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Methylmalonyl Coenzyme A Selectivity of Cloned and Expressed Acyltransferase and β-Ketoacyl Synthase Domains of Mycocerosic Acid Synthase from *Mycobacterium bovis* BCG

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Methyl-branched fatty acids and polyketides occur in a variety of living organisms. Previous studies have established that multifunctional enzymes use methylmalonyl coenzyme A (CoA) as the substrate to generate methyl-branched products such as mycocerosic acids and polyketides. However, we do not know which of the component activities show selectivity for methylmalonyl-CoA in any biological system. A comparison of homologies of the domains of the multifunctional synthases that selectively use malonyl-CoA or methylmalonyl-CoA suggested that the acyltransferase (AT) and β-ketoacyl synthase (KS) domains might be responsible for the substrate selectivity. To test this hypothesis, we expressed the AT and KS domains of the mycocerosic acid synthase (MAS) gene from *Mycobacterium bovis* BCG in *Escherichia coli* and examined whether they confer to synthases that normally do not use methylmalonyl-CoA the ability to incorporate methylmalonyl-CoA into fatty acids. Both the AT and the KS domains of MAS showed selectivity for methylmalonyl-CoA over malonyl-CoA. Acyl carrier protein (ACP)-dependent elongation of the *n*-C_{12} acyl primer mainly by one methylmalonyl-CoA unit was catalyzed by an *E. coli* fatty acid synthase preparation only in the presence of the expressed MAS domains. An ACP-dependent elongation of the *n*-C_{20} acyl primer by one methylmalonyl-CoA extender unit was catalyzed by fatty acid synthase from *Mycobacterium smegmatis* only in the presence of the expressed MAS domains. These results show methylmalonyl-CoA selectivity for the AT and KS domains of MAS. These domains may be useful in producing novel polyketides by genetic engineering.

Methyl-branched natural products are found in a variety of organisms (1, 7, 9). Examples of such products include polyketides (polyethers, macrolides, and macrolactams) (11, 17, 21) and multiple-methyl-branched-chain fatty acids (1, 19) of mycobacteria and of specialized glands in vertebrates (9). Different molecular strategies are used to produce such branched natural products. Some microbes use C methylation at an olefin with *S*-adenosylmethionine to produce methyl branches (9). In vertebrate glands, fatty acid synthase (FAS) is inherently capable of using methylmalonyl coenzyme A (CoA) in the absence of malonyl-CoA to produce multiple-methyl-branched fatty acids (4, 8, 14). In actinomycetes and mycobacteria, the use of methylmalonyl-CoA instead of malonyl-CoA leads to methyl branches in the polyketide backbone and multiple-methyl-branched mycocerosic acids, respectively (11, 17, 22).

Two different types of synthases are used to generate the methyl-branched natural products. When the synthesis of multiple-methyl-branched natural products involves repetition of the full complement of reactions involved in chain elongation, one multifunctional protein catalyzes several cycles of reactions to generate the final product (18). For this type of synthesis, each elongation involves condensation, ketoreduction, dehydration, and enoyl reduction followed by acyl transfer. On the other hand, when the synthesis of different segments of natural products involves different substrates or requires partial and/or different complements of chain-elongation reactions, each elongation cycle is catalyzed by a different enzyme module containing the appropriate combination of domains (11, 15, 17). Thus, some synthases may selectively use methylmalonyl-CoA in some cycles and malonyl-CoA in others to generate natural products with methyl branches in specific locations on the backbone (2, 11). The specific domain(s) of the synthase that confers selectivity for methylmalonyl-CoA in any of the synthases and the molecular basis of this selectivity are unknown.

Mycocerosic acid synthase (MAS) is the multifunctional enzyme which catalyzes the elongation of *n*-fatty acyl-CoA by specifically using methylmalonyl-CoA as the chain-extender unit to produce multimethyl-branched mycocerosic acids. These mycocerosic acids are found uniquely in pathogenic mycobacteria that are the causative agents of major infectious diseases, such as tuberculosis and leprosy, that kill millions of humans each year (3, 20). This enzyme has been purified and characterized (22), and the gene encoding this enzyme was cloned from *Mycobacterium bovis* BCG (18). The active site areas of the β-ketoacyl synthase (KS) domain and the acyltransferase (AT) domain of MAS show 93 and 83% homology, respectively, to their counterparts in the *eryA* gene, which encodes a polyketide synthase (PKS) specific for methylmalonyl-CoA, and a significantly lower degree of homology to the corresponding domains of the FASs that use malonyl-CoA to generate *n* fatty acids. The other domains of MAS did not show such uniquely high degrees of homology to the corresponding domains of methylmalonyl-CoA-utilizing synthases. Therefore, it was suggested that the AT and KS domains might be responsible for selectivity for methylmalonyl-CoA (18).

In this paper we report the results of experiments to test this possibility. We report the expression of the AT and KS domains of MAS in *Escherichia coli*. We demonstrate that these domains manifest a selectivity for methylmalonyl-CoA and confer the ability to incorporate methylmalonyl-CoA into fatty

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acids to the fatty acid synthases of *E. coli* and *Mycobacterium smegmatis* that normally do not use methylmalonyl-CoA. This is the first experimental demonstration of methylmalonyl-CoA-selective domains in nature.

MATERIALS AND METHODS

Expression of AT and KS domains. The AT and KS domains were cloned by PCR with *M. bovis* BCG genomic DNA as the template and the following primers. For KS, which comprises nucleotides 1 to 1023, the primers used were 5′ GCGCGAATTCATGGAATCACGTGTCACTCCC 3′ (forward primer) and 5′ CGATCCAAGCGCGCAGGGGGTGCC 3′ (reverse primer). For AT, which comprises nucleotides 1522 to 2671, the primers used were 5′ CTCGTCGAGG GTTTGCGC 3′ (forward primer) and 5′ GGCACCTTGTGCCCGCTGTTC TTG 3′ (reverse primer). The reaction mixture was cycled 40 times as follows: for 30 s at 92°C, for 30 s at 68°C, and for 30 s at 72°C. Products of the expected sizes were cloned into the TA cloning vector (Invitrogen) by the manufacturer's protocol to give constructs pKS and pAT. Both constructs were sequenced with Sequenase, version 2 (United States Biochemical and Amersham), to verify that no mutation had been introduced by the PCR procedure. Expression constructs were then created by introducing the KS domain into the *Eco*RI site of pET21c (Novagen) to give expression plasmid pMKS and the AT domain into pET21b (Novagen) with the *Not*I and *Hin*dIII sites to give expression plasmid pMAT. pMKS and pMAT were then individually introduced into strain BL21(DE3) pLysS (Novagen), and their expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h according to the manufacturer's protocol. The cells were lysed by following the manufacturer's protocol, and aliquots of the extract were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained.

Immunoblotting. Extracts from the cultures expressing AT and KS were subjected to SDS-PAGE. The proteins were blotted onto a Nytran membrane. Immunoblotting was done as previously described (6) with polyclonal antibodies raised against MAS in rabbit (22) and ^{125}I-protein A for detection.

Purification of enzymes. *M. smegmatis* FAS was purified as described previously (25). The expressed AT and KS domains were purified by utilizing the His.Tag purification system (Novagen) according to the manufacturer's protocol.

Methylmalonyl-CoA incorporation assay. The reaction mixture in a total volume of 500 μl contained 0.1 M phosphate buffer (pH 6.8), 1 mM NADH, 1 mM NADPH, 2 mM EDTA, 2 mM dithiothreitol, 100 μM acyl-CoA primer, 15 μM acyl carrier protein (ACP), and 25 μM [2-^{14}C]malonyl-CoA or [*methyl*-^{14}C] methylmalonyl-CoA (56 Ci/mol). To this mixture was added either (i) the *E. coli* extract containing the expressed domain, (ii) the extract containing the expressed domain and the *M. smegmatis* extract, or (iii) purified expressed enzyme with purified *M. smegmatis* FAS. Following a 60-min incubation at 37°C, the reaction was stopped by the addition of 250 μl of 10% NaOH, the mixture was heated in a boiling water bath for 10 min and acidified, and the lipids, which were extracted with chloroform-methanol (2:1), were subjected to thin-layer chromatography (TLC) on Silica Gel G plates with hexane-diethyl ether-formic acid (35:15:1, vol/vol/vol) as the solvent. After the plates were sprayed with a 0.1% ethanolic solution of 2,7-dichlorofluorescein, the positions of the UV-visible spots were recorded. External fatty acid standards were also used. The plates were then scanned for distribution of radioactivity with a Berthold LB285 Tracemaster automatic TLC-linear analyzer. Silica gel from the region corresponding to free fatty acids was scraped into a counting vial, mixed with Scintiverse scintillation fluid (Fisher Scientific), and assayed for radioactivity in a Beckman model LS3801 liquid scintillation system. Enzyme activity is expressed as total picomoles of methylmalonyl-CoA or malonyl-CoA incorporated into free fatty acids per milligram of the synthase.

Nature of the product. To determine if the product generated from methylmalonyl-CoA was free or esterified, a routine assay was performed and the chloroform-extractable products were removed and analyzed on a TLC plate as previously described. To determine if any of the product was protein bound (esterified), the aqueous phase that remained after the solvent extraction was treated by boiling for 30 min with 20% NaOH and acidification with 6 M HCl and the products were extracted with chloroform as indicated above and assayed for ^{14}C. Radiolabeled products recovered from the silica gel from the TLC plates were esterified by refluxing them with 14% BF_3 in methanol for 2 h at 55°C. The methyl esters recovered by extraction with chloroform were purified by TLC on Silica Gel G with hexane-ether-formic acid (35:15:1) as the solvent. Radio-gas-liquid chromatography of the methyl esters was performed with a coiled stainless steel column (0.3 by 300 cm) packed with 5% OV-1 (wt/wt) in Chrom W-HP 80/100 using a Varian model 3300 gas chromatograph with a 50 to 300°C temperature program at 20°C/min. The effluent from the column was passed through a LabLogic GC-RAM detector with Winflow (IN/US Systems) software. A statistical deconvolution algorithm (LabLogic) was used for statistical image enhancement. Chain lengths were determined by comparing retention times of the synthesized products to those of straight-chain fatty acid standards.

Partial reactions. Methylmalonyl-CoA or malonyl-CoA transacylase was measured by following the transfer of the methylmalonyl or malonyl group to ACP, as estimated by acid-precipitable radioactivity as previously described (24). The KS activity was assayed radiochemically by the condensation-CO_2 exchange reaction as previously described (16).

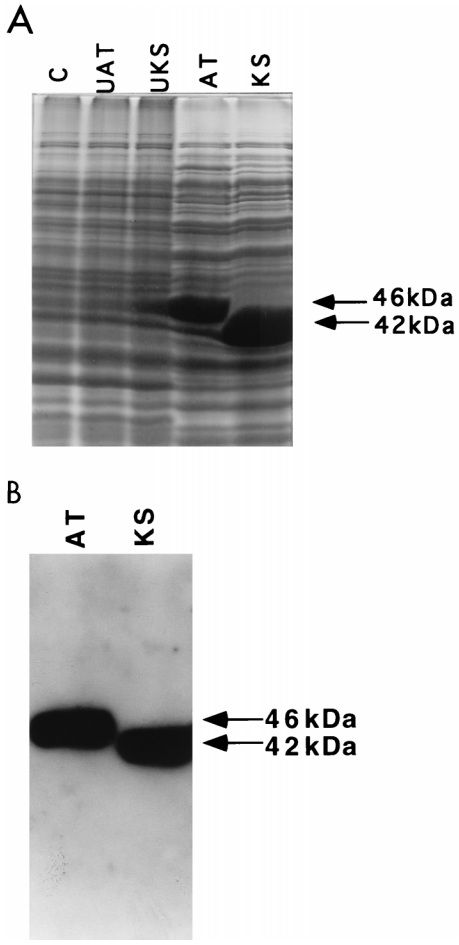

FIG. 1. (A) Coomassie blue-stained SDS-polyacrylamide gel of extracts of *E. coli* cells expressing the AT and KS domains of MAS. C, control of untransformed *E. coli* cells; UAT, uninduced extract of AT-expressing *E. coli* cells; UKS, uninduced extract of KS-expressing *E. coli* cells; AT, IPTG-induced extract of AT-expressing *E. coli* cells; KS, IPTG-induced extract of KS-expressing *E. coli* cells. (B) Autoradiogram of the immunoblot of the SDS-polyacrylamide gel shown in panel A with polyclonal antibodies raised against MAS and ^{125}I-labeled protein A. Molecular mass markers (in kilodaltons) are noted (arrows).

RESULTS

The uniquely high homology between the active sites of AT and KS of MAS with the corresponding domains in *eryA* suggested that the AT and KS domains may have selectivity for methylmalonyl-CoA (18). To test this possibility directly on the domains, the individual domains were produced by using recombinant DNA technology. The boundaries of the domains within the multifunctional synthase are not known. We chose to define the domains so as to include the active site of the enzyme in question plus a minimum of 100 amino acid residues on either side of that site. We used PCR to clone the AT and KS domains from the MAS gene. After verification of the clones by sequencing, these domains were expressed in the pET expression system in *E. coli.* SDS-PAGE of the cell extracts produced after a 4-h induction with IPTG showed that a 46-kDa protein was the dominant one in the extracts of cells expressing the AT domain and that a 42-kDa protein was the dominant one in the cells expressing the KS domain (Fig. 1A).

TABLE 1. Effects of the additions of the AT and KS domains of MAS from *M. bovis* BCG expressed in *E. coli* on the incorporation of methylmalonyl-CoA into fatty acids by FAS from *M. smegmatis*

Additions to reaction mixture[a]	Rate of incorporation of methylmalonyl-CoA (pmol/mg/h)
AT, C_{20}-CoA	54
KS, C_{20}-CoA	74
AT + KS, C_{20}-CoA	25
AT, C_{20}-CoA, ACP	95
KS, C_{20}-CoA, ACP	98
AT + KS, C_{20}-CoA, ACP	269
AT, C_{12}-CoA	289
KS, C_{12}-CoA	286
AT + KS, C_{12}-CoA	300
AT, C_{12}-CoA, ACP	464
KS, C_{12}-CoA, ACP	379
AT + KS, C_{12}-CoA, ACP	596
C_{12}-CoA, ACP	78

[a] Reaction mixtures contained NADH, NADPH, ATP, and labeled methylmalonyl-CoA. Assays were carried out as described in Materials and Methods.

The sizes of these expressed AT and KS domains matched those expected from the open reading frames introduced into the expression vector. Immunoblots showed that the expressed domains cross-reacted with polyclonal anti-MAS antibodies (Fig. 1B). AT and KS partial reactions were then performed under linear conditions with methylmalonyl-CoA and malonyl-CoA to determine the substrate selectivities of the expressed domains. Both the AT and the KS domains demonstrated selectivities for methylmalonyl-CoA over malonyl-CoA. The substrate selectivities of the KS domain were 24.5 and 40.7 pmol/mg for malonyl-CoA and methylmalonyl-CoA, respectively. The substrate selectivities of the AT domain were 5.1 and 18.9 pmol/mg for malonyl-CoA and methylmalonyl-CoA, respectively. The domains appeared to be correctly folded to be catalytically functional, as seen by the results obtained from these partial reaction assays. The primary structures of the domains are obviously sufficient to allow proper folding of these two domains to yield enzymatically active proteins.

To determine whether the expressed domains can confer the ability to incorporate methylmalonyl-CoA into fatty acids to enzymes that normally do not do so, extracts of *E. coli* and *M. smegmatis* were tested. *E. coli* reaction mixtures included the AT or KS expressed protein, both NADH and NADPH, ACP, the acyl-CoA primer, and either labeled methylmalonyl-CoA or malonyl-CoA. ACP was added to the reaction mixtures since ACP is thought to be a rate-limiting component in the synthesis of fatty acids by multicomponent FASs (18). ACP greatly increased the ability of the *E. coli* reaction mixture to incorporate methylmalonyl-CoA into fatty acids with C_{12}-CoA as the primer. The fact that the inability of *E. coli* extract to incorporate methylmalonyl-CoA, in the absence of expressed AT or KS, was not because of its inability to use C_{12} as a primer was shown by the observation that malonyl-CoA was elongated with the C_{12} primer (data not shown). Similar results were seen when C_{12}-CoA or C_{20}-CoA was used as the primer when *M. smegmatis* FAS was added to the reaction mixture (Table 1).

Effects of cofactors. The effects of several cofactors known to have an effect on the incorporation of methylmalonyl-CoA into fatty acids by the native MAS (22) were tested for incorporation of methylmalonyl-CoA into fatty acids with the *E. coli* reaction mixture in the presence of the expressed MAS domains (Table 2). The addition of ATP and Mg^{2+} to the reaction mixture caused a slight stimulation of methylmalonyl-CoA-incorporating activity. This result is similar to the effect of ATP on the activity of the native purified MAS. Bovine serum albumin (BSA) had no effect on methylmalonyl-CoA incorporation. The addition of 0.4 M KCl severely decreased the level of incorporation of methylmalonyl-CoA (Table 2). This result is in contrast to the effect of salt on MAS activity; with MAS, under certain conditions, in the absence of salt there was almost no elongation with methylmalonyl-CoA. The present results might be attributed to the effect of salt on the state of aggregation of *E. coli* type II FAS, which probably supplies the rest of the constituent enzymes for fatty acid synthesis. The addition of 0.4 M KCl severely decreased the level of incorporation of methylmalonyl-CoA into fatty acids in the presence of *M. smegmatis* FAS in the reaction mixture with C_{20}-CoA as the primer. This inhibition may be because of the disruption of the protein-protein interactions that may be crucial for the incorporation of methylmalonyl-CoA into fatty acids by the mixture of proteins.

Effect of exogenous primers. To determine the effect of primer length on the level of incorporation of methylmalonyl-CoA into fatty acids, and to optimize the primer concentration, we studied four different acyl-CoA primers, C_{12}-CoA, C_{16}-CoA, C_{18}-CoA, and C_{20}-CoA. In the absence of the expressed domains, the *E. coli* reaction mixture did not incorporate methylmalonyl-CoA into fatty acids, irrespective of the primer used. The effect of the chain length of the primer on the level of incorporation of methylmalonyl-CoA in the presence and absence of *M. smegmatis* FAS in the *E. coli* reaction mixture is shown in Table 3. The level of incorporation of methylmalonyl-CoA into fatty acids decreased as the primer chain length increased from C_{12}-CoA to C_{18}-CoA in both the presence and the absence of *M. smegmatis* FAS in the *E. coli* reaction mixture. There was insignificant incorporation of methylmalonyl-CoA with C_{20}-CoA primer in the absence of added *M. smegmatis* FAS. With the expressed AT or KS domain in the *E. coli* reaction mixture, the optimal concentration of C_{12}-CoA primer for incorporation of methylmalonyl-CoA was 100 μM. Interestingly, the incorporation of methylmalonyl-CoA was more with C_{20}-CoA as the primer than with C_{18}-CoA as the primer in the presence of *M. smegmatis* FAS. Arachidoyl-CoA was also used more efficiently as a primer than stearoyl-CoA by MAS (22). With C_{20}-CoA as the primer in the presence of *M. smegmatis* FAS in the *E. coli* extract, the optimal concentration of primer was also found to be 100 μM. Previous studies of MAS (22) had also shown similar optimal concentrations of primer.

The ability to incorporate methylmalonyl-CoA into fatty acids by *M. smegmatis* FAS in the presence of the expressed AT

TABLE 2. Effects of various cofactors on methylmalonyl-CoA incorporation by *E. coli* extracts with C_{12}-CoA as the primer

Additions to reaction mixture[a]	Rate of incorporation of methylmalonyl-CoA (pmol/mg/h)
AT, ACP	199
KS, ACP	92
AT, ACP, KCl	24
KS, ACP, KCl	28
AT, ACP, ATP	237
KS, ACP, ATP	186
AT, ACP, ATP, BSA	184
KS, ACP, ATP, BSA	126

[a] Reaction mixtures contained C_{12}-CoA, NADH, NADPH, and labeled methylmalonyl-CoA. Assays were performed as described in Materials and Methods.

TABLE 3. Primer dependence on incorporation of methylmalonyl-CoA into fatty acids by *E. coli* extracts containing the AT or KS domain of MAS, with or without *M. smegmatis* FAS

Addition(s) to reaction mixture[a]	Rate of incorporation of methylmalonyl-CoA (pmol/mg/h)
With *M. smegmatis* FAS	
AT, C_{12}-CoA	289
KS, C_{12}-CoA	286
AT, C_{16}-CoA	150
KS, C_{16}-CoA	150
AT, C_{18}-CoA	117
KS, C_{18}-CoA	94
AT, C_{20}-CoA	199
KS, C_{20}-CoA	178
C_{12}-CoA	24
C_{20}-CoA	13
Without *M. smegmatis* FAS	
AT, C_{12}-CoA	152
KS, C_{12}-CoA	193
AT, C_{16}-CoA	55
KS, C_{16}-CoA	83
AT, C_{18}-CoA	53
KS, C_{18}-CoA	56
AT, C_{20}-CoA	45
KS, C_{20}-CoA	38
C_{12}-CoA	17

[a] Reaction mixtures contained 100 μM acyl-CoA primer, ACP, ATP, NADH, NADPH, and labeled methylmalonyl-CoA. The assay was performed as described in Materials and Methods.

or KS domain with C_{20}-CoA as the primer probably results from the ability of *M. smegmatis* FAS to use the longer primer and the ability of the expressed AT and KS domains to use the branched precursor. Results confirming the role of FAS from *M. smegmatis* in the observed incorporation of methylmalonyl-CoA into fatty acids were obtained when the purified *M. smegmatis* FAS was used in an assay with purified AT or KS protein, NADPH, NADH, ACP, and ATP. Methylmalonyl-CoA was incorporated into fatty acids, and the addition of anti-FAS antibodies to this assay mixture resulted in a dramatic decrease in the level of incorporation of methylmalonyl-CoA into fatty acids (Table 4). When these antibodies were added to the *E. coli* reaction mixture, they showed little effect on the incorporation of methylmalonyl-CoA with C_{12}-CoA as the primer.

The inhibitory effect demonstrates that the ability to use methylmalonyl-CoA with the long-chain C_{20}-CoA primer is dependent on the *M. smegmatis* FAS, which provides the rest of the component enzymes required for fatty acid synthesis.

To identify the nature of the products, purified *M. smegmatis* FAS and the purified AT or KS protein of MAS were incubated with [^{14}C]methylmalonyl-CoA, NADH, NADPH, ACP, and ATP and the products were analyzed. About 10% of the total product formed was protein-bound (presumably ACP-bound) fatty acids, and the remainder was free fatty acids. Radio gas-liquid chromatography of the products derived from *E. coli* extracts containing the expressed AT or KS domain of MAS revealed that C_{12}-CoA primer was elongated mainly by one C_3 unit, yielding a C_{15} product (Fig. 2). Products derived from reaction mixtures containing partially purified *M. smegmatis* FAS in the presence of the AT or KS domain revealed elongation of the C_{20}-CoA primer by one methylmalonyl-CoA unit.

DISCUSSION

Pathogenic mycobacteria produce mycocerosic acids formed by elongation of an *n*-fatty acyl primer with methylmalonyl-CoA as the extender unit. The production of these unusual fatty acids is catalyzed by a unique multifunctional FAS-like enzyme called MAS, which has an atypical specificity for methylmalonyl-CoA (22). It is also known that the biosynthesis of polyketides by PKSs is mechanistically equivalent to the formation of long-chain fatty acids. Malonyl-CoA and methylmalonyl-CoA are two of the building blocks most frequently used by the PKSs. For PKSs, just as for MAS, the specific component enzyme responsible for selectively choosing the branched methylmalonyl-CoA and the molecular basis for this specificity remain unknown. The unique similarity of the AT and KS domains of MAS to the corresponding domains of the PKS suggested that AT or KS might have unique selectivity for methylmalonyl-CoA incorporation. Therefore, we investigated if either one of these domains from MAS could be responsible for conferring methylmalonyl-CoA specificity to MAS. Our results demonstrate that both the AT and KS domains from MAS have preference for methylmalonyl-CoA. We have demonstrated that both of these domains can confer the ability to incorporate methylmalonyl-CoA into fatty acids to *E. coli* and *M. smegmatis* FASs that normally do not use methylmalonyl-CoA as a substrate.

The results with the expressed AT and KS domains of MAS and *M. smegmatis* FAS show that the expressed domains are probably responsible for the ability of the *M. smegmatis* FAS to incorporate methylmalonyl-CoA into fatty acids. We have also demonstrated that when C_{20}-CoA is used as a primer, the incorporation of methylmalonyl-CoA into fatty acids cannot take place unless *M. smegmatis* FAS is present in the reaction mixture. The direct participation of *M. smegmatis* FAS is further demonstrated by the results obtained with antibodies that cross-react with *M. smegmatis* FAS. Such antibodies severely inhibited the incorporation of methylmalonyl-CoA with C_{20}-CoA as the primer. These results when considered together show that while the expressed domain of MAS provides the ability to use methylmalonyl-CoA as an extender unit, the rest of the component activities of fatty acid-synthesizing enzymes are provided by the *M. smegmatis* FAS for the synthesis of methyl-branched fatty acids.

In the multicomponent systems that involve ACP, expressed MAS domains and the *E. coli* or *M. smegmatis* FAS must all function together to synthesize branched fatty acids. We have observed a significant increase in incorporation of methylmalonyl-CoA into fatty acids in the presence of exogenous ACP. In other systems, foreign ACPs have been shown to be func-

TABLE 4. Effects of antibodies raised against *M. bovis* BCG FAS on incorporation of methylmalonyl-CoA into fatty acids by *M. smegmatis* FAS and purified AT or KS protein of MAS

Addition(s) to reaction mixture[a]	Rate of incorporation of methylmalonyl-CoA (pmol/mg/h)
AT, C_{20}-CoA	270
KS, C_{20}-CoA	280
C_{20}-CoA	76
AT, C_{20}-CoA, FAS antibody	70
KS, C_{20}-CoA, FAS antibody	60

[a] The reaction mixture contained purified *M. smegmatis* FAS, NADH, NADPH, ACP, ATP, and labeled methylmalonyl-CoA. Ten microliters of FAS antibody was used. The assay was carried out as described in Materials and Methods.

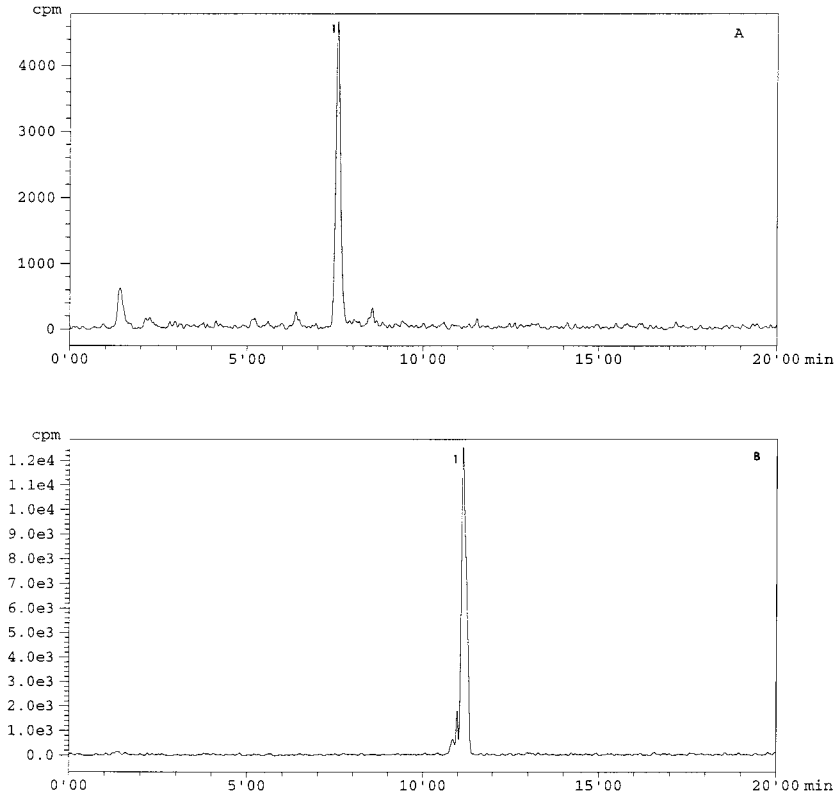

FIG. 2. (A) Radio-gas-liquid chromatograph of methyl esters of fatty acids synthesized from methylmalonyl-CoA by *M. smegmatis* FAS in the presence of the expressed AT domain with C_{12}-CoA as the primer. Peak 1 represents the methyl ester of the C_{15} methyl-branched fatty acid. (B) Radio-gas-liquid chromatograph of methyl esters of fatty acids synthesized from methylmalonyl-CoA by *M. smegmatis* FAS in the presence of the expressed AT domain with C_{20}-CoA as the primer. Peak 1 represents the methyl ester of the C_{23} methyl-branched fatty acid. e3 and e4 indicate 10^3 and 10^4, respectively.

tional and competent to interact with other components of the host PKS (12). In our multicomponent system, the ACP holoprotein, with its pantethenic cofactor, is the probable site at which individual methylmalonate units are thioesterified to be used as nucleophiles in the condensation reaction. The expressed AT domain can catalyze this thioesterification; following this transfer, condensation takes place between the acyl-CoA primer loaded at the active site of the *E. coli* or *M. smegmatis* FAS KS and the methylmalonyl-ACP. Once the expressed AT domain of MAS preferentially transfers the methylmalonyl group to ACP, condensation by the *M. smegmatis* FAS follows. It is unlikely that the malonyl-CoA–ACP transacylase of *E. coli* participated in the observed incorporated of methylmalonyl-CoA into fatty acids, because if the *E. coli* enzyme could catalyze such a transfer, the *E. coli* extract would have incorporated methylmalonyl-CoA into fatty acids without requiring the addition of the expressed AT domain. Thioesterification of the methylmalonyl group to ACP can presumably also be catalyzed by the expressed KS domain of MAS in a manner similar to that seen in the KS III enzymes from *E. coli* and spinach that are known to catalyze both transacylation and condensation (10, 23). Thus, either the expressed AT or KS domain from MAS can bring about methylmalonyl-CoA incorporation. When only the methylmalonyl-CoA-selective AT domain is added, methylmalonyl-CoA incorporation must depend for condensation on the FAS domain, which might be less efficient in using methylmalonyl-CoA. Similarly, when the KS domain alone is used, methylmalonyl-CoA incorporation depends on the methylmalonyl transferase activity of the KS domain, which may not be efficient. The addition of both domains therefore might show a synergistic effect when low concentrations of enzyme are used. In fact, such a synergy was observed; with both the AT and KS domains, methylmalonyl-CoA incorporation was twice that obtained with either domain alone in the *E. coli* extract (data not shown). Once the methyl-branched ketoacyl-ACP intermediate is formed, the ketoreductase, dehydratase, and enoyl reductase domains of either the *M. smegmatis* FAS or the corresponding individual enzymes in *E. coli* can complete the process to generate a fully reduced 2-methyl-branched fatty acid. The ability of the resident enoyl reductase, dehydratase, ketoreductase, and thioesterase component enzymes of *M. smegmatis* and *E. coli* FAS to use branched intermediates of fatty acid synthesis has never been tested, although vertebrate FAS is known to be able to produce methyl-branched fatty acids from methylmalonyl-CoA, albeit at a much lower rate than the rate of synthesis of *n* fatty acids (4, 5). The results obtained from this study show that both the mycobacterial and *E. coli* systems are able to utilize the alternative branched-chain intermediates that are generated when methylmalonyl-CoA is used instead of malonyl-CoA in fatty acid synthesis.

The difference between the *E. coli* synthase and the *M. smegmatis* synthase is in their abilities to use primers. The mycobacterial FAS is known to generate a bimodal distribution of products (13, 25) and is able to use short- and long-chain exogenous primers (13). Thus, *M. smegmatis* FAS could use the C_{20}-CoA primer whereas *E. coli* FAS could use only shorter primers to generate methyl-branched fatty acids. Both the multifunctional FAS (*M. smegmatis*) and the FAS multienzyme complex (*E. coli*) must function with the expressed MAS do-

main(s) and ACP. Physical interaction among these proteins is necessary to catalyze the series of reactions needed to produce the fully reduced methyl-branched acid. The observed inhibition of incorporation of methylmalonyl-CoA by a high concentration of salt might be due to the inhibition of such interactions.

Our results raise new possibilities for polyketide engineering. Novel polyketides can be produced by varying the chain length, the degree of β-keto reduction, and the choice of chain-building extender units. Since we have identified domains with a selectivity for the methylmalonyl-CoA extender unit, such domains can now be introduced at preselected points into any given polyketide gene. Such approaches may allow the introduction of methyl branches at preselected points on the polyketide backbone and thereby greatly increase the potential ranges of novel structures, possibly with unique activities.

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