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The *fabJ*-encoded β-ketoacyl-[acyl carrier protein] synthase IV from *Escherichia coli* is sensitive to cerulenin and specific for short-chain substrates

(antibody/condensing enzyme/fatty acid synthase)

MADS SIGGAARD-ANDERSEN*, MARGIT WISSENBACH*, JO-ANNE CHUCK*, IB SVENDSEN†, JOHAN GOTTHARDT OLSEN*‡, AND PENNY VON WETTSTEIN-KNOWLES*‡

Departments of *Physiology and †Chemistry, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Valby, Denmark; and ‡Genetics Department, Institute of Molecular Biology, University of Copenhagen, Øster Farimagsgade 2A, DK-1353 Copenhagen K, Denmark

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ABSTRACT **A fourth fatty acid condensing enzyme was isolated from *Escherichia coli* by its ability to restore elongating activity to a protein extract which had been treated with cerulenin, a condensing enzyme-specific inhibitor. The purified β-ketoacyl-[acyl carrier protein] (ACP) synthase IV [3-oxoacyl-ACP synthase; acyl-ACP:malonyl-ACP *C*-acyltransferase (decarboxylating), EC 2.3.1.41] (KAS IV) is specific for short-chain acyl-ACP substrates. The enzyme is stable at 43°C and very sensitive to cerulenin (50% inhibition at 3 μM), which binds covalently. A condensing enzyme-specific antibody raised to an expressed open reading frame from barley was used to identify KAS IV protein in Western blots, and the sequence obtained for 30 amino-terminal residues. This led to the isolation of the *fabJ* gene located in the *fab* cluster at 24.8 min of the *E. coli* chromosome. The *fabJ* gene encodes a polypeptide of 413 amino acids and molecular mass 43 kDa that shows 38% identity and 64% similarity to the *fabB*-encoded KAS I. The amino acid sequence of KAS IV, however, is more similar to all other published condensing enzyme sequences than the KAS I sequence is. A specialized putative function for this enzyme is to supply the octanoic substrates for lipoic acid biosynthesis. We predict that an analogue of KAS IV with the same function will be found in plant mitochondria. The described complementation assay can be used to detect condensing enzymes with other substrate specificities by supplementing the cerulenin-treated extract with appropriate purified KAS enzymes.**

Three β-ketoacyl-ACP synthases (EC 2.3.1.41) (KASs) participating in fatty acid synthesis have been described in *Escherichia coli* (1). They have different chain-length specificities and sensitivities to cerulenin, a specific inhibitor of condensing enzymes (2). KAS III carries out the cerulenin-insensitive condensation of acetyl-CoA and malonyl-ACP to yield acetoacetyl-ACP in the first elongating reaction of the fatty acid biosynthetic pathway (3). KAS I, which is inhibited 50% by 1 μM cerulenin, operates over a broader substrate range, utilizing saturated C_2–C_{14} and unsaturated $C_{10:1}$–$C_{14:1}$ acyl-ACPs as substrates for condensation with a C_2 unit derived from malonyl-ACP (4). The final extension step of unsaturated fatty acid synthesis ($C_{16:1}$ to $C_{18:1}$) is carried out by KAS II, which utilizes $C_{14:0}$ and $C_{12:1}$–$C_{16:1}$ acyl-ACPs in a reaction reportedly inhibited at higher cerulenin concentrations (4). We have previously purified KAS I and sequenced the corresponding *fabB* gene (5). The *fabH* gene, encoding KAS III, has also been purified and sequenced (6), while *fabF* (KAS II) has been mapped to 24–25 min of the *E. coli* genome (4), just downstream from a cluster of fatty acid biosynthetic (*fab*) genes (7). We here report the purification of another member of the KAS family, KAS IV, with a substrate specificity between those of KAS III and KAS I. The enzyme is inhibited 50% by 3 μM cerulenin in an irreversible reaction, as previously found for cerulenin binding to KAS I (5) and reported herein for KAS II. KAS IV is encoded by *fabJ*, a member of the *fab* gene cluster.§

MATERIALS AND METHODS

Purification of KASs I and IV. *E. coli* K-12 (400 g of frozen paste) was suspended in 20 mM Tris·HCl, pH 8.0/2 mM dithiothreitol/1 mM EDTA (buffer A) containing 100 mM NaCl to give a final volume of 800 ml. Cells were ruptured by a single passage through a French pressure cell at 150 MPa, after which soluble proteins were recovered in a 45-min centrifugation at 135,000 × *g*. The protein was dialyzed against buffer A and then loaded on a 5 × 18-cm column of Q-Sepharose (Pharmacia). After the column was washed with 700 ml of the same buffer, proteins were eluted in a 91 mM/liter linear gradient of NaCl. Two peaks of condensing enzyme activity were eluted at 150 and 300 mM NaCl and were individually pooled and dialyzed against buffer A. Both proteins were further purified on a 2.6 × 30-cm column of Q-Sepharose equilibrated in buffer A. After the protein was loaded on the column, the buffer was changed to 20 mM piperazine·HCl, pH 6.0/2 mM dithiothreitol/1 mM EDTA. Elution was in a 333 mM/liter linear gradient of NaCl. For the sample containing KAS I, the anion-exchange step in buffer A was repeated using a 1 × 10-cm column of Mono Q (Pharmacia) and a 1 M/liter linear gradient of NaCl. Both enzymes were then subjected to hydrophobic-interaction chromatography on a 1 × 10-cm column of alkyl-Superose (Pharmacia). The samples were adjusted to 2 M $(NH_4)_2SO_4$, loaded on the column, which had been equilibrated in 50 mM Tris·HCl, pH 8.0/2 mM dithiothreitol/1 mM EDTA/2 M $(NH_4)_2SO_4$, and eluted in a linear gradient from 2 to 0 M $(NH_4)_2SO_4$ in 240 ml. Active fractions were pooled and loaded on a 1.6 × 100-cm column of Sephacryl S-300 (Pharmacia) which was equilibrated and eluted in buffer A at 200 μl/min.

Amino-Terminal Sequencing of KAS IV. After purification as described above, the KAS IV was separated from contaminating proteins by SDS/PAGE (8) and blotted to a poly(vinylidene difluoride) (PVDF) membrane (Immobilon P; Millipore) in 50 mM sodium borate buffer at pH 8.0, with a transfer current of 0.8 mA/cm² for 1 hr in a semidry transfer

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Abbreviations: ACP, acyl carrier protein; KAS, β-ketoacyl-ACP synthase; ORF, open reading frame; PVDF, poly(vinylidene difluoride).

§The *fabJ* sequence reported in this paper has been deposited in the GenBank data base (accession no. Z34979).

apparatus (Bio-Rad). An initial yield of 80 pmol allowed identification of 30 amino-terminal residues on an Applied Biosystems 470 amino acid sequencer.

Western Blots. Proteins were transferred to PVDF as described above and then incubated in 50 mM Tris·HCl, pH 7.5/0.9% (wt/vol) NaCl/0.2% (vol/vol) Tween 20 (TTBS) containing 1% (wt/vol) skim milk powder for 15 hr. The membrane was washed in TTBS before addition of polyclonal antibodies in rabbit serum diluted 1:15,000 in TTBS. After 3 hr at 25°C the filter was washed in TTBS and incubated with an alkaline phosphatase-conjugated swine anti-rabbit immunoglobulin antibody (Dakopatts, Copenhagen) for 2 hr. Following a wash in TTBS excluding Tween, bands of interest were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma).

Complementation Enzyme Assay. Fractions were tested for KAS activity by their ability to restore fatty acid synthesis in a cerulenin-treated *E. coli* protein extract. Such extracts were prepared by incubating crude soluble *E. coli* proteins at 10 mg/ml in 50 mM Tris·HCl (pH 8.0) with 200 μM cerulenin for 1 hr at 4°C, after which excess inhibitor was removed on a column of Sephadex G-25 superfine (Pharmacia) with the same buffer including 2 mM dithiothreitol, to bind any remaining active cerulenin. Assay mixtures contained 20 μg of cerulenin-treated *E. coli* protein, 0.2–2 μg of protein from fractions being assayed, 10 μM *E. coli* ACP (Sigma), 10 μM acetyl-CoA, 60 μM [^{14}C]malonyl-CoA (18.3 GBq/mol), 1 mM NADPH, 1 mM NADH, 2 mM dithiothreitol, and 50 mM potassium phosphate buffer at pH 6.8 in a total volume of 500 μl. After incubation for 60 min at 37°C, assays were stopped by addition of NaOH to 2 M, and hydrolysis was allowed to proceed for 30 min at 80°C. Samples were then acidified with H_2SO_4 and free fatty acids were extracted with three 2-ml portions of *n*-hexane. The radioactive content was determined after direct addition of scintillant. Assays were linear to ≈25% incorporation of labeled substrate (≈9000 dpm) with a background of <300 dpm.

Product Identification. Assays were carried out as described above, except that the specific activity of [^{14}C]malonyl-CoA was increased to 122 GBq/mol and assay volumes were decreased to 100 μl with protein amounts adjusted accordingly. At the end of the incubation period, acyl-ACPs were precipitated by addition of an equal volume of ice-cold 8% (wt/vol) trichloroacetic acid. The pellet was suspended in 75 mM Tris·HCl (pH 6.8), and the proteins were separated by electrophoresis at 15°C in a 13% polyacrylamide gel containing urea (9). Proteins were electroblotted to a PVDF membrane for 30 min as described above, and the radioactive products were detected by use of a PhosphorImager (Molecular Dynamics). For gas chromatographic analysis of radioactive products, free fatty acids were recovered as described above and converted to methyl esters by use of BF_3/methanol (10). The resulting esters were separated on a column of HT-5 (SGE), and quantitated with a radiochromatography detector (Radiomatic Instruments and Chemicals, Tampa, FL).

Cerulenin and Heat Sensitivities. [^{3}H]Cerulenin labeling was carried out as described (5), with 4.8 μM cerulenin for 1 hr at 25°C. For sensitivity assays, cerulenin (Sigma) was dissolved in acetone, aliquoted to the assay tubes, and dried under a stream of nitrogen. The assay components, except condensing enzyme, were then added in a volume of 490 μl and the tubes were thoroughly shaken. After 5 min the condensing enzyme to be tested was added in a volume of 10 μl, and the assays were run as described above. The heat lability of KAS I and IV activities was tested by preincubation in 50 mM potassium phosphate buffer, pH 6.8/2 mM DTT/1 mM EDTA at 43°C for the indicated times, followed immediately by complementation assay at 37°C as described.

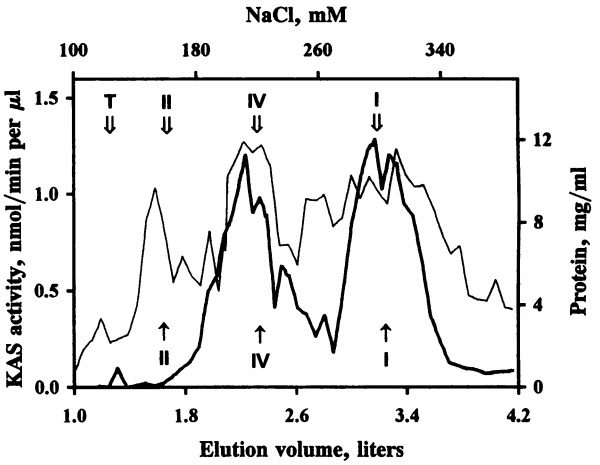

FIG. 1. Anion-exchange chromatography of crude *E. coli* proteins, with protein concentration (thin line) and KAS activity (thick line) indicated. Activities identified in product-specific assays (see Fig. 3) are indicated with thin arrows (↑) at the bottom. Open arrows (⇓) show elution positions of [^{3}H]cerulenin-binding proteins (see Fig. 4), of which IV and I have previously been demonstrated (10). T, thioredoxin; II, IV, and I, KASs II, IV, and I, respectively.

Assays were also carried out at 30°C, 37°C, and 43°C with condensing enzymes that had not been heated.

Cloning and Sequencing of the *fabJ* Gene. Of the three genes determining condensing enzymes known in *E. coli* at the start of the work, only *fabF* had not yet been sequenced. Believing that the KAS activity being purified was coded for by *fabF*, mapped downstream from *acpP* by cotransduction at ≈25 min on the *E. coli* genome (4), a polymerase chain reaction (PCR) approach was selected for gene isolation. DNA from *E. coli* K-12 served as template. A sense primer was synthesized to correspond to the junction of the coding and noncoding 3′ end of *acpP* (7), while a degenerate antisense primer was designed to hybridize with a nucleotide sequence encoding the amino acid motif NSFGFGG. The latter is highly conserved and close to the carboxyl-terminal end of plant and *E. coli* condensing enzymes (11). Thirty cycles of PCR (94°C for 1 min, 55°C for 2 min, 72°C for 3 min) with *Taq* polymerase (Perkin–Elmer/Cetus) were carried out. The resulting 1.3-kb amplified product was purified and then blunt-end cloned into plasmid pUC18 at the *Sma* I site. The insert was sequenced in both directions by primer walking, initially using the pUC universal primers and thereafter selected synthetic primers. To obtain the missing 3′ end of the open reading frame (ORF), estimated to be ≈50 bases by comparison with known sequences (11), appropriate sequencing primers were de-

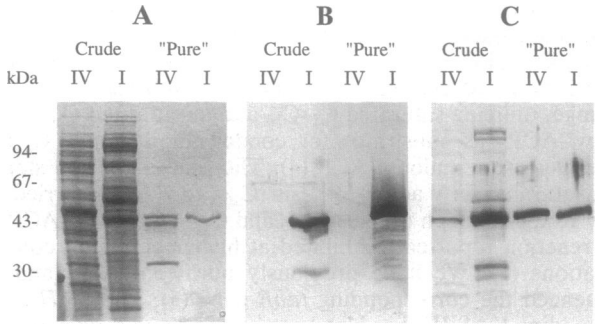

FIG. 2. Western blotting of KASs I and IV from fractions following separation by anion-exchange (crude) and at the final purity ("pure"). The gels were stained with Coomassie brilliant blue (*A*), or proteins were blotted to PVDF for probing with antibodies directed to the *E. coli* KAS I (*B*) or to an expressed condensing-enzyme-homologous ORF from barley (*C*).

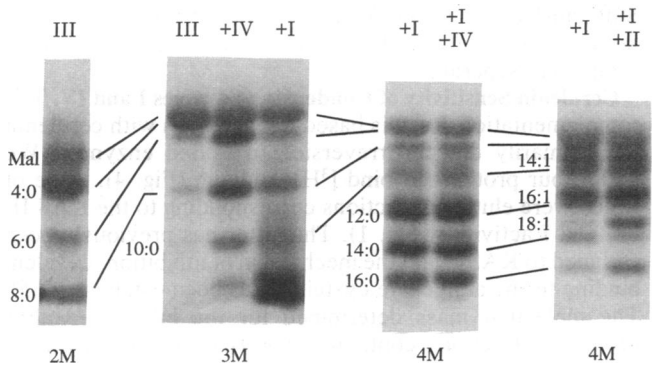

FIG. 3. Acyl-ACPs separated by urea/PAGE and detected by autoradiography. Urea concentrations are shown below the autoradiograms. III, complementation assay background generally attributed to KAS III; +I, +II, and +IV, products formed after addition of KASs I, II, and IV. Identification of acyl chains $\geq C_{10}$ was confirmed by radio gas chromatography (data not shown). Results shown in the rightmost panel were obtained for a different cerulenin-treated protein extract, with increased $C_{16:1}$ synthesis in the presence of KAS I.

signed to continue downstream. Kohara's λ clone 236 of ≈44.5 kb was used as template (12). By using DNA from this clone the entire ORF and its 5′ upstream region were then resequenced to ensure that no PCR errors had been introduced during the initial amplification. Sequencing was accomplished by the dye terminator method on an Applied Biosystems sequenator with the manufacturer's recommended parameters.

RESULTS AND DISCUSSION

Purification of Two KAS Enzymes. Two condensing enzymes that are separable by anion-exchange chromatography can be detected in the complementation assay (Fig. 1). Their elution positions coincide with those of [^{3}H]cerulenin-binding proteins (10), thus indicating that the condensing enzymes which are capable of restoring overall fatty acid synthesis to a cerulenin-treated enzyme extract can also bind the inhibitor covalently. This has previously been confirmed for the second eluted peak (Fig. 1, I), which was identified as KAS I, and the corresponding *fabB* gene was isolated and sequenced (5). In this work, we used a complementation assay to monitor the purification of the other activity through several rounds of anion-exchange chromatography followed by hydrophobic-interaction and gel filtration chromatography. As

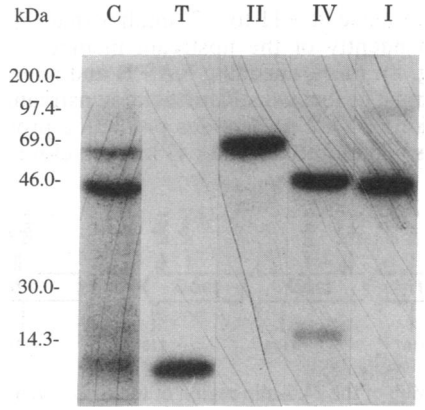

FIG. 4. [^{3}H]Cerulenin-binding proteins of *E. coli* analyzed by SDS/PAGE and fluorography before fractionation (C) and after anion-exchange (T, II, IV and I, thioredoxin and KASs II, IV and I; *cf.* Fig. 1, open arrows).

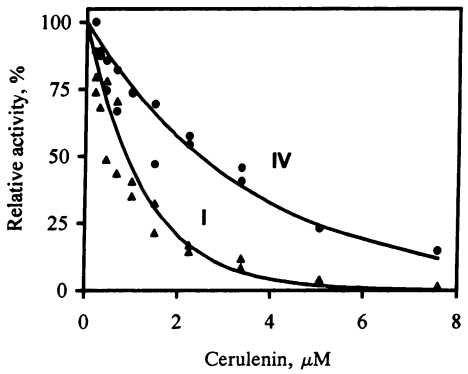

FIG. 5. Cerulenin inhibition of KAS I and IV activity. Assays were carried out as described, with 100% activity equaling 123 pmol/min (8100 dpm per assay) for KAS I and 100 pmol/min (6600 dpm per assay) for KAS IV.

judged from Coomassie-stained SDS/polyacrylamide gels, the final preparation contained several bands (Fig. 2*A*, "pure" lane IV) of which all the major proteins were amino-terminally sequenced. They were identified as pantothenate synthase (31.6 kDa), dihydroorotase (38.8 kDa), aspartate aminotransferase (43.6 kDa), and a condensing-enzyme homolog (44 kDa) of which the 30 amino-terminal residues were obtained. We infer the KAS activity to be associated with the latter protein and have given it the name KAS IV because several of its properties are different from those of the three previously characterized *E. coli* condensing enzymes. KAS IV crossreacts with antibodies raised to an expressed ORF from barley, homologous to condensing enzymes (Fig. 2*C*) (13), but not with antibodies raised to the native *E. coli* KAS I (Fig. 2*B*). Interestingly, a similar lack of crossreactivity was observed between KAS I antibodies and purified KAS II (4). As judged from the relative band intensities in Coomassie-stained gels (Fig. 2*A*) and Western blots (Fig. 2*C*), the antibody raised to the barley condensing enzyme shows similar affinities for *E. coli* KAS I and IV. After initial separation of KAS I and KAS IV (Fig. 1), use of this antibody on aliquots revealed KAS I to be a more abundant protein than KAS IV (Fig. 2 *A* and *C*, crude). Given that the KAS IV and I activities loaded in the same lanes were 1.8 and 2.3 nmol/min, it may also be estimated that KAS IV is a much more active enzyme in our assay than is KAS I. All following analyses were carried out on fractions from the final stage of column chromatographic purification (Fig. 2*A*, pure).

Substrate Specificities of the Condensing Enzymes. Conformationally sensitive gels were used to identify the acyl-ACP products (9) formed in the complementation assay by each of

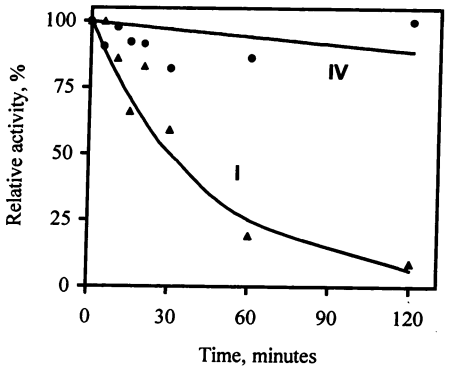

FIG. 6. Heat stability of KASs I and IV. Each enzyme was incubated at 43°C for the specified times and immediately assayed for residual activity at 37°C. The reference activities were 55 pmol/min (3600 dpm per assay) for KAS I and 41 nmol/min (2700 dpm per assay) for KAS IV.

the two activities (Fig. 3). This analysis revealed that the cerulenin-inhibited protein extracts produced mainly $C_{4:0}$ acyl-ACP, believed to be due to the presence of the cerulenin-insensitive KAS III (14). The two activities analyzed carried out elongations resulting in the synthesis of $C_{6:0}$ and $C_{8:0}$ and of $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, and $C_{16:1}$ (Fig. 3). The latter was consistent with the expected behavior of KAS I, while the former represented a KAS with unexpected substrate specificity—that is, the ability to elongate C_4 and possibly C_6 to yield C_8 as final product. Since the standard complementation assay (Fig. 1) did not detect other KASs carrying out the C_4-to-C_6 elongation, assays for the $C_{16:1}$- to $C_{18:1}$-elongating KAS II activity were carried out by including purified KAS I in the assay mix to produce a $C_{16:1}$ background. Analysis of formed acyl-ACPs revealed a KAS II activity in fractions (Fig. 3, rightmost lane; Fig. 1, II↑) different from those

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 s      CGTAAGTGAACATCTCCAGG→
-73 CACCAGGCGTAAGTGAACATCTCCAGGCGGTCGTTCGACCGCC-TGAGTT
      H  Q  A  *
-50 TTATCTTTTTGTCCCACTAGAATCATTTTTTCCCTCCCTGGAGGACAAAC
  1 GTGTCTAAGCGTCGTGTAGTTGTGACCGGACTGGGCATGTTGTCTCCTGT
  1 V  S  K  R  R  V  V  V  T  G  L  G  M  L  S  P  V
 51 CGGCAATACCGTAGAGTCTACCTGGAAAGCTCTGCTTGCCGGTCAGAGTG
 18   G  N  T  V  E  S  T  W  K  A  L  L  A  G  Q  S
101 GCATCAGCCTAATCGACCATTTCGATACTAGCGCCTATGCAACGAAATTT
 34 G  I  S  L  I  D  H  F  D  T  S  A  Y  A  T  K  F
151 GCTGGCTTAGTAAAGGATTTTAACTGTGAGGACATTATCTCGCGCAAAGA
 51 A  G  L  V  K  D  F  N  C  E  D  I  I  S  R  K  E
201 ACAGCGCAAGATGGATGCCTTCATTCAATATGGAATTGTCGCTGGCGTTC
 68   Q  R  K  M  D  A  F  I  Q  Y  G  I  V  A  G  V
251 AGGCCATGCAGGATTCTGGCCTTGAAATAACGGAAGAGAACGCAACCCGC
 84 Q  A  M  Q  D  S  G  L  E  I  T  E  E  N  A  T  R
301 ATTGGTGCCGCAATTGGCTCCGGGATTGGCGGCCTCGGACTGATCGAAGA
101   I  G  A  A  I  G  S  G  I  G  G  L  G  L  I  E  E
351 AAACCACACATCTCTGATGAACGGTGGTCCACGTAAGATCAGCCCATTCT
118   N  H  T  S  L  M  N  G  G  P  R  K  I  S  P  F
401 TCGTTCCGTCAACGATTGTGAACATGGTGGCAGGTCATCTGACTATCATG
134 F  V  P  S  T  I  V  N  M  V  A  G  H  L  T  I  M
451 TATGGCCTGCGTGGCCCGAGCATCTCTATCGCGACTGCCTGTACTTCCGG
151 Y  G  L  R  G  P  S  I  S  I  A  T  A  C  T  S  G
501 CGTGCACAACATTGGCCATGCTGCGCGTATTATCGCGTATGGCGATGCTG
168   V  H  N  I  G  H  A  A  R  I  I  A  Y  G  D  A
551 ACGTGATGGTTGCAGGTGGCGCAGAGAAAGCCAGTACGCCGCTGGGCGTT
184 D  V  M  V  A  G  G  A  E  K  A  S  T  P  L  G  V
601 GGTGGTTTTGGCGCGGCACGTGCATTATCTACCCGCAATGATAACCCGCA
201   G  G  F  G  A  A  R  A  L  S  T  R  N  D  N  P  Q
651 AGCGGCGAGCCGCCCGTGGGATAAAGAGCGTGATGGTTTCGTACTGGGCG
218   A  A  S  R  P  W  D  K  E  R  D  G  F  V  L  G
701 ATGGTGCCGGTATGCTGGTACTTGAAGAGTACGAACACGCGAAAAAACGC
234 D  G  A  G  M  L  V  L  E  E  Y  E  H  A  K  K  R
751 GGTGCGAAAATTTACGCTGAACTCGTCGGCTTTGGTATGAGCAGCGATGC
251   G  A  K  I  Y  A  E  L  V  G  F  G  M  S  S  D  A
801 TTATCATATGACGTCACCGCCAGAAAATGGCGCAGGCGCAGCTCTGGCGA
268   Y  H  M  T  S  P  P  E  N  G  A  G  A  A  L  A
851 TGGCAAATGCTCTGCGTGATGCAGGCATTGAAGCGAGTCAGATTGGCTAC
284 M  A  N  A  L  R  D  A  G  I  E  A  S  Q  I  G  Y
901 GTTAACGCGCACGGTACTTCTACGCCGGCTGGCGATAAAGCTGAAGCGCA
301   V  N  A  H  G  T  S  T  P  A  G  D  K  A  E  A  Q
951 GGCGGTGAAAACCATCTTCGGTGAAGCTGCAAGCCGTGTGTTGGTAAGCT
318   A  V  K  T  I  F  G  E  A  A  S  R  V  L  V  S
1001 CCACGAAATCTATGACCGGTCACCTGTTAGGTGCGGCGGGTGCAGTAGAA
334 S  T  K  S  M  T  G  H  L  L  G  A  A  G  A  V  E
1051 TCTATCTACTCCATCCTGGCGCTGCGCGATCAGGCTGTTCCGCCAACCAT
351   S  I  Y  S  I  L  A  L  R  D  Q  A  V  P  P  T  I
1101 CAACCTGGATAACCCGGATGAAGGTTGCGATCTGGATTTCGTACCGCACG
368   N  L  D  N  P  D  E  G  C  D  L  D  F  V  P  H
 as                    ←AAYWSXTTYGGX
1151 AAGCGCGTCAGGTTAGCGGAATGGAATACACTCTGTGTAACTCCTTCGGC
384 E  A  R  Q  V  S  G  M  E  Y  T  L  C  N  S  F  G
 as TTYGGXGG
1201 TTCGGTGGCACTAATGGTTCTTTGATCTTTAAAAAGATCTAAGTTGTCAT
401   F  G  G  T  N  G  S  L  I  F  K  K  I  *
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FIG. 7. Sequence of the *E. coli fabJ* gene and the encoded KAS IV. Sense (s) and antisense (as) primers used for the initial PCR are indicated. Amino acids shown in bold were also identified by sequencing of the purified protein. Nucleotides in bold were identical to the 3′ noncoding region of the *acpP* gene (7), except at position −56, where a single T rather than TT was found. The terminal 3′ coding and noncoding region of *acpP* is shown for reference. Nucleotide ambiguity codes are according to the International Union of Biochemistry (18).

containing KASs I and IV, which showed no KAS II elongating activity. Thus, the KAS II and IV activities were completely separable.

Cerulenin Sensitivity of Condensing Enzymes I and IV. The complementation assay is based on inhibition with cerulenin and primarily detects irreversibly inhibited enzymes. We found four proteins to bind [^{3}H]cerulenin (Fig. 4), three of which were eluted in fractions corresponding to the KAS II, IV, and I activities (Fig. 1). The latter has previously been assigned to KAS I, and the mechanism of inhibition, covalent binding to the active-site cysteine, has been established (5). The molecular mass determined for the inhibitor-binding protein in fractions containing the KAS IV activity is in agreement with that expected for a labeled KAS IV (Fig. 4). We conclude that the irreversibly inhibited KAS in this fraction is indeed KAS IV. KAS II, which the above assays also showed to be irreversibly sensitive to cerulenin, may correspond to a 55-kDa inhibitor-binding protein found in these fractions (Figs. 1 and 4), an inference in conflict with the previously reported molecular mass of 44 kDa for this enzyme (4). The 11.8-kDa [^{3}H]cerulenin-binding protein (Fig. 4) that did not correspond to a KAS activity was identified as thioredoxin by amino-terminal sequencing. Under the described experimental conditions, 1 and 3 μM concentrations of cerulenin were required for 50% inhibition of KASs I and IV, respectively (Fig. 5). Since it is possible to completely inhibit the KAS I-dependent unsaturated fatty acid synthesis with little effect on overall KAS activity (5, 15), KAS II must be somewhat less sensitive than KAS I, and hence also less sensitive than KAS IV.

Heat Sensitivity. The proportion of unsaturated fatty acids in lipids of *E. coli* is temperature dependent (16, 17) and is regulated by the activity of KAS II (4). To determine whether KAS IV is also involved in this regulation at the protein level, the temperature dependency of its activity was analyzed. No change in activity was observed after 2-hr preincubations at 43°C, whereas KAS I retained only 10% of its original activity (Fig. 6). Complementation assays were also carried out for 30 min at 30°C, 37°C, and 43°C. Only small changes in the relative activities of KAS IV versus KAS I were noted (data not shown). We conclude that KAS IV does not play an important role in temperature control of *E. coli* fatty acid synthesis.

Sequence of *fabJ* and KAS IV. The first nine amino acids of KAS IV were identical to those which can be translated from an incomplete ORF found 3′ of *acpP* (7). PCR with primers derived from *acpP* and a well-conserved region of condensing enzymes led to the complete sequencing of *fabJ*, encoding KAS IV (Fig. 7). The presence of a putative transcription terminator just downstream from *acpP* (7), plus the Shine-Dalgarno sequence at −11 to −7, implies that *fabJ* is translated independently of the upstream members of the *fab* cluster (Fig. 8). *fabF*, encoding KAS II and mapped to this region (4), must be located still further downstream. The *fabJ* coding region of 1239 nucleotides predicts a protein of 413 amino acids and molecular mass 43 kDa, in close agreement

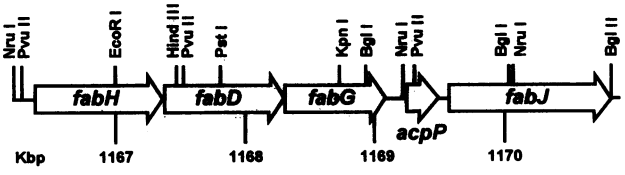

FIG. 8. Map of the 24.8-min region of the *E. coli* genome relative to the *Eco*RI restriction site at 1167 kbp (12). The genes *fabH*, *fabD*, *fabG*, *acpP*, and *fabJ* encode KAS III, malonyl-CoA:ACP transacylase, β-ketoacyl-ACP reductase, ACP, and KAS IV, respectively. *fabB*, encoding KAS I, is found at 50 min on the *E. coli* map (5, 19).

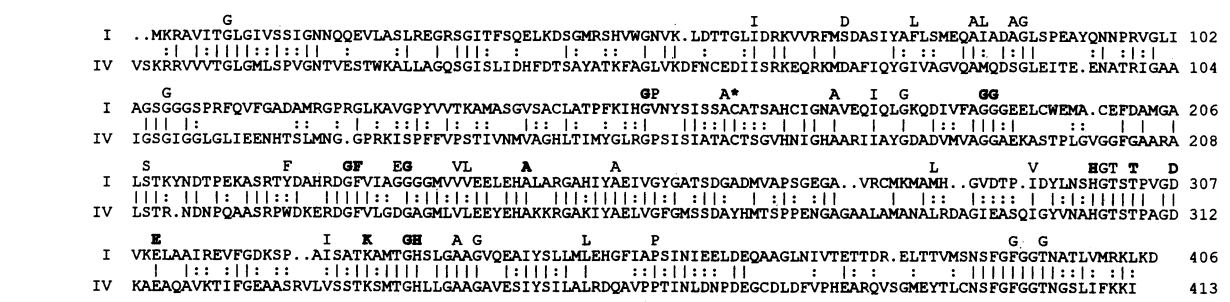

FIG. 9. Comparison of the primary structures of *E. coli* KASs I and IV. Amino acid identities are indicated by vertical lines and conservative substitutions by colons. For the latter assignments, amino acids were placed in groups: AGSTP, ILMV, FYW, EDNQ, and HKR. Residues found to be highly conserved among 45 condensing enzymes and homologs (11) are shown above the alignment, where a boldface type indicates nearly complete conservation. The substrate-binding cysteine is indicated with an asterisk.

with the 44-kDa estimate obtained upon SDS/PAGE (Fig. 2). When compared with the *fabB*-encoded KAS I (5), it is 38% identical and has an additional 26% conserved residues, totaling a similarity of 64% (Fig. 9). In all known cases, KAS IV is more similar to other condensing enzymes than is KAS I. For example, KAS IV shares 45% and KAS I 35% identical residues with the expressed barley protein used in preparing antibodies for this work (13).

Why KAS IV? Synthesis of the fatty acyl chains in *E. coli* destined for the membrane phospholipids (C_{16}, $C_{16:1}$, and $C_{18:1}$) and the lipid A (β-OH-C_{14}) component of the lipopolysaccharides of the outer membrane has been extensively studied over the years. A major focus of interest has been to determine the contributions of temperature regulation and the roles of KASs I, II, and III in determining the final fatty acyl composition of the phospholipids and hence membrane fluidity. Given the picture which has emerged from this work (1, 20), one can ask, why KAS IV? We suggest that the function of this condensing enzyme with its preference for short chains is to provide the C_8 precursors for lipoic acid (21), and hence the lipoamide cofactor. Given the endosymbiont theory for the origin of plant plastids and the extensive similarities between synthesis of fatty acids in *E. coli* and plants, we predict that an analogue of *E. coli* KAS IV will be found in plants. However, the location of the plant KAS IV is likely to be in the mitochondria rather than the plastids. That is, the glycine decarboxylase complex with its lipoamide cofactors which functions in photorespiration has been estimated to amount to about one-third of the total soluble mitochondrial proteins in green pea leaves (22). The need for the large number of C_8 fatty acyl chains provides a potential function in fatty acid synthesis for the ACP detected in plant mitochondria (23, 24). Will another KAS IV occur in plastids? The answer may be of interest with respect to the industrial goal of producing short-chain fatty acids in oil seeds.

Note Added in Proof. Further sequencing of the region 3′ of *fabJ* by A. R. Stuitje's group has revealed that *fabJ* is the last fatty acid biosynthetic gene in this cluster (personal communication). *pabC*, encoding aminodeoxychorismate lyase (25), begins 118 bp after the stop codon of *fabJ*.

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