Conversion of a β-Ketoacyl Synthase to a Malonyl Decarboxylase by Replacement of the Active-Site Cysteine with Glutamine†

Andrzej Witkowski,‡ Anil K. Joshi,‡ Ylva Lindqvist,§ and Stuart Smith*,‡

Children's Hospital Oakland Research Institute, Oakland, California 94609, and Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden

Received April 30, 1999; Revised Manuscript Received July 2, 1999

ABSTRACT: β-Ketoacyl synthases involved in the biosynthesis of fatty acids and polyketides exhibit extensive sequence similarity and share a common reaction mechanism, in which the carbanion participating in the condensation reaction is generated by decarboxylation of a malonyl or methylmalonyl moiety; normally, the decarboxylation step does not take place readily unless an acyl moiety is positioned on the active-site cysteine residue in readiness for the ensuing condensation reaction. Replacement of the cysteine nucleophile (Cys-161) with glutamine, in the β-ketoacyl synthase domain of the multifunctional animal fatty acid synthase, completely inhibits the condensation reaction but increases the uncoupled rate of malonyl decarboxylation by more than 2 orders of magnitude. On the other hand, replacement with Ser, Ala, Asn, Gly, and Thr compromises the condensation reaction without having any marked effect on the decarboxylation reaction. The affinity of the β-ketoacyl synthase for malonyl moieties, in the absence of acetyl moieties, is significantly increased in the Cys161Gln mutant compared to that in the wild type and is similar to that exhibited by the wild-type β-ketoacyl synthase in the presence of an acetyl primer. These results, together with modeling studies of the Cys → Gln mutant from the crystal structure of the *Escherichia coli* β-ketoacyl synthase II enzyme, suggest that the side chain carbonyl group of the Gln-161 can mimic the carbonyl of the acyl moiety in the acyl−enzyme intermediate so that the mutant adopts a conformation analogous to that of the acyl−enzyme intermediate. Catalysis of the decarboxylation of malonyl-CoA requires the dimeric form of the Cys161Gln fatty acid synthase and involves prior transfer of the malonyl moiety from the CoA ester to the acyl carrier protein domain and subsequent release of the acetyl product by transfer back to a CoA acceptor. These results suggest that the role of the Cys → Gln β-ketoacyl synthases found in the loading domains of some modular polyketide synthases likely is to act as malonyl, or methylmalonyl, decarboxylases that provide a source of primer for the chain extension reactions catalyzed by associated modules containing fully competent β-ketoacyl synthases.

β-Ketoacyl synthases play critical roles in the biosynthesis of a variety of natural products, including fatty acids, the polyketide precursors of commercially important pharmacological agents, and the mycolic acid precursors for the cell wall of disease-causing mycobacteria. These enzymes have been identified as targets for the development of new drugs for fighting cancer (*1*−*4*) and tuberculosis (*5*) and for the engineering of transgenic plants that could produce seed oils with unique compositions (*6*). The β-ketoacyl synthases catalyze the formation of new carbon−carbon bonds by condensation of a variety of acyl chain precursors with an elongating carbon source, usually malonyl or methylmalonyl moieties, that is covalently attached via a thioester linkage to an ACP.[1] The β-ketoacyl synthases exist in two different molecular forms. In plants and the majority of prokaryotes, the enzymes consist of approximately 400 residues and typically form homodimers and are called monofunctional polypeptide, or type II, systems (*7*, *8*). On the other hand, in the fatty acid-synthesizing systems of animals and the structurally related modular polyketide systems of prokaryotes, the β-ketoacyl synthases constitute one of the catalytic domains of large molecular complexes, called multifunctional polypeptide, or type I, systems (*9*−*11*). In the type II FAS systems of plants and microorganisms, multiple forms of β-ketoacyl synthases have been described that have different substrate specificities. Thus, β-ketoacyl synthases I and II can effectively elongate short-chain precursors to the 14-carbon stage, but further elongation, in particular the elongation of C16:1-ACP to C18:1-ACP, is more efficiently catalyzed by β-ketoacyl synthases II (*8*, *12*−*14*). Type II β-ketoacyl synthases recently identified in *Mycobacterium tuberculosis*, named β-ketoacyl synthases A and B, apparently specialize in elongating acyl-ACPs containing more than 26 carbon atoms and play important roles in the production of mycolic acids by this organism (*5*). In the

† This work was supported by Grant DK 16073 from the National Institutes of Health (to S.S.) and by grants from the Swedish Natural Science Research Council and the Swedish Foundation for Strategic Research (to Y.L.).

* To whom correspondence should be addressed: Children's Hospital Oakland Research Institute, 747 52nd St., Oakland, CA 94609. Telephone: (510)428-3523. Fax: (510)428-3608. E-mail: ssmith@mail.cho.org.

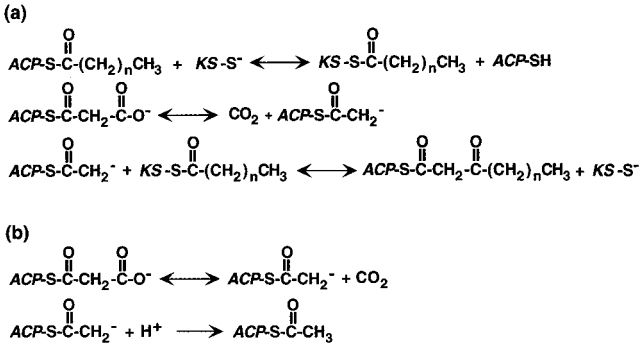
‡ Children's Hospital Oakland Research Institute.

§ Karolinska Institutet.

[1] Abbreviations: ACP, acyl carrier protein; FAS, fatty acid synthase; KS^Q, β-ketoacyl synthase domain in which the usual active-site cysteine residue is replaced with glutamine.

10.1021/bi990993h CCC: $18.00
Published on Web 08/18/1999

Scheme 1: Reactions Catalyzed by the β-Ketoacyl Synthase Domain of (a) Wild-Type FAS and (b) the C161Q FAS Mutant



animal type I FAS, a single β-ketoacyl synthase catalyzes all of the condensation reactions that are necessary for elongation of a two-carbon precursor to palmitic acid (*15*−*17*). The type I modular polyketide synthases utilize a different β-ketoacyl synthase for each of the elongation steps, leading to the production of complex polyketides, whereas their type II counterparts utilize the same β-ketoacyl synthases for each condensation reaction that is required for the production of aromatic polyketides.

All of the various members of the β-ketoacyl synthase family of enzymes likely utilize the same basic reaction mechanism involving, successively, transfer of the first substrate from the ACP phosphopantetheine to a cysteine nucleophile, decarboxylation of the second substrate, either malonyl- or methylmalonyl-ACP, to yield the carbanion, and finally a nucleophilic attack of the carbanion on the carbonyl of the cysteine-bound acyl substrate, resulting in the formation of a new carbon−carbon bond (Scheme 1). Although the identity of the cysteine nucleophile is well-established, other residues that have been implicated in the reaction mechanism have not been unambiguously identified, this despite the recent determination of the crystal structure of one of the *Escherichia coli* β-ketoacyl synthases II (*18*).

The residues promoting decarboxylation of malonyl (or methylmalonyl)-ACP are particularly difficult to pinpoint since, in the absence of the first substrate, decarboxylation of malonyl moieties takes place at a rate that is far slower than when accompanied by a condensation reaction. Presumably, this phenomenon reflects the importance to the enzyme of generating the reactive carbanion species only when a recipient acyl chain is positioned on the active-site cysteine, thus avoiding the loss of malonyl moieties and establishing a futile cycle. In seeking a means of uncoupling the decarboxylation and condensation reactions, while maintaining the full capacity of the former, we were intrigued by the discovery that several modular polyketide synthases possess a β-ketoacyl synthase domain in which the active-site cysteine residue is replaced with glutamine. These unusual β-ketoacyl synthases, termed KS^Q domains, are found at the N-terminus of a loading module, the role of which is to provide the starter substrate for the first condensation reaction catalyzed by the adjacent fully competent module (*19*−*21*). In other modular polyketide synthases, loading modules are specialized either to facilitate the transfer of unusual primers, for example, a dihydrocyclohexylcarbonyl moiety in the FK506 (*22*) and rapamycin (*23*) polyketide synthases, or to contain only an acyltransferase and ACP domain, for example, in the 6-deoxyerythronolide B polyketide synthase (*11*), or are lacking entirely, as in the pyoluteorin polyketide synthase (*24*).

A role for the KS^Q enzymes has yet to be proposed. Since replacement of the cysteine nucleophile with glutamine would preclude catalysis of the condensation reaction, we reasoned that the role of a KS^Q domain might be to increase the efficiency of primer delivery by the loading module. Modification of the active-site cysteine residue of β-ketoacyl synthases by iodoacetamide is known to increase the rate of uncoupled malonyl decarboxylase activity in yeast and fungal type I FASs (*25*, *26*). Therefore, on the basis of the structural similarity between the Gln side chain ($CH_2CH_2CONH_2$) and that of the iodoacetamide-modified cysteine side chain (CH_2-SCH_2CONH_2), we hypothesized that KS^Q domains might provide the primer substrate for polyketide synthesis by promoting decarboxylation of malonyl, or methylmalonyl, moieties. The objective of this study, then, was to test the hypothesis that replacement of the cysteine nucleophile in a β-ketoacyl synthase would convert the condensing enzyme into a potent malonyl decarboxylating enzyme.

EXPERIMENTAL PROCEDURES

Construction of cDNAs Encoding His_6- and FLAG-Tagged FASs and Expression of the Proteins in Sf9 Cells. The strategies for construction of cDNAs encoding the wild-type FAS, single domain-specific mutants and for introduction of His_6 or FLAG tags have been described in detail previously (*27*−*30*). The Cys161 mutant FASs were constructed by first generating mutated partial cDNA fragments by polymerase chain reaction amplification, using pFAS 74.20 (partial FAS cDNA in pUCBM20) as the template together with the appropriate primers (*27*). Oligonucleotide FAS1152B (5′-cac tag aat tcT TCA GGG TTG GGG TTG TGG AAA TGC; uppercase letters correspond to bp 1152−1176 of the rat FAS cDNA, and lowercase letters correspond to nucleotides added for the introduction of restriction sites) was used as the antisense primer. The sequence of the sense primers was 5′-TCA AAG GAC CCA GCA TTG CCC TGG ACA CAG CC**N NN**T CCT CTA GCC T (bp 530−575 of rat FAS cDNA), **NNN** being GBA for Cys161Ala/Gly (B denotes C, G, or T), ASC for Cys161Ser/Thr (S denotes C or G), AAT for Cys161Asn, and CAA for the Cys161Gln mutant.

The authenticity of the amplification products was confirmed by DNA sequencing, and the appropriate fragments were moved stepwise into the full-length, wild-type construct (*27*, *32*). The final constructs for Cys161Ser/Gln/Asn mutants also encoded a C-terminal His_6 affinity tag, and those for the Cys161Ala/Gly mutants encoded a C-terminal FLAG tag, although the presence of the tags was not exploited in these studies. These FAS cDNA constructs, in the context of the pFASTBAC 1 vector, were used to generate recombinant baculovirus stocks by the transposition method employing the BAC-to-BAC baculovirus expression system according to the manufacturer's instructions. *Sf*9 cells were then infected with the purified recombinant viruses and cultured for 48 h at 27 °C. The tagged FAS proteins were partially purified from the cytosols as described previously (*27*) and then subjected to final purification by affinity chromatography (*30*); glycerol (10%, v/v) was included in all the buffers that were used for chromatography.

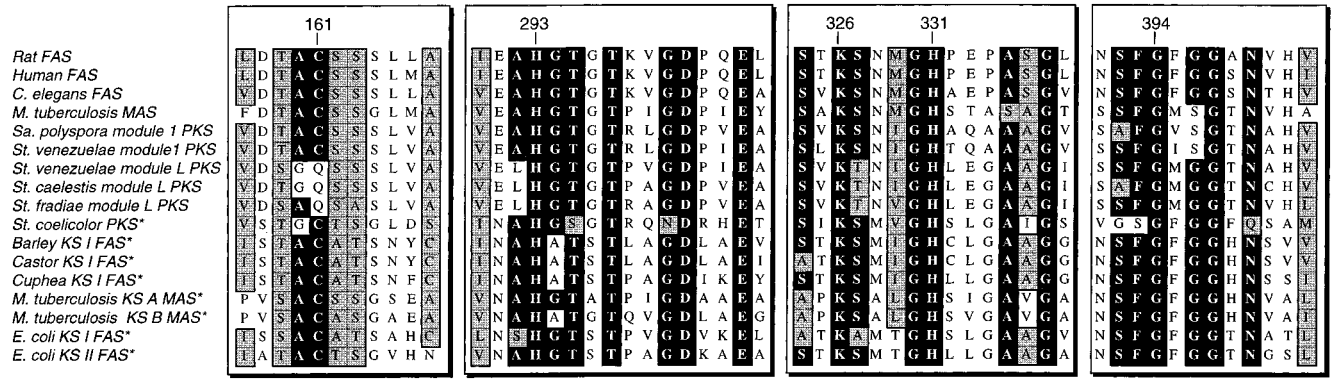


FIGURE 1: Alignment of the amino acid sequences from four regions of β-ketoacyl synthases. Those β-ketoacyl synthases associated with multifunctional (type I) and monofunctional (type II) polypeptides are distinguished by asterisks. MAS, mycolic acid synthesis; PKS, polyketide synthesis; *C., Caenorhabditis*; *M., Mycobacterium*; *Sa., Saccharopolyspora*; *St., Streptomyces*; *E., Escherichia.* The numbering system is that for the rat FAS. Consensus residues, defined by 75% compliance, are denoted by black shading and conservative replacements by gray shading. The GenBank protein ID numbers for the sequences, in the order in which they are listed, are (1) 66561, (2) 2117715, (3) 3876624, (4) 547900, (5) 416965, (6 and 7) 3800834, (8) 2558838, (9) 2317860, (10) 729876, (11) 119784, (12) 294666, (13) 3800749, (14) 1261947, (15) 1261948, (16) 119783, and (17) 729460.

Estimation of the Proportion of Monomers and Dimers in FAS Preparations. Proteins were chromatographed on a SigmaChrom GFC-1300 column (0.75 cm × 30 cm) in 0.25 M potassium phosphate (pH 7) and 1 mM EDTA, employing a flow rate of 0.5 mL/min at 20 °C, and were detected by monitoring the absorbance of the effluent at 280 nm. Blue dextran, FAS dimers, and FAS monomers were eluted with retention times of 9.0, 10.5, and 11.5 min, respectively.

Dissociation and Reassociation of FAS Subunits. FASs [in 50 mM potassium phosphate buffer (pH 7.0), 1 mM DTT, 1 mM EDTA, and ~1% glycerol] were aged at 4 °C for 7−9 days to promote dissociation into their component subunits (*31*). Spontaneous reassociation of the subunits was induced by adjustment of the solvent to approximately 0.2 M potassium phosphate (pH 7.0) and 10% glycerol and incubation at 30 °C for 75 min.

Assay for Decarboxylation of Malonyl Moieties. Decarboxylase activity was assayed by quantification of acetyl-CoA, β-ketobutyryl-CoA, and triacetic acid lactone formed from malonyl-CoA. Enzymes were incubated at 37 or 10 °C for 1−2 min with 110 μM [2-^{14}C]malonyl-CoA and 50 μM CoASH in 0.2 M potassium phosphate buffer (pH 6.6); CoASH was omitted from the reaction mixture when the activity of the wild-type FAS was assayed. Reactions were quenched with perchloric acid (*28*), and the products were identified by reversed-phase HPLC (*32*). Triacetic acid lactone (retention time of 9.3 min) was identified in the eluate by comparison with the elution position of a chemically synthesized standard. The overall decarboxylase activity was calculated on the basis of the total amount of acetyl units formed from malonyl-CoA, and includes those released from the enzyme by transfer to CoASH as well as those utilized as primers for condensation and subsequently released as either β-ketobutyryl-CoA or triacetic acid lactone. A unit of activity is equivalent to 1 μmol of malonyl moieties decarboxylated per minute at the specified temperature.

Overall Fatty Acid Synthesizing Activity. Activity was measured spectrophotometrically at 37 or 10 °C (*33*). A unit of activity is equivalent to 1 μmol of NADPH oxidized per minute at the specified temperature.

Modification of FAS by Iodoacetamide. The FAS storage buffer was replaced with 0.25 M potassium phosphate (pH 5.8) containing 1 mM EDTA and 0.5 mM tris(2-carboxyethyl)phosphine (Calbiochem-Novabiochem Corp., San Diego, CA) by centrifugation through a BioGel P-30 (Bio-Rad, Hercules, CA) gel filtration column (*34*). The modification reaction was carried out at 20 °C using 0.73 mM iodoacetamide; to protect the phosphopantetheine thiol from modification, 0.96 mM malonyl-CoA was included in the reaction mixture. The reaction was quenched by addition of mercaptoethanol to a final concentration of 10 mM, and the reaction buffer was replaced with storage buffer by repeated dilution and concentration in a Centricon-100 device (Amicon, Inc., Beverly, MA).

Modeling of Mutant Structures. Crude models of the various β-ketoacyl synthase mutants were generated from the crystal structure of β-ketoacyl synthase II from *E. coli* (*18*, *35*), using the mutate option in the graphics program O (*36*) and manual optimization using the torsion command.

RESULTS AND DISCUSSION

Effect of Replacement of the β-Ketoacyl Synthase Active-Site Cysteine Residue upon Catalysis of the Malonyl Decarboxylation Reaction. Recently, it has become apparent that, with the possible exception of the β-ketoacyl synthase type III enzymes, all of the β-ketoacyl synthases involved in the biosynthesis of fatty acids, polyketides, and mycolic acid precursors share appreciable sequence similarity and are clearly related both structurally and evolutionarily (*37*). Thus, multiple sequence alignments provided the first clues about the possible identities of residues that play critical roles in catalysis (*37*). In addition to the cysteine nucleophile (Cys-161 in the rat FAS), three basic residues are universally conserved (corresponding to His-293, Lys-326, and His-331 in the rat FAS) that likely play important roles either catalytically or structurally (Figure 1). A conserved glycine-rich region near the C-terminus has also been implicated as facilitating the entry of substrates into the active-site pocket (*18*). With the exception of the cysteine nucleophile, all of these regions are also well-conserved in the KS^{Q} domains that are associated with the loading modules of the modular polyketide synthases responsible for the synthesis of the macrolides 10-deoxymethynolide and narbonolide (*Streptomyces venezuelae*), tylactone (*Streptomyces fradiae*), and the macrolide

portion of niddamycin (*Streptomyces caelestis*). If the role of the KS^Q domains were to increase the efficiency of primer delivery, we surmised that replacement of the cysteine nucleophile alone might be sufficient to induce malonyl decarboxylase activity in the β-ketoacyl synthases. Several different amino acid replacements, including glutamine, were engineered into position 161 of the rat FAS and the recombinant proteins purified.

As anticipated, the Cys161Gln mutant is inactive in the spectrophotometric assay for fatty acid synthesis (Figure 2a). In this assay, the activity of the wild-type FAS is dependent on the presence of the cosubstrate, acetyl-CoA, as well as malonyl-CoA and NADPH (Figure 2, compare traces b and f). However, when the Cys161Gln mutant FAS is included in the same assay together with the wild-type FAS, the dependency on added acetyl-CoA is eliminated (Figure 2, compare trace b with traces c−e). In reaction mixtures containing the highest levels of the Cys161Gln mutant, the initial rate of reaction is similar to that observed in the presence of added acetyl-CoA but drops to zero after about 8 min (Figure 2e); the addition of more malonyl-CoA at this time sparks the reaction back to the original rate (denoted by arrow). These results are consistent with the Cys161Gln mutant having catalyzed the decarboxylation of malonyl-CoA, thus providing a supply of acetyl moieties required by the wild-type FAS. In the presence of high levels of the mutant, the availability of malonyl-CoA eventually becomes rate-limiting for the wild-type FAS (Figure 2e). These conclusions were confirmed by subsequent characterization of the Cys161Gln mutant and comparison of its properties with those of the other Cys-161 mutants.

Replacement of the cysteine nucleophile with Gln, Ser, Ala, Asn, Gly, or Thr completely eliminates the ability to catalyze the condensation reaction and consequently eliminates overall FAS activity (Table 1). Only replacement of Cys-161 with Ser produces a β-ketoacyl synthase with residual catalytic activity, as described previously (*38*). Introduction of the Cys161Gln mutation increases malonyl decarboxylase activity 150-fold compared to that of the wild-type FAS, whereas replacement of Cys-161 with other residues either reduces, or only slightly increases, malonyl decarboxylase activity. In the case of the wild-type FAS, most of the acetyl moieties produced by decarboxylation of malonyl moieties are condensed with either one or two malonyl moieties, resulting ultimately in the release of either β-ketobutyryl-CoA or triacetic acid lactone from the enzyme. However, in the case of the Cys161Gln mutant, since no condensation can take place, all of the acetyl moieties produced are released as acetyl-CoA (Table 1).

Decarboxylation of Malonyl Moieties Requires Prior Translocation from CoA to ACP Thioester. The malonyl decarboxylase activity associated with the Cys161Gln mutant is stimulated approximately 2-fold by the addition of 20−50 μM CoASH (data not shown), indicating that the malonyl moieties likely are decarboxylated following transfer to the FAS, so that release of the acetyl product requires the addition of CoASH as an acceptor. In the case of the wild-type FAS, where the rate of decarboxylation is relatively slow, most of the product is released as triacetic acid lactone and β-ketobutyryl-CoA. For each equivalent of triacetic acid lactone that is formed, 3 equiv of CoASH is released, and for each equivalent of β-ketobutyryl-CoA that is formed, 2 equiv of CoASH is released and only 1 equiv is utilized. Presumably, this net overproduction of CoASH, together with the slow rate of decarboxylation, ensures that CoA availability does not become rate-limiting for product release by the wild-type FAS. The fact that the decarboxylation of malonyl moieties requires the initial loading of malonyl moieties onto the phosphopantetheine thiol attached to Ser-2151, via the active-site serine residue of the malonyl/acetyltransferase, Ser-581, is also supported by the observation that mutation of either of these serine residues to alanine eliminates decarboxylase activity (Table 1).

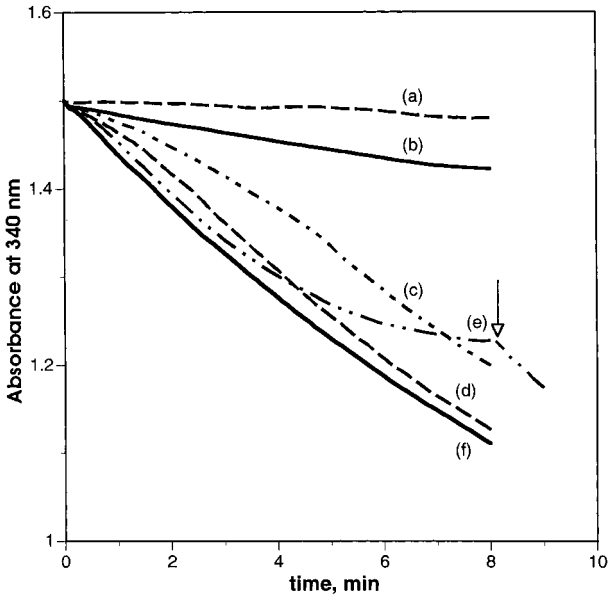


FIGURE 2: Elimination of the requirement for acetyl-CoA by the wild-type FAS in the presence of the Cys161Gln FAS mutant. Spectrophotometric assays were performed at 37 °C. All reaction mixtures contained, in a volume of 0.2 mL, 0.1 M potassium phosphate (pH 6.6), 0.25 mM NADPH, 135 μM malonyl-CoA, and 50 μM CoASH. In addition, individual assays contained (a) 10 μg of Cys161Gln FAS and 65 μM acetyl CoA, (b) 1.5 μg of wild-type FAS (no acetyl-CoA added), (c) wild-type and Cys161Gln FASs, 1.5 μg each (no acetyl-CoA added), (d) 1.5 μg of wild-type and 4.5 μg of Cys161Gln FASs (no acetyl-CoA added), (e) 1.5 μg of wild-type and 10 μg of Cys161Gln FASs (no acetyl-CoA added), with additional malonyl-CoA (200 μM) added at the time marked by the arrow, and (f) 1.5 μg of wild-type FAS and 65 μM acetyl-CoA.

Since no condensation products are produced by the Cys161Ala, Cys161Asn, and Cys161Gly mutants, which lack an appropriate nucleophile at position 161, only acetyl-CoA is recovered as a product, although in these cases the rate of formation of acetyl-CoA is much slower than for the Cys161Gln mutant (Table 1). For comparison, the properties of the iodoacetamide-treated FAS were also studied. As has been reported for the yeast FAS (*25*), both the β-ketoacyl synthase and overall fatty acid synthesizing activity of the rat FAS are markedly lowered by the modification, whereas the malonyl decarboxylase activity is increased; most of the acetyl moieties that are produced are released by transfer to a CoA acceptor. However, the increment in decarboxylase activity is modest when compared to that produced by the Cys161Gln mutation.

To determine whether the dimeric form of the Cys161Gln FAS is required for catalysis of the decarboxylation reaction, we again utilized the spectrophotometric assay, in which the presence of an active malonyl decarboxylase activity obviates

Table 1: Characterization of FASs Mutated or Chemically Modified at Cys-161[a]

FAS	FAS synthesis (milliunits/mg)	β-ketobutyryl-CoA synthesis (milliunits/mg)	malonyl decarboxylation			
			activity (milliunits/mg)	products		
				TAL[b] (%)	β-ketobutyryl-CoA (%)	acetyl-CoA (%)
wild-type	2029 ± 43	130 ± 10	3.3 ± 0.2	49	49	2
Cys161Gln	0	0	495 ± 16	0	0	100
iodoacetamide-treated wild-type	53 ± 10	2.8 ± 0.1	6.4 ± 0.3	0	15	85
Cys161Ser	13 ± 1	5.8 ± 0.7	1.3 ± 0.2	7	42	51
Cys161Ala	0	0	7.0 ± 0.1	0	0	100
Cys161Asn	0	0	1.5 ± 0.1	0	0	100
Cys161Gly	0	0	0.2 ± 0.0	0	0	100
Cys161Thr	0	0	0			
Ser2151Ala (ACP-)	0	0	0.0 ± 0.0			
Ser581Ala (MAT-)	0	0	0.0 ± 0.1			

[a] All assays were performed at 37 °C. [b] TAL is triacetic acid lactone.

the need for added acetyl-CoA in the synthesis of fatty acids by the wild-type FAS. In these experiments, the spectrophotometric assays were performed at 10 °C so that monomers added to the incubation system would not undergo reassociation during the course of the assay (*31*). Thus, in the absence of added acetyl-CoA, Cys161Gln FAS that had been dissociated into monomers, by aging in the cold, did not support fatty acid synthesis by the wild-type FAS (details not shown). Only when the subunits were reassociated prior to the assay did the Cys161Gln FAS support fatty acid synthesis by the wild-type FAS in the absence of added acetyl-CoA. The fact that the decarboxylation reaction is catalyzed only by the dimeric form of FAS was confirmed by direct assay. Thus, malonyl decarboxylase activities of Cys161Gln monomers and dimers, assayed directly at 10 °C, were 0.8 ± 0.1 and 64.5 ± 0.7 milliunits/mg, respectively, whereas activities of wild-type monomers and dimers were 0.03 ± 0.02 and 0.65 ± 0.01 milliunits/mg, respectively. These results are consistent with a mechanism that requires the proper juxtaposition of the malonyl/acetyltransferase, ACP, and β-ketoacyl synthase domains to allow transfer of the malonyl moiety to the ACP domain, interaction of the malonyl-S-ACP with the KS^Q domain, and release of the acetyl moiety by transfer back to a CoA acceptor. This critical juxtaposition of domains, existing only in the dimeric form of the enzyme, is also essential for catalysis of the condensation of acetyl and malonyl moieties by the wild-type FAS (*39*).

Mechanism of Induction of Malonyl Decarboxylase in the Cys161Gln Mutant. In the wild-type FAS, decarboxylation of an ACP-bound malonyl moiety normally takes place at an appreciable rate only when the cysteine nucleophile of the β-ketoacyl synthase domain, Cys-161, is occupied by an acyl moiety so that formation of the carbanion is tightly coupled to its reaction with the carbonyl group of the acyl moiety and little or no opportunity arises for reaction of the carbanion with a proton. It has not been demonstrated directly whether the decarboxylation reaction and addition of the carbanion to the carbonyl follow a concerted or stepwise pathway, although on the basis of theoretical considerations (*40*) and mechanistic similarities with other Claisen-type condensations (*41*), it has been inferred that the β-ketoacyl synthases also employ a stepwise path. In the Cys161Gln mutant, where acylation of residue 161 cannot take place, the only option for the carbanion is to react with a proton (Scheme 1). Since, in the Cys161Gln mutant, decarboxylation of malonyl moieties can take place at a rate that is at least as great as that associated with the condensation reaction catalyzed by the wild-type FAS, it would appear that a proton is readily accessible.

We surmised that the elevated malonyl decarboxylase activity associated with the Cys161Gln mutant might result from an increase in the affinity of the β-ketoacyl synthase for malonyl moieties and devised an experimental system for examining the effect of the malonyl-S-ACP concentration on the rate of decarboxylation catalyzed by the wild type and the Cys161Gln mutant. Because the concentration of malonyl-S-ACP cannot be altered directly in the multifunctional form of FAS, we altered the level of saturation of the ACP with malonyl moieties by varying the concentration of malonyl-CoA in the presence of free CoASH; the latter compound influences the equilibrium between the enzyme-bound and CoA-bound forms of the substrate (*42*). The wild-type FAS and Cys161Gln mutant exhibited markedly different kinetics with respect to malonyl-CoA concentration. Whereas the rate of decarboxylation catalyzed by the Cys161Gln mutant almost reached saturation at 40 μM, the rate of decarboxylation catalyzed by the wild-type FAS was essentially a linear function of malonyl-CoA concentration over the range of 0−40 μM (Figure 3). Acetyl-CoA was the sole product formed by the Cys161Gln mutant, whereas the wild-type FAS produced a mixture of β-ketobutyryl-CoA (83%) and triacetic acid lactone (17%) under these conditions. Apparently, in the case of the wild-type FAS, acetyl moieties formed on the phosphopantetheine by decarboxylation of malonyl moieties are preferentially translocated to the Cys-161 thiol and utilized in the condensation reaction rather than being translocated back to the CoA acceptor, despite the high concentration of the latter used in these assays. At 40 μM malonyl-CoA, the rate of decarboxylation of malonyl moieties by the Cys161Gln mutant was 2 orders of magnitude higher than that catalyzed by the wild-type FAS. For comparison, we also examined the effect of malonyl-CoA concentration on the rate of β-ketobutyryl-CoA formation by the wild-type FAS in the presence of non-rate-limiting concentrations of acetyl-CoA (Figure 3). In contrast to the results obtained in the absence of added acetyl-CoA, 40 μM malonyl-CoA was sufficient to nearly saturate the system. The product, mainly β-ketobutyryl-CoA (triacetic acid lactone accounted for less than 3% of the products), was formed at a rate approaching that observed for the decarboxylation of malonyl moieties catalyzed by the

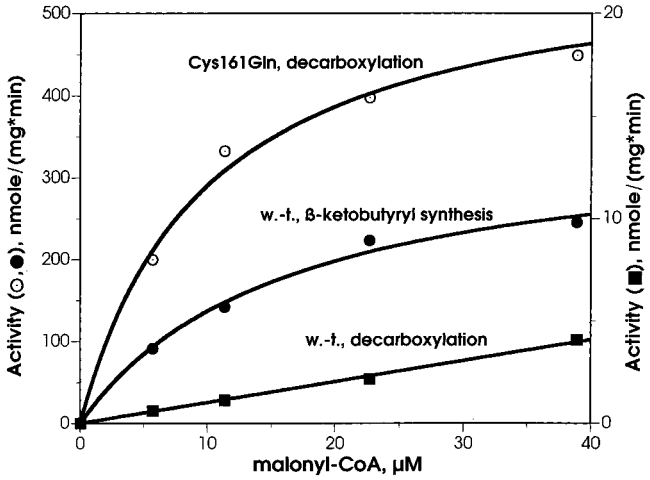


FIGURE 3: Kinetics of decarboxylation of malonyl-CoA by wild-type and Cys161Gln FAS. To assay decarboxylase activity, Cys161Gln (6.2 nM, ○) and wild-type FASs (0.47 μM, ■) were incubated at 37 °C with radioactive malonyl-CoA in the presence of 50 μM CoASH for 1 min (see Experimental Procedures for more details). The β-ketobutyryl-CoA synthesizing activity of wild-type FAS (at 39 nM, ●) was assayed in the presence of 70 μM acetyl-CoA and 50 μM CoASH at 37 °C for 1 min. The reactions were stopped with perchloric acid and analyzed as described in Experimental Procedures. Cys161Gln and wild-type FASs were diluted with the storage buffer containing 0.75 mg/mL BSA, and as a consequence, these assays contained 37.5 μg/mL BSA. BSA by itself had no activity in either assay (data not shown). The V_{max} values calculated from the corresponding double-reciplocal plots were 360 (wild-type, β-ketobutyryl synthesis) and 580 nmol min^{-1} mg^{-1} (Cys161Gln, decarboxylation).

Cys161Gln mutant FAS. The malonyl-CoA concentration required to achieve a rate that equals half of the V_{max} value was similar for both reactions, indicating that the condensing wild-type FAS and the Cys161Gln FAS mutant interact similarly with the malonyl substrate. These results appear to be consistent with the hypothesis that the presence of a glutamine side chain at position 161 mimics the effect produced by the formation of an acyl-S-Cys-161 by the wild-type FAS. It may be significant that, of all the amino acid substitutions introduced at residue 161, only the glutamine side chain ($CH_2CH_2CONH_2$) introduces a carbonyl moiety at a position similar to that formed by the acyl-S-Cys intermediate (CH_2-S-COR).

More than 20 years ago, Lynen and co-workers (*25*) speculated that the β-ketoacyl synthase domain would exist in at least two distinct conformations, one favoring the acylation of the active-site cysteine and the other favoring the formation of the carbanion from malonyl-ACP; switching between conformations would be determined by the presence or absence of an acyl moiety on the cysteine nucleophile. Our results are consistent with this hypothesis since, in the absence of added acetyl-CoA, the β-ketoacyl synthase exhibits only weak affinity for malonyl moieties and decarboxylation occurs inefficiently. In contrast, in the presence of added acetyl-CoA, a relatively strong malonyl-binding site is created so that decarboxylation, coupled with condensation, takes place efficiently.

Structural Implications of the Cys → Gln Replacement. To probe possible structural effects of replacements of the active-site cysteine residue, the various mutants were modeled in the context of the structure of the β-ketoacyl synthase II of *E. coli* (*18*, *35*). All mutations could easily be accommodated in the substrate pocket with the exception of the Gln replacement (residue 163 in the *E. coli* enzyme, Figure 4). This substitution gives rise to close contacts with the side chain of a conserved phenylalanine (Phe-400 in the *E. coli* enzyme). However, it has been observed that this side chain changes conformation upon covalent attachment of cerulenin to the cysteine nucleophile, resulting in an opening of the substrate pocket both toward the surface and toward the dimer interface (*35*). This structural change might well influence the decarboxylase activity by facilitating access to the active-site pocket. The most striking feature of the Cys → Gln replacement, however, is that it is very homologous to an acyl intermediate in that the carbonyl groups occupy the same spatial position. When this intermediate is formed, it has to go through a tetrahedral transition state with a negative charge on the oxygen that has to be stabilized through protein interactions. From the crystal structure, one could surmise that this stabilization may be obtained through hydrogen bonds to the peptide NH group of the phenylalanine discussed above and perhaps by charge interactions with the second conserved histidine in the active site (*18*). Thus, this "oxy-anion binding pocket" would be open in the free enzyme, while in the acyl intermediate the acyl carbonyl oxygen would occupy this pocket. In the Cys → Gln mutant, the side chain could occupy this pocket in a similar way (Figure 4). It is very likely that in the case of the native free enzyme the negatively charged carboxyl group of the malonyl moiety would bind in this oxy-anion binding pocket in a fashion which is nonproductive for decarboxylation, while for the acyl intermediate or the Cys → Gln enzyme, the pocket is blocked and the malonyl moiety instead is bound in a productive way. Clearly, on the basis of our results to date, we cannot rule out unequivocally the possibility that decarboxylation of malonyl moieties by the Cys161Gln mutant occurs via a mechanism uniquely different from that which is operative in the wild-type enzyme when the reaction is coupled directly with condensation. Nevertheless, the predictions based on modeling of the Cys → Gln mutant in the context of the *E. coli* β-ketoacyl synthase II are entirely consistent with our observation that introduction of the glutamine side chain at position 161 creates a high-affinity binding site for malonyl moieties (Figure 3). Thus, identification of the Cys → Gln replacement as being particularly effective in uncoupling the decarboxylation and condensation steps of the β-ketoacyl synthase reaction may provide a unique model system that can be exploited to identify residues that are required for promotion of the decarboxylation step in the FAS reaction sequence.

The multifunctional FAS has proven to be a valuable paradigm for elucidation of the functional organization and programming rules that enable the modular polyketide synthases to synthesize a wide variety of macrolide products (*11*, *45*). An immediate and important consequence of this study with a multifunctional FAS is the identification of a likely role for the KS^Q domains that are found characteristically at the N-termini of the loading modules of some multifunctional polyketide synthases. On the basis of the extensive sequence similarity of the β-ketoacyl synthases associated with polyketide and fatty acid synthesizing systems (Figure 1), it is reasonable to expect that these KS^Q domains also possess potent malonyl decarboxylase activity. Their location in the specialized loading modules represents

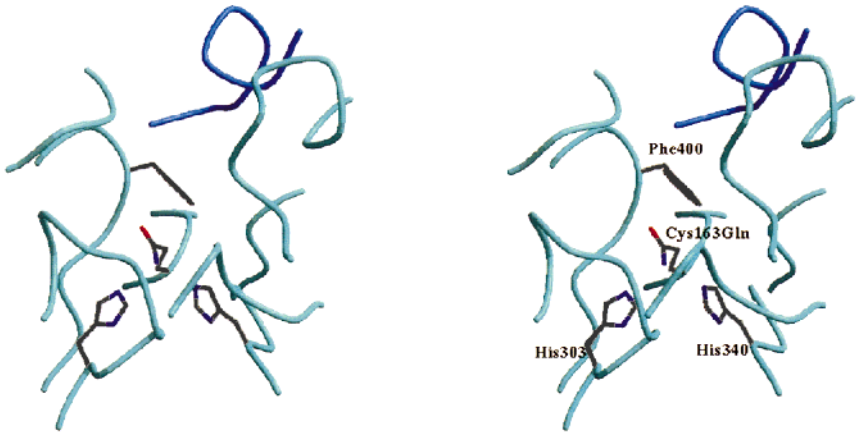


FIGURE 4: Stereoview of the active-site pocket in a model of the *E. coli* β-ketoacyl synthase II Cys163Gln mutant. Residues that are discussed are shown as ball-and-stick models and labeled according to the sequence numbering of the *E. coli* enzyme. The polypeptide chain of the surroundings is cyan, and part of the second subunit in the dimer is blue. This figure was prepared using Molscript (*43*) and Raster3d (*44*).

Proposed consensus for malonyl	**ETGYA**	**QxAxFGLL**	**GHSxG**
Niddamycin	$^{-33}$**RTEYT**	$^{-21}$**QTALYRTL**	**GHSVG**
Proposed consensus for methylmalonyl	**RVDVV**	**MxSxAAxW**	**GHSQG**
Tylactone	$^{-33}$**RVDVV**	$^{-21}$**MVSLARYW**	**GHSQG**
10-Deoxymethynolide/narbonolide	$^{-33}$**RVDVV**	$^{-21}$**MVSLARVW**	**GHSQG**

FIGURE 5: Comparison of conserved motifs within acyltransferase domains adjacent to KS^Q domains in the loading modules of several modular polyketide synthases. Consensus sequences for methylmalonyl- and malonyl-specific acyltransferases were derived by sequence analysis of a large number of polyketide synthase domains (not including any KS^Q domains) that catalyze the transacylation of either methylmalonyl-CoA or malonyl-CoA onto ACP. Regions were identified in which the acyltransferase sequences diverged according to whether they were specific for malonyl-CoA or methylmalonyl-CoA (*46*). The locations of the conserved motifs are identified relative to the active-site serine within the GHSxG motif, which is designated position 1.

compelling circumstantial evidence indicating that this unique ability of the KS^Q domain to catalyze decarboxylation of the substrate normally used for chain extension has been exploited to optimize primer availability. Thus, the acetyl, or propionyl, moieties formed on the ACP domain by decarboxylation of malonyl, or methylmalonyl, moieties could be transferred directly to the ketoacyl synthase associated with the adjacent module responsible for performing the first elongation step. Further support for this hypothesis can be derived from an analysis of the amino acid sequences of the acyltransferase domains associated with loading modules that contain KS^Q domains (Figure 5). An earlier sequence analysis of acyltransferase domains associated with modular polyketide synthases revealed distinct differences in the sequences of those enzymes that exhibit specificity for malonyl and methylmalonyl moieties (*46*). Those acyltransferase domains that lie adjacent to the KS^Q domains of the modular polyketide synthases responsible for the synthesis of tylactone, in *S. fradiae*, and 10-deoxymethynolide and narbonolide, in *S. venezuelae*, contain the conserved sequence elements typical of the methylmalonyl-specific acyltransferases, whereas that associated with the niddamycin polyketide synthase, in *S. caelestis*, contains the sequence elements characteristic of the malonyl-specific class of acyltransferases. These specificities are exactly as anticipated if these acyltransferases were to supply the substrate for decarboxylation by the adjacent KS^Q domain. Thus, on the basis of their chemical structures, it can be deduced that tylactone (*19*) and 10-deoxymethynolide and narbonolide (*21*) must be synthesized from a propionyl primer whereas niddamycin (*20*) is synthesized from an acetyl primer.

Conceivably, in the microenvironment surrounding the modular polyketide synthases, the substrate required for the multiple chain elongation steps may be present in excess over that required for the single priming step. The ability of the KS^Q domain to generate the primer substrate from the chain extender substrate moiety at precisely the right location could ensure that delivery of the priming substrate does not become rate-limiting for the overall reaction sequence.

Discovery of the modular nature of the polyketide synthases responsible for production of complex macrolides has resulted in the emergence of novel strategies for the engineering of new pharmacological agents by genetic manipulation of the polyketide synthase modules. It is possible that the introduction of KS^Q domains into the loading modules of both engineered and natural polyketide synthases may offer an additional strategy for optimizing yields of macrolide products.

ACKNOWLEDGMENT

We thank Dr. Vangipuram Rangan for purifying some of the enzymes used in this study and for helpful discussions.

REFERENCES

1. Kuhajda, F. P., Jenner, K., Wood, F. D., Hennigar, R. A., Jacobs, L. B., Dick, J. D., and Pasternak, G. R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 6379−6383.
2. Pizer, E. S., Wood, F. D., Pasternack, G. R., and Kuhajda, F. P. (1996) *Cancer Res. 56*, 745−751.
3. Pizer, E. S., Wood, F. D., Heine, H. S., Romantsev, F. E., Pasternack, G. R., and Kuhajda, F. P. (1996) *Cancer Res. 56*, 1189−1193.
4. Pizer, E. S., Jackisch, C., Wood, F. D., Davidson, N. E., Pasternack, G. R., and Kuhajda, F. P. (1996) *Cancer Res. 56*, 2745−2747.
5. Mdluli, K., Slayden, R. A., Zhu, Y., Ramaswamy, S., Pan, X., Mead, D., Crane, D. D., Musser, J. M., and Barry, C. E. I. (1998) *Science 280*, 1607−1610.
6. Dehesh, K., Edwards, P., Fillatti, J., Slabaugh, M., and Byrne, J. (1998) *Plant J. 15*, 383−390.
7. Somerville, C., and Browse, J. (1991) *Science 252*, 80−87.

8. Magnuson, K., Jackowski, S., Rock, C. O., and Cronan, J. E. (1994) *Microbiol. Rev. 57*, 522−542.
9. Wakil, S. J. (1989) *Biochemistry 28*, 4523−4530.
10. Smith, S. (1994) *FASEB J. 8*, 1248−1259.
11. Donadio, S., Staver, M. J., McAlpine, J. B., Swanson, S. J., and Katz, L. (1991) *Science 252*, 675−679.
12. Clough, R. C., Matthis, A. L., Barnum, S. R., and Jaworski, J. G. (1992) *J. Biol. Chem. 267*, 20992−20998.
13. Tsay, J.-T., Oh, W., Larson, T. J., Jackowski, S., and Rock, C. O. (1992) *J. Biol. Chem. 267*, 6807−6814.
14. Edwards, P., Nelsen, J. S., Metz, J. G., and Dehesh, K. (1997) *FEBS Lett. 402* (1), 62−66.
15. Libertini, L. J., and Smith, S. (1979) *Arch. Biochem. Biophys. 192*, 47−60.
16. Anderson, V. E., and Hammes, G. G. (1985) *Biochemistry 24*, 2147−2154.
17. Witkowski, A., Joshi, K. A., and Smith, S. (1997) *Biochemistry 36* (51), 16338−16344.
18. Huang, W., Jia, J., Edwards, P., Dehesh, K., Schneider, G., and Lindqvist, Y. (1998) *EMBO J. 17*, 1183−1191.
19. Kuhstoss, S., Huber, M., Turner, J. R., Paschal, J. W., and Rao, R. N. (1996) *Gene 183* (1−2), 231−236.
20. Kakavas, S. J., Katz, L., and Stassi, D. (1997) *J. Bacteriol. 179* (23), 7515−7522.
21. Xue, Y., Zhao, L., Liu, H.-W., and Sherman, D. H. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 12111−12116.
22. Motamedi, H., and Shafiee, A. (1998) *Eur. J. Biochem. 256*, 528−534.
23. Schwecke, T., Aparicio, J. F., Molnar, I., König, A., Khaw, L. E., Haydock, S. F., Oliynyk, M., Caffrey, P., Cortes, J., Lester, J. B., Böhm, G. A., Staunton, J., and Leadlay, P. F. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 7839−7843.
24. Nowak-Thompson, B., Gould, S. J., and Loper, J. E. (1997) *Gene 204*, 17−24.
25. Kresze, E.-B., Steber, L., Oesterhelt, D., and Lynen, F. (1977) *Eur. J. Biochem. 79*, 191−199.
26. Tomoda, H., Kawaguchi, A., Omura, S., and Okuda, S. (1984) *J. Biochem. 95*, 1705−1712.
27. Joshi, A. K., and Smith, S. (1993) *Biochem. J. 296*, 143−149.
28. Witkowski, A., Joshi, A. K., and Smith, S. (1996) *Biochemistry 35*, 10569−10575.
29. Joshi, A. K., Witkowski, A., and Smith, S. (1998) *Biochemistry 37*, 2515−2523.
30. Joshi, A. K., Rangan, V. S., and Smith, S. (1998) *J. Biol. Chem. 273*, 4937−4943.
31. Smith, S., and Abraham, S. (1971) *J. Biol. Chem. 246*, 6428−6435.
32. Joshi, A. K., and Smith, S. (1993) *J. Biol. Chem. 268*, 22508−22513.
33. Smith, S., and Abraham, S. (1975) *Methods Enzymol. 35*, 65−74.
34. Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891−2899.
35. Moche, M., Schneider, G., Edwards, P., Dehesh, K., and Lindqvist, Y. (1999) *J. Biol. Chem. 274*, 6031−6034.
36. Jones, T. A., Zou, J.-Y., Cowan, S., and Kjeldgaard, M. (1991) *Acta Crystallogr. A47*, 100−119.
37. Siggaard-Andersen, M. (1993) *Protein Sequences Data Anal. 5*, 325−335.
38. Joshi, A. K., Witkowski, A., and Smith, S. (1997) *Biochemistry 36*, 2316−2322.
39. Kumar, S., Dorsey, J. A., Muesing, R. A., and Porter, J. W. (1970) *J. Biol. Chem. 245*, 4732−4744.
40. Dewar, M. J. S., and Dieter, K. M. (1988) *Biochemistry 27*, 3302−3308.
41. Clark, J. D., O'Keefe, J. O., and Knowles, J. R. (1988) *Biochemistry 27*, 5961−5971.
42. Stern, A., Sedgwick, B., and Smith, S. (1982) *J. Biol. Chem. 257*, 799−803.
43. Kraulis, P. (1991) *J. Appl. Crystallogr. 24*, 946−950.
44. Merrit, E. A., and Murphy, M. E. P. (1994) *Acta Crystallogr. D50*, 869−873.
45. Donadio, S., and Katz, L. (1992) *Gene 111* (1), 51−60.
46. Haydock, S. F., Aparicio, J. F., Molnar, I., Schwecke, T., Khaw, L. E., Konig, A., Marsden, A. F., Galloway, I. S., Staunton, J., and Leadlay, P. F. (1995) *FEBS Lett 374*, 246−248.

BI990993H