Cloning of the \textit{fabF} gene in an expression vector and in vitro characterization of recombinant \textit{fabF} and \textit{fabB} encoded enzymes from 
\textit{Escherichia coli}

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Abstract Analysis of the \(\beta\)-ketoacyl-ACP synthase (KAS) encoded by the \textit{fabF} gene of \textit{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 \textit{fabF} gene. This enzyme has the properties ascribed to KAS II and not those of a putative KAS IV reported to be encoded by \textit{fabJ}, a genomic clone with DNA sequence identical to that of \textit{fabF}. We also characterize active protein from a recombinant \textit{fabB} gene and suggest that this method may have a general utility for analysis of KAS enzymes.

\textit{Key words: FabF; FabJ; Affinity purification; Recombinant enzyme; Thermal regulation; \(\beta\)-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 \(\beta\)-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 \textit{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, and in its membrane [9]. KAS II is 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 \(\alpha\)-linolenic acid (C18:3). \textit{E. coli} responds to a temperature down-shift by increasing the \(\alpha\)-linolenate 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 \textit{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 \(\mu\)M cerulein. 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 \textit{fabJ} [11]. Later, Magnuson et al. [12] reported cloning of \textit{fabF}, a gene with DNA sequence identical to that of \textit{fabJ}. These authors utilized various molecular and genetic data to show that the \textit{fabJ} gene is the previously reported \textit{fabF} gene, which had been associated with KAS II. They also noted that an active enzyme product had not been obtained from the cloned \textit{fabF} gene.

To determine directly the enzyme activity encoded by the \textit{fabF/\textit{fabJ}} sequence, we have produced corresponding recombinant protein as well as that encoded by the related \textit{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 \textit{\textit{fabB}} gene has the properties assigned to KAS I. Analysis of the \textit{\textit{fabF/\textit{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 \(\beta\)-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 \textit{E. coli} as described by Alberts et al. [14].

2.2. Cloning and expression of the \textit{fabB} and \textit{fabF} genes and purification of the encoded proteins

The DNA from \textit{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 \textit{\textit{fabB}} and \textit{\textit{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 \textit{E. coli} phage T5 promoter with N-terminal 6Xhis-
2.5 Enzyme assays

2.4 ACP and acyl-ACP substrates

2.3 Size exclusion chromatography

2.2.3 Methods

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

The PCR products of the fabB gene were cut with BamHI and Sall restriction enzymes and cloned into the corresponding sites in pQE-30 vectors (pCGN4850). The PCR products of the fabF gene were cut with SphI and HindIII 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 OD490 of 1.0, then were induced with 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 1 h. Cells were sedimented by centrifugation (14000 × g, 10 min), resuspended in 50 mM KPO4, 10% (w/v) glycerol, 300 mM NaCl and 10 mM imidazole, pH 7.8, and lyzed by three 5-s sonications. Debris was sedimented by centrifugation (17500 × 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 [18] prior to electrophoresis on 406 and 401 amino acids with predicted molecular masses of 40.6 and 39.1 kDa, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Transformed bacteria with either these plasmids, or vector alone as the control, were grown at 37°C to an OD600 of 1.0, then were induced with 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 1 h. Cells were sedimented by centrifugation (14000 × g, 10 min), resuspended in 50 mM KPO4, 10% (w/v) glycerol, 300 mM NaCl and 10 mM imidazole, pH 7.8, and lyzed by three 5-s sonications. Debris was sedimented by centrifugation (17500 × 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 [18] prior to electrophoresis on 406 and 401 amino acids with predicted molecular masses of 40.6 and 39.1 kDa, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

The PCR products of the fabB gene were cut with BamHI and Sall restriction enzymes and cloned into the corresponding sites in pQE-30 vectors (pCGN4850). The PCR products of the fabF gene were cut with SphI and HindIII 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 OD600 of 1.0, then were induced with 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 1 h. Cells were sedimented by centrifugation (14000 × g, 10 min), resuspended in 50 mM KPO4, 10% (w/v) glycerol, 300 mM NaCl and 10 mM imidazole, pH 7.8, and lyzed by three 5-s sonications. Debris was sedimented by centrifugation (17500 × 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 [18] prior to electrophoresis on 406 and 401 amino acids with predicted molecular masses of 40.6 and 39.1 kDa, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

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 recombinant proteins appear 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 pI 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 fabB encoded protein and 46 kDa for the fabF 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 C16:0-ACP saturated substrates, have substantially less activity with C14:0-ACP and have little capacity for elongation of C16:0-ACP. Further-
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
\text{Abs}_{380}\text{nm} peak with an estimated mass of 72 kDa (Fig. 2A). Under identical conditions, the majority of the affinity purified fabF 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 synthetase 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 gene product.

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
\text{Abs}_{380}\text{nm} x 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-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.](image-url)

![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 (●—●).](image-url)
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 FabF 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