formamide, 20 mM-EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol) was added, and a 4 μ l aliquot analysed on a 6% sequencing gel with appropriate size markers.

2.7. Screening of the λ EMBL4 clones

Isolated, nick-translated (39) *pfabB1* was used as a probe to screen the λ EMBL4 clones. Plaque hybridizations were made as described by BEN-TION and DAVIS (3).

2.8. Cerulenin labelling of crude protein extract

E. coli B was grown at 37 °C in LB medium and recovered at stationary phase by low speed centrifugation. After washing once in a buffer containing 20 mM-Tris-HCl (pH 8.0), 10 mM-2-

mercaptoethanol and 100 mM-NaCl, the cells were resuspended in a minimum volume of the same buffer, and run twice through a French press at 150 MPa. Soluble proteins were recovered by a 90 min centrifugation at 135,000 $\cdot$ g, and filtering of the supernatant (220 nm GV membrane, Millipore).

[³H]-cerulenin was prepared by ³H exchange in ³H₂O by Amersham. Although several labelled components could be separated by thin layer chromatography, it was not possible to show any differences in their binding specificities. The preparation was therefore used without purification.

4.2 g of crude *E. coli* protein extract at 30 g $\cdot$ l⁻¹ was labelled by adding 15.5 MBq of [³H]-cerulenin (57 TBq $\cdot$ mol⁻¹) in ethanol and stirring for 12 h at 4 °C. Non labelled cerulenin dissolved in ethanol was added, bringing the total concentration of cerulenin to 100 μ M. This was done to ensure binding of cerulenin at all potential binding sites, since it was unknown whether bound cerulenin would affect the chromatographic behavior of the protein of interest. The preparation was stirred for 5 h at 4 °C before purification.

2.9. Enzyme purification

[³H]-cerulenin labelled protein (15.5 MBq, 4.2 g) was dialyzed against 20 mM-Tris-HCl (pH 8.0) containing 10 mM-2-mercaptoethanol. A precipitate was removed by centrifugation and the supernatant (9.3 MBq, 3.9 g protein) was loaded onto a 200 $\times$ 26 mm column of Q-sepharose FF (anion exchanger), equilibrated with 20 mM-Tris-HCl (pH 8.0). The column was washed with 10 volumes of equilibration buffer, and then eluted with a 2 l linear gradient from 0 to 1 M-NaCl at 3 ml $\cdot$ min⁻¹. Radioactive fractions corresponding to [³H]-cerulenin labelled β -ketoacyl-ACP synthase I eluting at 300 mM-NaCl were combined (1.8 MBq, 480 mg protein), and dialyzed into 20 mM-piperazine-HCl (pH 6.0). A precipitate was removed by centrifugation and the supernatant (550 kBq, 116 mg protein) loaded onto the same Q-sepharose column as described above. Chromatographic conditions were identical to those described for the previous run, except that the

buffer was 20 mM-piperazine-HCl (pH 6.0). Radioactive fractions were combined (410 kBq, 20 mg protein, 230 mM-NaCl), and the salt removed by dialysis before loading the sample on a 50×5 mm column of Mono-Q (anion exchanger) equilibrated with 20 mM-piperazine-HCl (pH 6.0). Elution was with a 100 ml linear gradient from 0 to 1 M of NaCl, at a flow rate of 1 ml·min⁻¹. ³H-labelled fractions were combined (205 kBq, 1.5 mg protein, 140 mM-NaCl).

2.10. Electrophoretic and amino acid sequencing

The proteins were further separated electrophoretically on a 20 cm long and 1 mm thick 7.5-15% polyacrylamide gel in the presence of SDS, essentially according to LAEMMLI (38). Lanes were 10 mm wide and each lane was loaded with 67 µg protein (9.1 kBq). The radioactively labelled protein was identified by fluorography and judged to be sufficiently separated from contaminating proteins, as visualized by Coomassie brilliant blue staining, to allow complete purification by elution from a gel. Therefore, the proteins were electrophoretically transferred from a SDS-polyacrylamide gel to an activated glass fiber filter (PCGM-1, Janssen Life Sciences Products) using a 50 mM-Na-borate buffer (pH 8.0) at 8 mA·cm⁻² for 1 h and a semidry transfer apparatus (JKA Biotech, Copenhagen).

The bands were visualized in UV light by staining with 1 mg·l⁻¹ fluorescamine in acetone, and the one of interest was cut from the filter. Half of this filter was used for sequencing of the NH₂-terminal residues by automated Edman degradation in an Applied Biosystems 477A instrument equipped with a model 120A on-line HPLC. The yield of methionine as first amino acid was 34 pmol.

2.11. Characterization of a cerulenin binding peptide

1.5 mg of [³H]-cerulenin labelled protein (240 kBq) purified through the ion exchange steps essentially as described above was incubated with 50% (w/w) of trypsin in 50 mM-Tris-HCl (pH 8.0) for 20 h at 30 °C. A single, short

labelled peptide could not be obtained under milder conditions as judged on the basis of SDS-PAGE. The peptides were then separated by two runs on a 50×5 mm column of PEP-RPC (C1/C8). The gradient in the first run was 0-70% acetonitrile in water, containing 0.1% trifluoroacetic acid, in a total volume of 50 ml at a flow rate of 500 µl·min⁻¹. Fractions containing the ³H-labelled peptide were combined (235 kBq), and rechromatographed under identical conditions, except that the elution volume was increased to 140 ml. The pooled radioactive fractions (105 kBq) were freeze dried, and 105 kBq was taken up in 100 µl of 100 mM-HCl and gel filtrated in 100 mM-HCl on a 305×10 mm column of Superose-12 at 250 µl·min⁻¹. The radioactive fractions were pooled (40.8 kBq) and concentrated by freeze drying. The sample was used for two runs of automated Edman degradation: one in which the NH₂-terminal residues of the peptide were identified using an amount of peptide equalling 13.6 kBq, and one in which the fractions from each cycle were collected and the radioactivity measured. The amount of peptide used for this second run equalled 2.2 kBq.

3. RESULTS

3.1. Isolation and sequencing of the *fabB* gene

To isolate the *fabB* gene a genomic library was constructed by inserting Sau3A partially digested DNA from *E. coli* B into the BamHI site of pUC13. Aliquots of the ligation mixture were used to transform *E. coli* DM 86, which harbors the *fabB*^{ts} allele. Among the resulting ampicillin resistant transformants one colony able to grow under selective conditions (minimal medium, 42 °C) was identified. The recombinant plasmid (designated pfabB1) isolated from the colony was 5.7 kb in size and complemented the *fabB*^{ts} allele when transformed into DM 86. This result suggested that pfabB1 carried a wild type *fabB* allele.

A series of deletions encompassing the 3.0 kb insert in pfabB1 was obtained by using exonuclease III and S1 nuclease (24). Nucleotide sequence analyses of 17 plasmid subclones revealed an open reading frame (ORF) of 1218 bp. The complementary strand of this ORF and its 3' flanking region were sequenced directly from

	GCTCGT	C AAAATCTCG	GAACAGGTG	TACCCTCAGC	-201
ATTAAATTCG AGGTTGGCAC GTTGTAATGGA GTAGTGTTTT ACGTAAGSTA CTCGCCTTAC AGGCCGGTGGC TCGATCTTAG CGATGTGTGT AAGSCTGC GC -101					
AATTTCTCT ATTAATAGGC TGATCCGACT G-35 TTGTCGGCGT ACAAGGTGAC GA-TATTGTS C ATTCAAACCT TACTCTATGT GCGACTTACA RBS GAGGTATTGA -1					
ATG AAA CGT GCA GTG ATT ACT GGC CTG GGC ATT GTT TCC AGC ATC GGT AAT AAC CAG CAG GAA GTC CTG GCA TCT CTG CGT GAA GGA CGT 90 MET LYS ARG ALA VAL ILE THR GLY LEU GLY ILE VAL SER SER ILE GLY ASN ASN GLN Glu Val Leu Ala Ser Leu Arg Glu Gly Arg 30					
TCA GGG ATC ACT TTC TCT CAG GAG CTG AAG GAT TCC GGC ATG CGT AGC CAC GTC TGG GGC AAC GTA AAA CTG GAT ACC ACT GGC CTC ATT 180 Ser Gly Ile Thr Phe Ser Gln Glu Leu Lys Asp Ser Gly Met Arg Ser His Val Trp Glu Asn Val Lys Leu Asp Thr Thr Gly Leu Ile 60					
GAC CGC AAA GTT GTG CGC TTT ATG AGC GAC GCA TCC ATT TAT GCA TTC CTT TCT ATG GAG CAG GCA ATC GCT GAT GCG GGC CTC TCT CCG 270 Asp Arg Lys Val Val Arg Phe Met Ser Asp Ala Ser Ile Tyr Ala Phe Leu Ser Met Glu Glu Ala Ile Ala Asp Ala Gly Leu Ser Pro 90					
GAA GCT TAC CAG AAT AAC CCG CGC GTT GGC CTG ATT GCA GGT TCC GGC GGC GGC TCC CCG CGT TTC CAG GTG TTC GGC GCT GAC GCA ATG 360 Glu Ala Tyr Gln Asn Asn Pro Arg Val Gly Leu Ile Ala Gly Ser Gly Gly Gly Ser Pro Arg Phe Gln Val Phe Gly Ala Asp Ala Met 120					
CGC GGC CCG CGC GGC CTG AAA GCG GIT GGC CCG TAT GTG GTC ACC AAA GCG ATG GCA TCC GGC GTT TCT GCC TGC CTC GCC ACC CCG TTT 450 Arg Gly Pro Arg Gly Leu Lys Ala Val Gly Pro Tyr Val Val Thr Lys Ala Met Ala Ser Gly Val Ser Ala Cys Leu Ala Thr Pro Phe 150					
AAA ATT CAT GGC GTT AAC TAC TCC ATC AGC TCC GCG TGT GCG ACT TCC GCA CAC TGT ATC GGT AAC GCA GTA GAG CAG ATC CAA CTG GGC 540 Lys Ile His Gly Val Asn Tyr SER ILE SER SER ALA Cys ALA THR SER ALA HIS Cys Ile Gly Asn Ala Val Glu Gln Ile Gln Leu Gly 180		*			
AAA CAG GAC ATC GTG TTT GCT GGC GGC GGC GAA GAG CTG TGC TGG GAA ATG GCT TGC GAA TTC GAC GCA ATG GGT GCG CTG TCT ACT AAA 630 <u>Lys</u> Gln Asp <u>Ile</u> Val Phe Ala <u>Gly Gly</u> Gly Glu Glu Leu Cys Trp <u>Glu</u> Met Ala Cys <u>Glu Phe</u> Asp Ala <u>Met</u> Gly <u>Ala</u> Leu <u>Ser</u> Thr Lys 210					
TAC AAC GAC ACC CCG GAA AAA GCC TCC CGT ACT TAC GAC GCT CAC CGT GAC GGT TTC GTT ATC GCT GGC GGC GGC GGT ATG GTA GTG GTT 720 Tyr Asn Asp Thr Pro Glu Lys Ala Ser Arg Thr Tyr Asp Ala His Arg Asp Gly Phe Val Ile Ala Gly Gly Gly Gly Met Val Val Val 240					
GAA GAG CTG GAA CAC GCG CTG GCG CGT GGT GCT CAC ATC TAT GCT GAA ATC GTT GGC TAC GGC GCA ACC TCT GAT GGT GCA ATG GTT 810 Glu Glu Leu Glu His Ala Leu Ala Arg Gly Ala His Ile Tyr Ala Glu Ile Val Gly Tyr Gly Ala Thr Ser Asp Gly Ala Asp Met Val 270					
GCT CCG TCT GGC GAA GGC GCA GTA CCG TGC ATG AAG ATG GCG ATG CAT GGC GTT SAT ACC CCA ATC GAT TAC CTG AAC TCC CAC GGT ACT 900 Ala Pro Ser Gly Glu Gly Ala Val Arg Cys Met Lys Met Ala Met His Gly Val Asp Thr Pro ile Asp Tyr Leu Ser Ser His Gly Thr 300					
TCG ACT CCG GTT GGC GAC GTG AAA GAG Leu Ala Ala Ile Arg Glu Val Phe Gly Asp Lys 990 Ser Thr Pro Val Gly Asp Val Lys Glu Leu 310					
ACC GGT CAC TCT CTG GGC GCT GCT GGC GTA CAG GAA GCT ATC TAC TCT CTG CTG ATG CTG GAA CAC GGC TTC ATC GCC CCG AGC ATC AAC 1080 Thr Gly His Ser Leu Gly Ala Ala Gly Val Gln Glu Ala Ile Tyr Ser Leu Leu Met Leu Glu His Gly Phe Ile Ala Pro Ser Ile Asn 360					
ATT GAA GAG CTG GAC GAG CAG GCT GCA GGT CTG AAC ATC GTG ACC GAA ACG ACC GAT GAT CCG GAA CTG ACC ACC GTT ATG TCT AAC AGC TTC 1170 Ile Glu Glu Leu Asp Glu Gln Ala Ala Gly Leu Asn Ile Val Thr Glu Thr Thr Asp Arg Glu Leu Thr Thr Val Met Ser Asn Ser Phe 390					
				REP	
GGC TTC GGC GGC ACC AAC GCC ACG CTG GTA ATG CGC AAG CTG AAA GAT TAA TT GC CCTAGGTT CGGGTAAGGC GGCGCAGGCT CGCATCCGAC 1263 Gly Phe Gly Gly Thr Asn Ala Thr Leu Val Met Arg Lys Leu Lys Asp 400 406					
GTACCGCGCC AATCGCGECT CCGGCACTAA CACAAAAAGG GAACCCGATG GTTCCCCTTT TCACATCATT GACAATCGCC TGGTTCCAGG CAACCTCCCC 1363					
GCTTTGTGCA TTTCTTCTG AAAAGACGTA CGCGTTAAAT CDTGCCAACG CACTGTAAAC CTGA AACCG AGAGATGAGA CGGGGATACT CCTCGCCTTG 1463					
CGCTGCATTC TGGAGTAATG CATGACTGCT GTAAGECAAAA CCGAAACAC 1512					

Figure 1. Nucleotide sequence of the *fabB* gene and the derived amino acid sequence of β -ketoacyl-ACP synthase I. The NH₂-terminal and active site sequences determined from the purified enzyme are shown in capital letters. The cerulenin binding Cys 163 is denoted by an asterisk. The underlined amino acids between residues 162-208 are identical to those in a region of the β -ketoacyl synthase domain of fatty acid synthetase in *S. cerevisiae* (54). RBS designates a putative ribosome binding site for the *fabB* gene. The transcription start site is marked by an arrow, and the potential -10 and -35 promoter sequences are boxed. The sequence labelled REP in the 3' flanking region is homologous to the repetitive extragenic palindromic consensus sequence. An inverted repeat is indicated by horizontal arrows.

Figure 2. Mapping of the *fabB* gene to a nine kb fragment of the *E. coli* genome, and its probable location therein. Six λ EMBL4 clones covering a 70 kb long segment of the *E. coli* chromosome were screened using *pfabB1* as a probe. The individual clones represented by horizontal bars are positioned under the physical map of the *E. coli* genome of KOHARA et al. (32) with the *hisT* gene located at 50.2 min. Format of the restriction map from top to bottom line is BamHI, HindIII, EcoRI, EcoRV, BglI, KpnI, PstI and PvuII. No sites for EcoRV were established above the dotted line. Clones hybridizing to the probe are indicated by thick bars. Deduced restriction sites are shown below for the sequenced segment of the probe (broad bar) which contains the coding region of the *fabB* gene (stippled area). The sites marked with a triangle are consistent with those in the physical map above. This suggests that the *fabB* gene is located as shown and transcribed in the direction indicated by the arrow.

pfabB1 using appropriately positioned oligonucleotide primers.

3.2. The *fabB* gene

The ORF codes for a 406 amino acid polypeptide with a molecular weight of 42,619 (Fig. 1). Both the amino acid composition and the molecular weight calculated from the deduced sequence agree well with those of β -ketoacyl-ACP synthase I (17). This supports the notion that we had cloned the *fabB* gene.

If this is true, then *pfabB1* should hybridize to one or more of the λ EMBL4 clones including the 50 min region of the *E. coli* chromosome: the region to which *fabB* has been mapped by cotransduction (6). The hybridization results are schematized in Figure 2. Within the approximately nine kb overlapping segments of the hybridizing clones 12F12 and 2G11 one HindIII and one EcoRI site are found. Single sites for these two enzymes also occur at the same relative distance from one another in the coding region of the presumed *fabB* gene (Fig. 2).

Furthermore, a BglI site in the 3' flanking region can be matched to a BglI site in the physical map, while BamHI, KpnI and PvuII sites are lacking in both sequences. These observations infer that the *fabB* gene is located at 2455 kb on the physical map, and is transcribed in the counter-clockwise direction as illustrated in Figure 2. This hypothesis awaits confirmation as our sequence contains a PstI and an additional BglI site not present in the physical map.

Previously pLC39-16 from the Clarke and Carbon gene bank containing 13 kb of *E. coli* DNA was shown to complement *fabB^{ts}* strains (42). When this plasmid was digested with HindIII, 0.9, 3.4 and 14 kb fragments resulted. A single HindIII site occurs in the 9 kb segment to which we have localized the *fabB* gene (Fig. 2). While it might only be a coincidence the two HindIII fragments to the left of this site (Fig. 2) are remarkably similar in size to the two smaller ones obtained upon digestion of pLC39-16. Subsequent subcloning of the latter showed that the 0.9 kb fragment was unnecessary for complementation of *fabB^{ts}* strains. Thus MENDOZA et al.'s (42) observations are in accord with our hypothesis.

The 5' end of *fabB* mRNA was determined by the primer extension method. Figure 3 reveals that transcription initiates at nucleotide position -36, and is preceded by potential -10 and -35 promoter sites (Fig. 1) homologous to the consensus elements of *E. coli* promoters (23, 50).

The putative ribosome binding site (Fig. 1) consists of five nucleotides complementary to the 3' end of *E. coli* 16 S rRNA (19, 58). The stop codon is followed by a sequence (nucleotides 1228-1263, Fig. 1) homologous to the repetitive extragenic palindromic (REP) consensus sequence (2). This sequence element, originally described by HIGGINS et al. (26), is common to several intercistronic regions of bacterial operons (2, 62) and can form a stable stem and loop structure. While several possible functions have been proposed for REP sequences, the exact physiological role is still unknown (2, 62). An inverted repeat (nucleotides 1297-1324, Fig. 1) further downstream may function as a rho-independent terminator (47, 50). Whether it plays a role in *fabB* transcription is unknown.

The pattern of codon usage in the *fabB* gene

Figure 3. Determination of the transcription start site of the *fabB* gene. The primer complementary to nucleotides 42-81 in the *fabB* gene was hybridized to total RNA from *E. coli* and extended to the 5' end of the hybridized mRNA. The resulting cDNA was analyzed on a 6% sequencing gel. The same primer was used to sequence one of the deleted *pfabB1* subclones (section 2.5) extending into the 5' region. The resulting sequence ladders (lanes A, C, G and T) were used to locate the 5' end (arrow) of the *fabB* mRNA at position -36. The pUC13 sequence starts above nucleotide -41.

places it in the highly expressed category as defined by SHARP and LI (57). Of the ten codons rarely used by such genes, seven are not used at all and the other three once each. With respect to the other codons, the single discrepancy with the compiled data is the preferential use of GGC over GGU for glycine. The only other gene in the same biosynthetic pathway for which data is available is *fabA* which falls into the moderately expressed category (7).

Figure 4. *E. coli* proteins at various stages of β -ketoacyl-ACP synthase I purification were separated by SDS-PAGE and stained with Coomassie brilliant blue. Lane 1, crude protein extract; 2, after anion exchange at pH 8; 3, after anion exchange at pH 6; 4, after FPLC anion exchange at pH 6.

3.3. Purification of β -ketoacyl-ACP synthase I

Cerulenin, an antibiotic produced by the fungus *Cephalosporium caerulens* (41), is known to specifically inhibit a number of β -ketoacyl-thioester synthases (8, 67) by an irreversible mechanism (8). Therefore, [^3H]-cerulenin was used to tag such enzymes in crude protein extracts of *E. coli* before purification. By anion exchange two major peaks of radioactivity were observed (59) of which the second

corresponds to [^3H]-cerulenin labelled β -ketoacyl-ACP synthase I. This labelled protein was then further purified by two rounds of ion exchange as described in section 2.9. The purity at each step was examined by SDS-PAGE as illustrated in Figure 4. The enzyme was separated from contaminating proteins by SDS-PAGE and eluted from the gel. From this purified protein the sequence of the 19 NH_2 -terminal residues was obtained (Fig. 1). Since they are identical to those deduced from the 5' end of the ORF, our deduction that we had cloned and sequenced the *fabB* gene coding for β -ketoacyl-ACP synthase I is upheld.

In the first ion exchange step 1.8 MBq of radioactivity equalling 31 nmol of [^3H]-cerulenin was recovered with β -ketoacyl-ACP synthase I. Assuming that the cerulenin was bound to a monomer of the latter in a one to one molar ratio, the label corresponds to 1.3 mg of the enzyme. Since only 24-48% of the cerulenin binding peptide was radio labelled (section 3.4), the actual amount of cerulenin binding enzyme may be 5.7 mg. The enzyme is 100% inhibited by 20 μM -cerulenin *in vitro* (59) under conditions similar to those used in these experiments, suggesting that most likely all of the β -ketoacyl-ACP synthase I has bound the inhibitor. The estimate is therefore also valid for the total amount of this enzyme. The crude protein extract (4.2 g protein) thus contained 0.032-0.14% of β -ketoacyl-ACP synthase I. This is somewhat less than earlier estimates that the enzyme represents 0.6% of total protein (17, 25), and may reflect the different media used for growing the cells. A considerable loss of radioactive label occurred at most steps of this purification. Whether this is caused by decomposition of the protein, by the cerulenin being detached from the protein or by the [^3H]-cerulenin losing its ^3H -label due to hydrogen exchange is unknown. Only the latter would affect the estimate of enzyme concentration, and this would be toward the lower value.

3.4. Analysis of a cerulenin binding peptide

To define the binding site of cerulenin, [^3H]-cerulenin labelled β -ketoacyl-[ACP] synthase I was proteolytically digested using trypsin, and

Figure 5. Identification of the amino acid residue to which [^3H]-cerulenin is attached. 2.2 kBq of a radioactively labelled peptide, derived from [^3H]-cerulenin labelled β -ketoacyl-ACP synthase I by tryptic digestion, was Edman degraded. Radioactivity corresponding to each amino acid position was determined by liquid scintillation counting. The amino acid sequence of the peptide had previously been determined (Fig. 1).

the radioactive fragment purified and sequenced. The amino acid sequence obtained, Ser-Ile-Ser-Ser-Ala-x-Ala-Thr-Ser-Ala-His, matches the derived one in positions 158-168 (Fig. 1). For the first amino acids 360-380 pmol were recovered, suggesting that the sample contained 500-1000 pmol of peptide. The content of [^3H]-cerulenin was 240 pmol as calculated from the absolute (13.6 kBq) and specific (57 MBq $\cdot$ mol $^{-1}$) activities, indicating that only 24-48% of the peptide was labelled with [^3H]-cerulenin. The rest is assumed to be labelled with cold cerulenin. The sixth residue of the peptide could not be identified, possibly because cerulenin was bound to it. The recovery of alanine in the tenth cycle was 47 pmol, while 2 pmol of histidine were found in the next cycle. Since the nucleotide sequence indicates a histidine at this position, the peptide is believed to include this amino acid as its last residue, although this is not a normal site of cleavage for the protease. A tyrosine is found immediately preceding the peptide in the derived sequence of β -ketoacyl-ACP synthase I, indicating that a chymotryptic type cleavage gave rise to the NH_2 -terminal end of the peptide.

To determine to which amino acid the cerulenin was bound, the fractions from Edman

degradation of the labelled peptide were collected, and the radioactivity corresponding to each residue determined (Fig. 5). These data lead to the conclusion that cerulenin binds to Cys 163.

4. DISCUSSION

Fatty acid synthetases are divided into two groups on the basis of whether the participating functional domains are part of individual polypeptides as in *E. coli* or represent distinct domains of one or two multifunctional polypeptides as in *S. cerevisiae* or *C. caerulens*. Both the latter have an $\alpha_6\beta_6$ structure (54, 64). The catalytic mechanisms appear to be very similar whereby the condensation reaction is carried out in these three organisms (1, 9, 20, 29, 33, 34, 64, 66). Briefly, two partial reactions take place: the acyl component of an activated acyl primer is transferred to a cysteine residue of the β -ketoacyl synthase and then condensed with an activated malonyl donor with the concomitant release of CO_2 and the free enzyme. In *S. cerevisiae* this catalytic activity is a function of the α polypeptide determined by the *FAS2* gene which has recently been sequenced (54). When its derived primary structure is compared to that determined by the *E. coli fabB* gene an identical sequence of five amino acids (Ala-Cys-Ala-Thr-Ser) is found (Fig. 1). That this sequence contains the active site cysteine in the *S. cerevisiae* enzyme was concluded (54) from a comparison of the deduced primary structure (54) and a previously published sequence of carboxamidomethylated enzyme fragments (33). We believe from the results presented in this paper that this five amino acid sequence is also the active site of the *E. coli fabB* enzyme. If one continues another 42 residues toward the COOH-terminal ends of the enzymes an additional 14 identical residues occur (Fig. 1). Combined this gives a 47 amino acid segment with 40% similarity which is the only region of homology between the two enzymes at the primary structure level.

By contrast characterization of acetylated enzyme fragments from *C. caerulens* (65) has revealed a very different active site primary structure. That is, *S. cerevisiae* Thr-Pro-Val-Gly-Ala-Cys-Ala-Thr-Ser-Val-Glu-Ser versus *C. caerulens* Tyr-Gln-Val-Glu-Ser-Cys-Pro-Ile-

<i>FAS2</i>			GLY	ALA	CYS 1305	ALA	THR	SER	val
<i>fabB</i>	ile	SER	SER	ALA	CYS 163	ALA	THR	SER	ala
CHS, RS	met	SER	SER GLY	ALA	CYS 345	val			

Figure 6. Amino acid sequences encompassing the active site cysteine residues of *FAS2* (*S. cerevisiae*) and *fabB* (*E. coli*) gene products and the presumed active site cysteine of plant chalcone synthase (CHS) and resveratrol synthase (RS). Prepared from: *FAS2*, nucleotide and partial amino acid sequences (33, 54); *fabB*, data herein; CHS, nucleotide sequences from *P. hortense* (49), *A. majus* (61), *P. vulgaris* CHS-1, 4, 14, and 17 (51), *A. thaliana* (14), *P. hybrida* CHS-A, J, and G (30, 31), *H. vulgare*, *Z. mays*, *R. acer* CHS-1 and 2, *M. liliflora* (46); and RS, nucleotide sequences from *A. hypogaea* (55).

Leu-Glu-Gly-Lys. The latter also shows no homology with the *E. coli fabB* determined sequence (Fig. 1). These observations indicate that more than one active site structure can carry out the same condensation reaction. The difference is thought to explain why *C. caerulens* is insensitive to the cerulenin it synthesizes (65). Considerable indirect evidence suggests that cerulenin inhibition of condensing enzymes is due to its irreversible binding to the active site cysteine (8, 15, 64, 65), a supposition which our data demonstrates to be correct. Reaction of cerulenin's cis epoxide ring with the cysteine thiol is highly dependent upon the presence of the two trans double bonds in the hydrophobic chain (8). Estimating the maximum length of cerulenin as 1.3 nm, then a limited number of amino acid residues in addition to the cysteine can interact with the cerulenin. Interestingly, in all cerulenin sensitive enzymes (Fig. 6) as one progresses away from the active site cysteine in one or both directions are a non polar residue, then two polar ones followed by a non polar. The primary structures of the analogous regions in *E. coli*'s less sensitive β -ketoacyl-ACP synthase II coded by *fabF* (5) and its totally insensitive acetoacetyl-ACP synthase (27) are thus of great interest.

Enzymes catalyzing condensation reactions similar to those occurring in fatty acyl chain formation have been demonstrated or invoked

to take place in the synthesis of other compounds such as flavonoids (37), stilbenes (e.g. resveratrol) (56), 6-methylsalicylic acid (10, 11), polyene macrolides (e.g. candicidin) (40), long chain epicuticular waxes (e.g. β -diketones and hydrocarbons) (69) as well as the bitter substances of hops (e.g. lupulone and humulone) (13). The most extensively studied of these is chalcone synthase, the key enzyme in flavonoid biosynthesis which catalyzes the sequential condensation of three acetate units donated by malonyl-CoA with the primer 4-coumaroyl-CoA. Except for the direct use of CoA esters the mechanism is believed to be analogous to that in fatty acyl chain synthesis (35, 37). Not only can chalcone synthase catalyze CO₂ exchange and decarboxylation of malonyl-CoA (36), but this enzyme is also very sensitive to cerulenin (35). These similarities led to the proposal some years ago that β -ketoacyl synthase and chalcone synthase share a common evolutionary origin (37).

We thus decided to compare the primary structures of the seven cysteine encompassing regions of chalcone synthase to that of the cerulenin binding, active site cysteines of the *FAS2* and *fabB* gene products. No homology occurs around the cysteine residues at positions 35, 65, 89, 169 and 195 (numbering after consensus sequence, reference 46). The sequence at Cys 135 reads Leu-x-Phe-Cys-Thr-Thr-Ser-Gly which has in common with the analogous *FAS2*

and *fabB* sequences the motif Cys-x-Thr-Ser. An exchange of threonine for alanine results in a polar residue adjacent to the cysteine. As mentioned above cerulenin binding may require a non polar residue adjacent to the cysteine. Does the presence of phenylalanine with an aromatic group in place of alanine on the other side of the cysteine perturb cerulenin binding? While such questions can not be answered today, the reason we eliminated this sequence is shown in Figure 6 which compares the sequence embedding Cys 345 with those of the active sites in the two fatty acyl condensing proteins. In addition to the four identical amino acids, the exchange of methionine for isoleucine is conservative (12), while both valine and alanine are non polar. No homology occurs between either *FAS2* or *fabB* and the chalcone synthase (resveratrol synthase) sequences on either side of these six amino acids. This is perhaps to be expected given the structural differences of the acetyl and 4-coumaroyl primers. On the other hand the methionine residue is preceded by three additional amino acids (Tyr-Gly-Asn) common to all chalcone synthases (resveratrol synthase) listed in the text of Figure 6. These observations are in accord with our proposal that the sequence shown in Figure 6 is not only the cerulenin binding domain but thereby also the functional condensing domain of these enzymes. Given that our deduction is correct, the petunia genes CHS-D and H (31) can not code for active enzymes as the essential cysteine at position 345 is replaced by serine. We question if CHS-B (31) codes for an active enzyme, even though a low level of transcription has been demonstrated, as a polar cysteine residue occurs adjacent to Cys 345.

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