Cloning of the *fabF* gene in an expression vector and in vitro characterization of recombinant *fabF* and *fabB* encoded enzymes from *Escherichia coli*

Patricia Edwards[1], Janet Sabo Nelsen, James G. Metz, Katayoon Dehesh*

Oils Division, Calgene, Inc., 1920 Fifth St., Davis, CA 95616, USA

Abstract Analysis of the β-ketoacyl-ACP synthase (KAS) encoded by the *fabF* gene of *Escherichia coli* has been hampered by a reported instability of the cloned gene. Here we describe biochemical characterization of purified, active protein from the recombinant *fabF* gene. This enzyme has the properties ascribed to KAS II and not those of a putative KAS IV reported to be encoded by *fabJ*, a genomic clone with DNA sequence identical to that of *fabF*. We also characterize active protein from a recombinant *fabB* gene and suggest that this method may have a general utility for analysis of KAS enzymes.

Key words: FabF; FabJ; Affinity purification; Recombinant enzyme; Thermal regulation; β-Ketoacyl-ACP synthase

1. Introduction

The chain elongation step of fatty acid synthesis is the condensation of an acyl group, bound to either acyl carrier protein (ACP) or CoA, with malonyl-ACP and is catalyzed by β-ketoacyl-ACP synthases (KAS). Until recently, three enzymes (KAS-I, -II and -III) were believed to accomplish all of the fatty acid condensation reactions in *E. coli*. Biochemical analyses of the purified enzymes indicated that they differ in their substrate specificities and in their sensitivities to cerulenin, a fungal product that is an inhibitor of condensing enzymes [1,2]. KAS III, the product of the *fabH* gene, is insensitive to cerulenin and catalyzes the condensation of acetyl-CoA with malonyl-ACP to form acetoacetyl-ACP [3]. KAS I, encoded by the *fabB* gene [4,5], is inhibited 50% by 1 μM cerulenin. Inhibition of KAS II, which has been associated with the *fabF* gene [6,7], requires higher concentrations of this compound. Both KAS I and KAS II catalyze the elongation of a wide range of saturated acyl-ACP. KAS I may be responsible for a condensation reaction in unsaturated fatty acid synthesis that cannot be catalyzed by KAS II [8], while KAS II is predominantly responsible for the elongation of palmitoleic acid (C16:1) required for synthesis of *cis*-vaccenic acid (C18:1). *E. coli* responds to a temperature down-shift by increasing the *cis*-vaccenate in its membrane [9]. KAS II is believed to play a key role in this thermal regulation of fatty acid synthesis [6,10]. With an in vitro assay of purified native enzyme, Garwin et al. [6] demonstrated that KAS II retains a disproportionate capacity to elongate C16:1-ACP at the lower temperatures. Both KAS I and KAS II enzymes are thought

to be active as dimers, and the possibility that one or both of these may be heterodimers has not been rigorously eliminated.

In 1994, Siggaard-Andersen et al. [11] reported identification of a fourth acyl-ACP condensing enzyme in *E. coli* with a substrate specificity for C4:0- and C6:0-ACP. They referred to this enzyme as KAS IV, and reported a 50% inhibition of its activity in the presence of 3 μM cerulenin. Proteins in a partially purified fraction containing the putative KAS IV activity were resolved by SDS-PAGE and N-terminal amino acid sequences of several of the polypeptides were determined. One of the sequences showed homology to known condensing enzymes and was subsequently used to clone the corresponding gene which they designated *fabJ* [11]. Later, Magnuson et al. [12] reported cloning of *fabF*, a gene with DNA sequence identical to that of *fabJ*. These authors utilized various molecular and genetic data to show that the *fabJ* gene is the previously reported *fabF* gene, which had been associated with KAS II. They also noted that an active enzyme product had not been obtained from the cloned *fabF* gene.

To determine directly the enzyme activity encoded by the *fabF/fabJ* sequence, we have produced corresponding recombinant protein as well as that encoded by the related *fabB*. Here we report the use of a tightly regulated expression system to obtain active, soluble, affinity purified protein products encoded by these genes. We show that the product of the recombinant *fabB* gene has the properties assigned to KAS I. Analysis of the *fabF/fabJ* gene product shows that it does not have the substrate specificity characteristics ascribed by Siggaard-Andersen et al. [11] to the putative KAS IV, but does have the properties previously associated with KAS II [13]. We provide additional chromatographic and substrate-range characterizations of these enzymes and demonstrate that thermal regulation of fatty acid synthesis is due solely to intrinsic properties of the KAS II enzyme.

2. Materials and methods

2.1. Chemicals and radiochemicals
Malonyl-CoA, fatty acids, cerulenin and β-mercaptoethanol were purchased from Sigma. Radiolabeled compounds were purchased from Amersham and New England Nuclear. Bovine serum albumin (BSA) was from Pierce. Malonyl-CoA:ACP *S*-malonyltransferase (EC 2.3.1.39) used in the KAS enzyme assays was partially purified from *E. coli* as described by Alberts et al. [14].

2.2. Cloning and expression of the fabB and fabF genes and purification of the encoded proteins
The DNA from *E. coli* K 12 was extracted as described by Miura [15] and used as the template in a standard polymerase chain reaction (PCR) for cloning of *fabB* and *fabF* genes employing published sequences [5,11]. For the in-frame fusions of these amplified genes into pQE-30 (QIAexpress, Qiagen Inc., Chatsworth, CA), the expression system driven by *E. coli* phage T5 promoter with N-terminal 6×his-

tidine affinity tags, the following synthetic oligonucleotide primers were designed:

```
fabB forward: GGATCCATGAAACGTGCAGTGATT
fabB reverse: GTCGACGAATTAATCTTTCAGCTT
fabF forward: GGATCCGCATGCGTGTCTAAGCGTCGTGTA
fabF reverse: GGATCCAAGCTTAACTTAGATCTTTTTAAA-
              GATCAA
```

The PCR products of the *fabB* gene were cut with *Bam*HI and *Sal*I restriction enzymes and cloned into the corresponding sites in pQE-30 vectors (pCGN4850). The PCR products of the *fabF* gene were cut with *Sph*I and *Hin*dIII restriction enzymes and cloned into the corresponding sites in pQE-30 vectors (pCGN4851). These plasmids were sequenced and their authenticities verified. To obtain soluble protein the M15[pREP4] strain of *E. coli* was transformed with these plasmids. Transformed bacteria with either these plasmids, or vector alone as the control, were grown at 37°C to an OD_{600} of 1.0, then were induced with 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 1 h. Cells were sedimented by centrifugation ($14000 \times g$, 10 min), resuspended in 50 mM KPO_4, 10% (w/v) glycerol, 300 mM NaCl and 10 mM imidazole, pH 7.8, and lysed by three 5-s sonications. Debris was sedimented by centrifugation ($17500 \times g$, 15 min) and the supernatant fraction was used for affinity purification of the recombinant protein over Ni-NTA resin according to instructions from Qiagen. The eluates from each chromatographic step were prepared for SDS-PAGE according to Laemmli [16] prior to electrophoresis on 12% acrylamide Tris-glycine gels (Novex). Proteins were visualized using a silver staining protocol [17], or by Coomassie Brilliant Blue R-250 staining. Protein concentrations were measured according to the method of Bradford [18] with BSA as a reference.

2.3. Size exclusion chromatography

Each of the Ni-NTA affinity-purified recombinant proteins was chromatographed over a Superdex 75 HR 10/30 size exclusion column (Pharmacia) in 25 mM Tris-HCl, pH 7.8, 10% (w/v) glycerol, 300 mM NaCl at 0.4 ml per min. Protein was monitored by absorbance at 280 nm and 0.2 ml fractions were collected. The fractions were analyzed by SDS-PAGE and assayed for KAS activity using C12:0-ACP as described below. Size exclusion standards (BioRad) were chromatographed under identical conditions and used to generate a molecular mass calibration curve.

2.4. ACP and acyl-ACP substrates

ACP was isolated from *E. coli* as described by Rock and Cronan [19] and stored at 4°C. The C6:0 to C12:0 acyl-ACP were synthesized with acyl-ACP synthetase from *Vibrio harveyi* according to the method of Shen et al. [20]. The C10:0 to C16:0 acyl-ACP were synthesized and purified according to Rock et al. [21], with acyl-ACP synthetase (EC 6.2.1.20) partially purified from *E. coli* as described by Rock and Cronan [22]. Even though the acyl-ACP did not need to be radiolabeled for the KAS assays, we used (1-^{14}C)-labeled fatty acids of very low specific activity (0.08 mCi per mmol) for synthesis of all substrates except C16:1 to facilitate their subsequent purifications. The acyl-ACP that eluted from the octyl Sepharose (Pharmacia) column was brought to near dryness in a SpeedVac Concentrator (Savant Instruments, Inc., Hicksville, NY) and the residue was diluted with deionized water. The substrates were stored at −20°C.

2.5. Enzyme assays

The KAS activities were assayed according to Garwin et al. [6] except that the dithiothreitol was replaced by 1.85 mM β-mercaptoethanol, the ACP concentration was increased to 60 μM, and the specific activity of the [2-^{14}C] malonyl-CoA was increased to 20 mCi per mmol. To ensure the linearity of the assay, we carried out the enzyme activity assays with a range of substrate concentrations, protein concentrations and incubation times (results not shown). Based on these findings we chose to use 30 μM acyl-ACP in our assays comparing the relative specific activities of the enzymes. 1 unit of KAS activity is defined as the incorporation of 1 μmol of ^{14}C from [2-^{14}C]malonate into borohydride-reduced product per min at 37°C. To determine the effect of temperature on enzyme activity, assays were incubated at 27 or 37°C, using C6:0- to C14:0-ACP and C16:1-ACP. For the data of Fig. 3, a clarified cell lysate of the *E. coli* control culture and the A_{280nm} peak fractions from size exclusion

columns were assayed over a range of acyl-ACP substrates. Authenticities of the assay products were verified by reverse phase TLC (Whatman KC 18), developed in methanol. Radioactive regions were detected and quantitated with an AMBIS 4000 Radioanalytic Imaging System (Scanalytics, Billerica, MA 01821).

2.6. Effects of cerulenin on KAS activity

1 ng of either the FabB or FabF protein was incubated at room temperature with 0, 5, 10, 50, 100 or 200 μM cerulenin for 7 min prior to initiation of the condensation reaction. A final volume of 25 μl contained 200 mM potassium phosphate buffer, pH 7.8, 0.6 mM β-mercaptoethanol, 1 mM EDTA, 50 μM ACP, 200 mM malonyl-CoA, 0.6 mU MTA and 30 μM C12:0-ACP.

3. Results

3.1. Cloning and expression of fabB and fabF genes, and affinity purification of the FabB and FabF proteins

Amplified fragments of *fabB* and *fabF* genes were cloned into the pQE-30 expression system (QIAexpress) and conditions under which the highest levels of soluble recombinant polypeptides could be obtained were identified. SDS-PAGE analyses of the affinity purified recombinant proteins indicate that, in both cases, up to 50% of the encoded proteins are present in the soluble fraction (result not shown). Each of the affinity purified recombinant proteins appears as a single band on a gel stained with Coomassie Blue (Fig. 1). This demonstrates that both enzymes have been purified to near homogeneity. A comparable fraction from control cells is devoid of any detectable proteins (Fig. 1).

The recombinant *fabB* and *fabF* genes encode polypeptides of 406 and 401 amino acids with predicted molecular masses of 42.6 and 41.7 kDa, and p*I* values of 5.20 and 5.28, respectively. Due to the cloning strategy and the presence of 6×histidine, an additional 0.77 and 0.99 kDa are added to the molecular masses predicted for the native *fabB* and *fabF* encoded polypeptides, respectively. To ensure the purity of the polypeptides the lanes on the SDS-PAGE were overloaded with proteins (Fig. 1). Estimation of the polypeptide sizes based on their mobilities on SDS-PAGE was performed using silver stained gels with less protein loaded per lane. An apparent mass of 50 kDa was obtained for the *fabF* encoded protein and 46 kDa for the *fabB* encoded protein (data not shown).

The affinity purified proteins were analyzed with a KAS assay using a range of saturated substrates, C6:0- to C16:0-ACP, and C16:1-ACP. Both affinity purified enzymes readily elongate the C6:0-ACP through C12:0-ACP saturated substrates, have substantially less activity with C14:0-ACP and have little capacity for elongation of C16:0-ACP. Further-

Table 1
Relative KAS activity at 27°C

Acyl-ACP[a] substrate	FabB[b]	FabF	Crude *E. coli* lysate
6:0	41	56	53
8:0	47	56	53
10:0	49	55	54
12:0	46	50	51
14:0	[c]	46	57
16:0	[c]	66	63

[a]Affinity purified FabB, FabF and clarified *E. coli* lysate assayed with enzymatically synthesized acyl-ACP at 27 and 37°C.
[b]Activity at 27°C is expressed as percent of the activity at 37°C with the corresponding substrate.
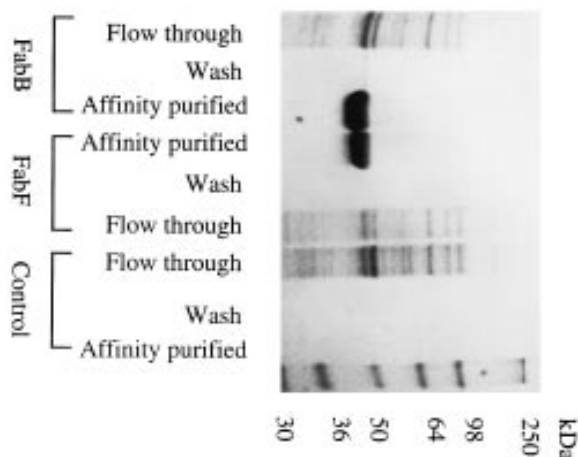[c]Below level of detection.

Fig. 1. SDS-PAGE of fractions from affinity chromatography of *fabB* and *fabF* encoded proteins. Lysates from cultures of transformants were chromatographed on Ni-NTA resin and proteins were resolved by SDS-PAGE and stained with Coomassie Blue. Control cells were transformed with vector alone. Affinity purified proteins were eluted with 300 mM imidazole buffer, pH 7.8.

more, the *fabB* gene product has little or no activity with C16:1-ACP, whereas the *fabF* gene product shows relatively good activity with this substrate. These data are not shown since they are qualitatively and quantitatively the same as those obtained using active fractions from the size exclusion columns (see below).

3.2. Size exclusion chromatography

On size exclusion chromatography in the presence of 300 mM NaCl, the *fabF* gene product elutes as a single A_{280nm} peak with an estimated mass of 72 kDa (Fig. 2A). Under identical conditions, the majority of the affinity purified *fabB* gene product elutes from the column as a peak with an apparent mass of 76 kDa. In addition, a smaller peak was observed with an apparent mass of 42 kDa. On SDS-PAGE the protein from each peak appears as a 46 kDa polypeptide (results not shown, but see Fig. 1). Presumably, the 76 kDa peak represents the dimeric form of the FabB protein, while that at 42 kDa is the monomer. Reapplication of a fraction from the dimer peak to the Superdex 75 column resulted in a similar profile, including the appearance of the peak at 42 kDa, indicating that dissociation of the dimer occurs during the chromatographic run. KAS activity (assayed using C10:0-ACP) is associated only with the dimeric form of the enzyme (Fig. 2B). The proportion of the FabB protein eluting in the two peaks is readily altered by either increasing or decreasing the salt concentration in the column buffer. For example, in the presence of 1 M NaCl, the majority of the protein was found to be in the monomeric form (Fig. 2C).

3.3. Substrate specificities of recombinant fabB and fabF encoded enzymes

The individual acyl-ACP substrates were prepared by enzymatic synthesis using synthetases from two bacterial sources. Neither acyl-ACP synthetase had activity over the entire range of fatty acids but they overlapped for C10:0 and C12:0. The acyl-ACPs of these two chain lengths prepared with each syn-

thetase gave comparable results in the KAS assays (results not shown).

The fraction from the dimer peak of each of the recombinant Fab proteins obtained from the size exclusion columns was used to characterize their substrate specificities. These fractions, as well as an *E. coli* lysate, were assayed using a range of saturated (C6:0- to C16:0-) acyl-ACP and C16:1-ACP (the only available unsaturated substrate) (Fig. 3A,B). As indicated above, the results are similar to those of analysis of the purified proteins prior to size exclusion chromatography. The homodimers of the *fabB* and *fabF* encoded enzymes have the highest levels of activity on the C6:0- to C12:0-saturated acyl-ACP and minimal levels of activity on C16:0-ACP. The activity measurements with C16:1-ACP show that the FabF dimer readily elongates palmitoleic acid while the FabB dimer does not. With all of the substrates tested and under the conditions employed for these assays, the *fabF* gene product has a higher relative specific activity than the *fabB*
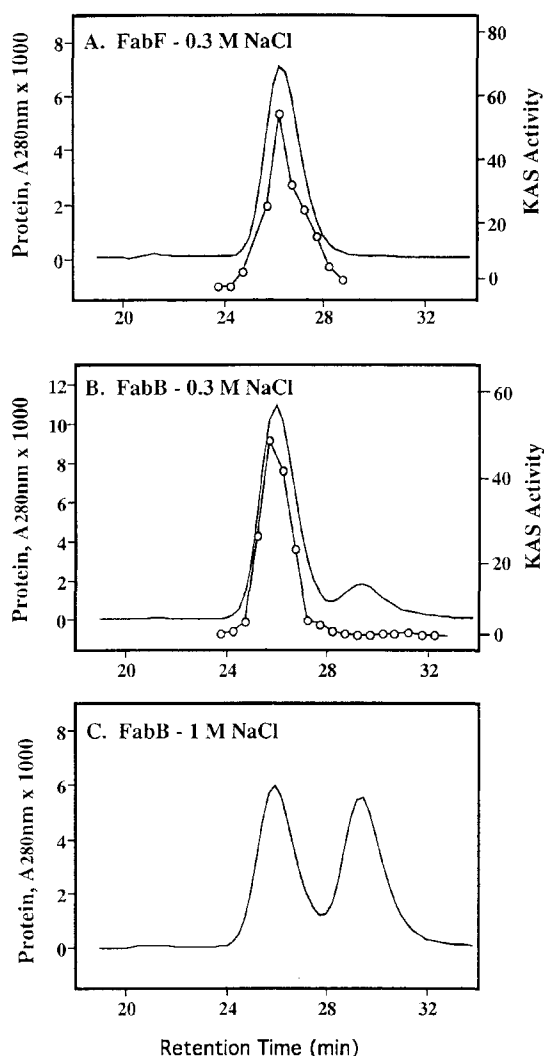

Fig. 2. Size exclusion chromatography of recombinant *fabF* and *fabB* encoded enzymes. The affinity purified *fabF* (A) and *fabB* (B,C) encoded proteins were chromatographed in the presence of either 300 mM NaCl (A and B) or 1 M NaCl (C) over a Superdex 75 HR column. The KAS activities were determined using C12:0-ACP as a substrate and are expressed as units per fraction (○—○). Protein is shown as $A_{280nm} \times 1000$ (———).

gene product. For example, the relative specific activities of *fabF* and *fabB* encoded enzymes with C10:0-ACP are 25.6 and 15.2 μU per μg protein, respectively. The most dramatic difference between these two enzymes, however, was that the specific activity of the *fabF* gene product was 9-fold more than that of the *fabB* gene product in elongating C16:1-ACP. In addition, radioscanning of the TLC plates indicates that 5–10% of total acyl product as the result of FabF protein activity is detected in a short chain product (data not shown). The exact chain length of this product is yet to be determined. Under no circumstances was such product detected on plates resolving acyl products resulting from KAS I activity.

3.4. Effect of temperature on enzyme activities

The KAS activities of purified *fabB* and *fabF* gene products and an *E. coli* cell lysate at 27 and 37°C were determined using a range of acyl-ACP substrates (Table 1). As expected, for all examined substrates KAS activities were lower at 27°C than at 37°C. This decrease in activity levels was greater for recombinant *fabB* gene product than for either recombinant *fabF* gene product or combined KAS activities in *E. coli* lysate. The activity least depressed by this temperature down-
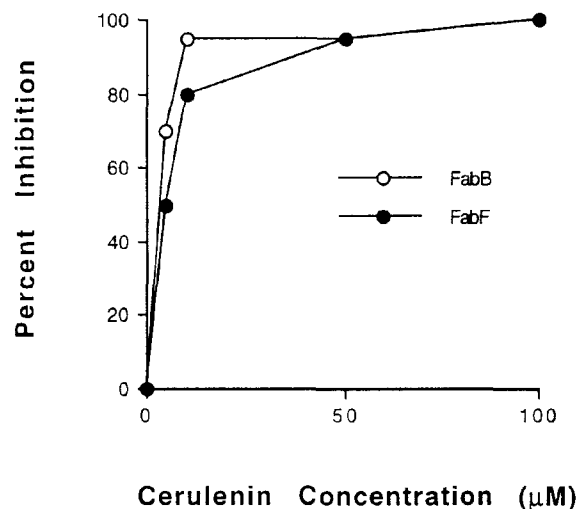
Fig. 4. Effects of cerulenin on FabB and FabF KAS activities. 1 ng of either enzyme was incubated with concentrations of cerulenin indicated prior to addition of C12:0-ACP. FabB (○—○), FabF (●—●).

shift was elongation of C16:1-ACP by the recombinant *fabF* gene product.

3.5. Cerulenin inhibition

Fig. 4 shows the effects of cerulenin, an irreversible inhibitor, on the KAS activities of the recombinant proteins. The KAS activity of 1 ng of FabB protein is 70% inhibited by 5 μM cerulenin. Under identical conditions, 1 ng of FabF protein is inhibited only 50%. Both enzymes are fully inactivated in the presence of 50 μM cerulenin.

4. Discussion

We report here the first use of an expression system to study the recombinant enzymes encoded by the *fabB* and *fabF* genes of *E. coli*. It has been established that the product of the *fabB* gene is KAS I, an enzyme which has been purified and biochemically characterized. We show that expression of the *fabB* gene using a system that includes the addition of six histidine residues to the N-terminus of the protein yields an enzyme which has properties described for native KAS I. The active enzyme is a dimer, capable of catalyzing the elongation of a range of saturated acyl-ACP substrates, but is inefficient in the elongation of C16:1-ACP.

We used the same expression system in order to resolve the conflicting reports concerning the nature of the enzyme encoded by *fabF* (called *fabJ* by Siggaard-Andersen et al. [11]). Our results show that the purified protein of *fabF* has the biochemical properties ascribed to the native KAS II. Its condensing activity is not restricted to short chain substrates and therefore it cannot, by itself, be associated with the putative KAS IV enzyme reported by Siggaard-Andersen et al. [11]. The purified protein exhibits an additional property which has been associated with KAS II, namely, involvement with thermal regulation of fatty acid synthesis. The effect of temperature on the activities of the purified *fabB* and *fabF* gene products is shown in Table 1 and the activity least depressed by this temperature down-shift is elongation of C16:0-ACP by the recombinant *fabF* gene product. This is identical to the
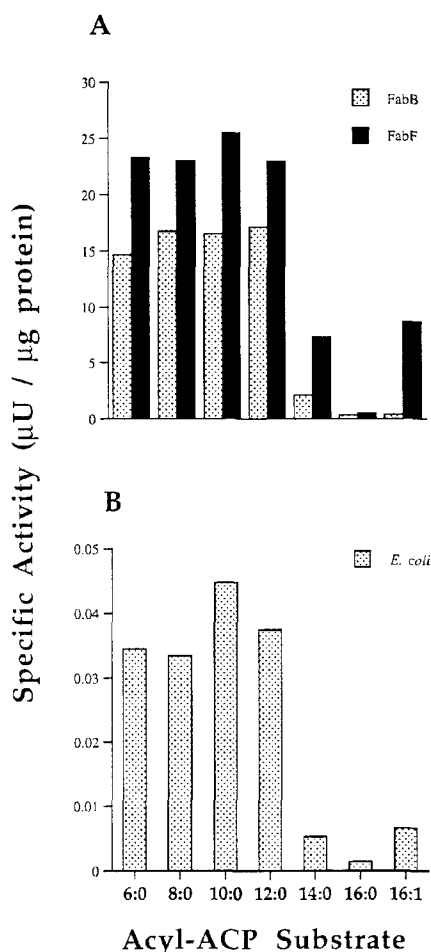
Fig. 3. Acyl-ACP substrate specificities of affinity purified recombinant *fabB* and *fabF* encoded proteins. The fraction corresponding to the dimer peak from each of FabB and FabF recombinant proteins (A), and the clarified cell lysate of an *E. coli* control culture (B), were assayed with 30 μM acyl-ACP substrates.

temperature response reported for native KAS II [6,10]. The effect of cerulenin on the activities of *fabB* and *fabF* encoded enzymes is also consistent with those reported for native KAS I and KAS II. When equivalent amounts of each protein are utilized in the assays, the KAS activity associated with FabB is more sensitive to low levels of cerulenin than is the KAS activity of FabF.

The expression system employed here provides a simple method for obtaining large amounts of purified active *E. coli* KAS I and KAS II enzymes. This should permit additional detailed analysis of their structures and functions and it may provide a general method for analysis of recombinant KAS enzymes. For example, the nature of the expression system demonstrates that both KAS I and KAS II are the products of single genes and eliminates the possibility that either one is composed of heterodimers.

It has been reported that native KAS II has broader pH optimum as well as higher temperature tolerance than KAS I [23]. We show that, under our isolation conditions, the dimeric form of the FabB protein is less stable than that of FabF. The FabF protein consistently chromatographs as a single 72 kDa peak on size exclusion columns, indicating all of the soluble protein is in the dimeric form. In contrast, preparations of the FabB protein always show the presence of some of the monomeric form. The amount of monomer observed in FabB preparations is readily influenced by the salt concentration present during chromatography while the FabF dimer is much more stable. Dissociation of the FabB dimer may account for some of the above mentioned relative differences in stability of KAS I and KAS II and may also contribute to the higher relative specific activity observed for the FabF protein in our preparations.

Involvement of KAS II and the *fabF* gene with temperature regulation of fatty acid composition in *E. coli* has been inferred from the results of physiological studies of mutants and activity measurements using purified native enzyme. In fact, the conclusion that *fabF* is the structural gene for KAS II was based on observation that mutants lacking temperature regulation (Cvc) also lack KAS II activity [6,10,13]. The same reports also indicate that the Cvc phenotype and lack of KAS II activity are due to a mutation in the same gene, designated *fabF*. Our use of the affinity purified *fabF* gene product demonstrates that this thermal response is due to an intrinsic property of a single enzyme and eliminates the possibility of involvement of some other protein factor.

Our data provide unequivocal molecular evidence that KAS II is the product of the *fabF* gene, yet the existence in *E. coli* of an enzyme with the properties ascribed to KAS IV remains a possibility. Siggaard-Andersen et al. [11] did not purify their putative KAS IV to homogeneity. The assay used in the KAS IV study was based on complementation of the fatty acid elongation system in cerulenin treated *E. coli* extracts and it is difficult to compare those data with our direct assay of the condensation reaction. However, we have extended the range of acyl-ACP substrates typically used in direct KAS assays by including those with acyl-chain lengths of 6, 8 and 10 carbons. This should have revealed an enzyme with the preference for short chain substrates ascribed to KAS IV. The substrate specificity profile of KAS enzymes in the crude lysate of *E. coli* appears to reflect the combined profiles of the FabB and FabF recombinant enzymes, suggesting that if a KAS IV is present, it is a relatively minor component.

This expression system should prove useful for further studies of KAS I and KAS II, as well as other related enzymes. The inherent features of the system permit one to resolve questions that are often difficult to address unambiguously with partially purified native enzymes. The system also provides a ready means to assess the effect of specific mutations in the KAS genes on their enzymatic activities.

References

[1] D'Agnolo, G., Rosenfeld, I.S., Awaya, J., Omura, S. and Vagelos, P.R. (1973) Biochim. Biophys. Acta 326, 155–166.

[2] Vance, D.E., Goldberg, I., Mitsuhashi, O., Bloch, K., Omura, S. and Nomura, S. (1972) Biochem. Biophys. Res. Commun. 48, 649–656.

[3] Tsay, J.-T., Oh, W., Larson, T.J., Jackowski, S. and Rock, C.O. (1992) J. Biol. Chem. 267, 6807–6814.

[4] De Mendoza, D., Garwin, J.L. and Cronan, J.E., Jr. (1982) J. Bacteriol. 151, 1608–1611.

[5] Kauppinen, S., Siggaard-Andersen, M. and Von Wettstein-Knowles, P. (1988) Carlsberg Res. Commun. 53, 357–370.

[6] Garwin J.L., Klages, A.L. and Cronan, J.E., Jr. (1980) J. Biol. Chem. 255, 11949–11956.

[7] Ulrich, A.K., De Mendoza, D., Garwin, J.L. and Cronan, J.E., Jr. (1983) J. Bacteriol. 154, 221–230.

[8] Cronan, J.E., Jr. and Rock, C.O. (1987) in: Escherichia coli and Salmonella typhimurium, Cellular and Molecular Biology (Neidhardt, F.C. ed.) pp. 474–497, American Society for Microbiology, Washington, DC.

[9] Nishihara, M., Iohinaga, M., Kato, M. and Kito, M. (1976) Biochim. Biophys. Acta 431, 54–61.

[10] Garwin, J.L., Klages, A.L. and Cronan, J.E., Jr. (1980) J. Biol. Chem. 255, 3263–3265.

[11] Siggaard-Andersen, M., Wissenbach, M., Chuck, J., Svendsen, I., Olsen, J.G. and Von Wettstein-Knowles, P. (1994) Proc. Natl. Acad. Sci. USA 91, 11027–11031.

[12] Magnuson, K., Carey, M.R. and Cronan, J.E., Jr. (1995) J. Bacteriol. 177, 3593–3595.

[13] Cronan, J.E. and Rock, C.O. (1996) in: Escherichia coli and Salmonella typhimurium, Cellular and Molecular Biology (Neidhardt, F.C. ed.) pp. 612–636, American Society for Microbiology, Washington, DC.

[14] Alberts, A.W., Majerus, P.W. and Vagelos, P.R. (1969) Methods Enzymol. 14, 53–56.

[15] Miura, K. (1967) Methods Enzymol. 12A, 543–545.

[16] Laemmli, U.K. (1970) Nature 227, 680–685.

[17] Blum, H., Beier, H. and Gross, H.J. (1987) Electrophoresis 8, 93–99.

[18] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.

[19] Rock, C.O. and Cronan, J.E., Jr. (1981) Methods Enzymol. 71, 341–351.

[20] Shen, Z., Fice, D. and Byers, D.M. (1992) Anal. Biochem. 204, 34–39.

[21] Rock, C.O., Garwin, J.L. and Cronan, J.E., Jr. (1981) Methods Enzymol. 72, 397–403.

[22] Rock, C.O. and Cronan, J.E., Jr. (1979) J. Biol. Chem. 254, 7116–7122.

[23] D'Agnolo, G., Rosenfeld, I.S. and Vagelos, P.R. (1975) J. Biol. Chem. 250, 5289–5294.