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Functional Analysis of Putative β-Ketoacyl:Acyl Carrier Protein Synthase and Acyltransferase Active Site Motifs in a Type II Polyketide Synthase of *Streptomyces glaucescens*

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The significance of potential active site motifs for acyltransferase and β-ketoacyl:acyl carrier protein synthase regions within the TcmK protein was investigated by determining the effects of mutations in the proposed active sites on the production of tetracenomycins F2 and C. In a *Streptomyces glaucescens tcmGHI JKLMNO* null mutant, plasmids carrying the S351A mutation produced high amounts of tetracenomycin F2 but plasmids carrying the C173A or C173S mutation or the H350L-S351A double mutation produced no detectable amount of any known intermediate. In a *tcmK* mutant, plasmids with the S351A mutation restored high production of tetracenomycin C and plasmids carrying the other mutations were able to complement the chromosomal defect to some extent. None of the mutations affected the amount of TcmK produced.

The carbon skeletons of polyketides such as tetracenomycin (TCM) C produced by *Streptomyces glaucescens* are synthesized by polyketide synthases (PKSs), which are classified into three types following the nomenclature established for fatty acid synthase (FAS) complexes. Type I comprises multifunctional enzyme complexes, while Type II systems are multienzyme complexes. Type III PKSs are significantly different, lacking the acyl carrier protein (ACP) function and acting directly on the coenzyme A (CoA) esters of carboxylic acids (10). In fatty acid biosynthesis a special β-ketoacyl:ACP synthase (KS) enzyme or domain performs the condensation reactions between the acylthioester intermediates, using a highly conserved cysteine residue at the active site to which the FAS inhibitor cerulenin can form a covalent adduct (14). The small fatty acid CoA substrates are loaded onto the FAS complexes by acyl-CoA acyltransferases (ATs), which exhibit preferences for different starter and extender units in different FAS systems. Comparisons of several such ATs has shown a high degree of sequence conservation, especially around a central GHSXG motif. This is believed to constitute the active site serine for substrate attachment (1, 6, 22, 27, 30), which is the residue that inhibitors like iodoacetamide and *p*-methylphenylsulfonyl-fluoride act on (40).

The TCM PKS, a Type II complex, consists of at least the TcmKLMN proteins (33) that produce TCM F2, the overall yield of which increases considerably when TcmJ is added to the complex (28, 34). Sequence analysis (2) revealed that TcmK contains two sequence motifs (around Cys-173 and Ser-351) highly similar to the KS and AT active site motifs of FASs and other PKSs (4, 7). It was also shown that the FAS inhibitors mentioned above strongly inhibit the TCM C PKS (28). These data led to the assumption that TcmK might be a bifunctional protein exhibiting both AT and KS activities (12, 28). Here we report the effect of mutations in the potential KS and AT active site residues on the activity of TcmK in vivo.

Construction of wild-type and mutant *tcmKLMN*^{177} and *tcmJKLMN* cassettes. To examine the significance of the above motifs, Cys-173 was replaced by Ala (primer 1, 5′-CGT CGA GGC CCG AGG TGG CGC CGG TCG ACA CCA CCG-3′) and Ser (primer 2, 5′-CGT CGA GGC CCG AGG TCG AGC CGG TCG ACA CCA CCG-3′), Ser-351 was replaced by Ala (primer 3, 5′-GTC CAT GAT CGG CCA CGC CCT CGG AGC GAT CGG C-3′), and His-350–Ser-351 was replaced by Leu-Ala (primer 4, 5′-CCA TCA AGT CCA TGA TCG GGC TAG CGC TCG GAG CGA TCG GCT CC-3′) by the method of Taylor et al. (36, 37) using the synthetic oligonucleotide primers given in parentheses. All mutations were obtained at a frequency of 60 to 80% and were sequenced for confirmation.

pWHM860 (Table 1), a derivative of the *Escherichia coli-Streptomyces* shuttle vector pWHM3 (39), was used to construct expression plasmids for use in *Streptomyces lividans* and *S. glaucescens*. The unmutated *tcm* gene cassettes were cloned into pWHM860 by cutting with *Bam*HI and *Eco*RI and subsequent ligation with the 3.77-kb *ermE**::*tcmKLMN*^{177} cassette from pWHM721 (33) or the 5.10-kb *ermE**::*tcmJKLMN* cassette from pWHM732 (33) to give plasmids pWHM861 and pWHM862, respectively (Table 1). Gene cassettes with the mutated *tcmK* genes were constructed, involving several intermediate plasmid constructs (not shown), to replace a homologous 2.07-kb *Eco*RI-*Mlu*I fragment in pWHM861 and a homologous 2.43-kb *Eco*RI-*Mlu*I fragment in pWHM862, yielding expression plasmids pWHM863 to -866 and pWHM875 to -878, respectively (Table 1).

Expression of mutant *tcmKLMN*^{177} and *tcmJKLMN* cassettes in *S. lividans* 1326 and *S. glaucescens* WMH1077. It was necessary to test the function of the *tcmKLMN*^{177} and *tcmJKLMN* mutant cassettes in either a background without a chromosomal copy of *tcmK* (*S. lividans* 1326) or one that did not express the chromosomal *tcmK* gene, as in *S. glaucescens* WMH1077, a *tcmGHIJKLMNO* null mutant (5, 23). Protoplasts of both strains were individually transformed with plasmids pWHM863 to -866 and pWHM875 to -878 and with pWHM861, pWHM862, and pWHM860 (Table 1) as positive and negative controls. Cultures of each of the transformants were assayed for TCM F2 production, the product of the TCM PKS (29), by procedures described previously (11, 33). pWHM865 and pWHM877, both containing the S351A mutation in *tcmK*, produced high amounts of TCM F2, as revealed by thin-layer chromatography analysis (Fig. 1, lanes 5 and 6,

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TABLE 1. Plasmids used in this study

Plasmid[a]	Characteristics	Reference
pWHM3	*E. coli-Streptomyces* shuttle vector, based on pIJ486 and pUC19	39
pWHM721	pIJ486 derivative with the *tcmKLMN*^{177} genes under control of the *ermE** promoter (9)	33
pWHM732	pIJ486 derivative with the *tcmJKLMN* genes under control of the *ermE** promoter	33
pWHM850	pGem7Zf(+) derivative with the *tcmKL* genes under control of the *ermE** promoter	32
pWHM860	pWHM3 carrying a 0.3-kb *Sph*I-fragment containing a transcriptional terminator cloned from pWHM721	This work
pWHM861	pWHM860 derivative with the *ermE**::*tcmKLMN*^{177} cassette from pWHM721 ligated into the single *Eco*RI-*Bam*HI sites	This work
pWHM862	pWHM860 derivative with the *ermE**::*tcmJKLMN* cassette from pWHM732 ligated into the single *Eco*RI-*Bam*HI sites	This work
pWHM863	pWHM861 derivative containing the *ermE**::*tcmKLMN*^{177} cassette; the tcmK gene was replaced by its mutated version derived from in vitro mutagenesis with primer 1	This work
pWHM864	pWHM861 derivative containing the *ermE**::*tcmKLMN*^{177} cassette; the *tcmK* gene was replaced by its mutated version derived from in vitro mutagenesis with primer 2	This work
pWHM865	pWHM861 derivative containing the *ermE**::*tcmKLMN*^{177} cassette; the *tcmK* gene was replaced by its mutated version derived from in vitro mutagenesis with primer 3	This work
pWHM866	pWHM861 derivative containing the *ermE**::*tcmKLMN*^{177} cassette; the *tcmK* gene was replaced by its mutated version derived from in vitro mutagenesis with primer 4	This work
pWHM875	pWHM862 derivative containing the *ermE**::*tcmJKLMN* cassette; the *tcmK* gene was replaced by its mutated version derived from in vitro mutagenesis with primer 1	This work
pWHM876	pWHM862 derivative containing the *ermE**::*tcmJKLMN* cassette; the *tcmK* gene was replaced by its mutated version derived from in vitro mutagenesis with primer 2	This work
pWHM877	pWHM862 derivative containing the *ermE**::*tcmJKLMN* cassette; the *tcmK* gene was replaced by its mutated version derived from in vitro mutagenesis with primer 3	This work
pWHM878	pWHM862 derivative containing the *ermE**::*tcmJKLMN* cassette; the *tcmK* gene was replaced by its mutated version derived from in vitro mutagenesis with primer 4	This work

[a] The italicized plasmids were used for expression and complementation experiments in *S. lividans* and *S. glaucescens* strains.

and Table 2), while the H350L-S351A TcmK double mutant, expressed by plasmids pWHM866 and pWHM878, resulted in completely inactive PKS complexes (Fig. 1, lanes 3 and 4, and Table 2). Although the H350L replacement introduces an amino acid side chain with a molecular volume larger than that of the imidazole ring in His, which might interfere with the correct folding of the nascent TcmK protein, it has been demonstrated in the case of isopenicillin N synthase that His→Leu replacements did not impair enzyme activity unless the His residue played an important role in the catalytic function of the enzyme (26). Hence, the fact that the TCM PKS complex made with the H350L-S351A TcmK double mutant is not functional indicates the importance of the His residue in the GHSXG motif of TcmK.

Inactive PKS complexes were also formed by plasmids carrying the C173A mutation (pWHM863 and pWHM875) or the C173S mutation (pWHM864 and pWHM876) (Fig. 1, lanes 7 to 10, and Table 2). Cys, Ala, and Ser have similar molecular volumes and intramolecular bonding interactions, which should minimize any perturbation of the three-dimensional structure of the TcmK protein. We thought that the C173S exchange might retain some activity due to the formation of *O*-ester bonds with the initial substrates and intermediates of the growing polyketide chain, as has been observed with a C89S mutant of biosynthetic thiolase enzymes from *Zoogloea ramigera* (21), whose catalytic mechanism resembles that proposed for the KSs. Instead, our results (Fig. 1, lanes 7 to 10, and Table 2) clearly demonstrate that both mutations led to completely inactive PKS complexes, indicating the critical role played by Cys-173 in the TcmK-catalyzed condensation reaction. We therefore conclude that TcmK functions like other kinds of type II KSs, with Cys-173 forming a thioester with the acetate starter unit and the β-ketoacyl intermediates resulting from the individual condensation reactions with malonyl*S*-ACP.

Expression of mutant *tcmKLMN*^{177} and *tcmJKLMN* cas-

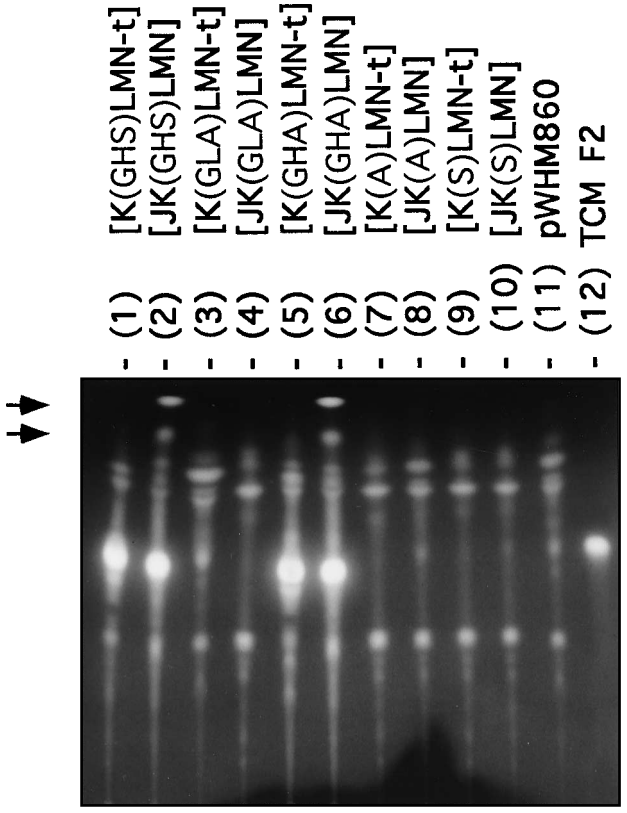

FIG. 1. Thin-layer chromatogram of ethyl acetate extracts prepared from *S. lividans* 1326 transformants carrying plasmids pWHM863 to -866 with the mutated *tcmKLMN*^{177} cassettes, plasmids pWHM875 to -878 with the mutant *tcmJKLMN* cassettes, and plasmids pWHM860, pWHM861, and pWHM862 as controls (Table 1). High-performance liquid chromatography-purified TCM F2 was used as a standard. The arrows on the left side indicate the positions of unknown metabolites seen in lanes 2 and 6. The material in lanes 3 and 11 with an R_f similar to that of TCM F2 is also an unknown metabolite.

TABLE 2. Results of expression of mutant *tcmK* genes in *S. lividans* and different *S. glaucescens* type strains

Plasmids	*S. lividans* 1326	*S. glaucescens*		
		WMH1077[a]	WMH1061[b]	WMH1068[c]
tcmKLMN[177]				
pWHM861 (Wild type)	++[d]	++	++	++
pWHM863 (Cys→Ala)	−[e]	−	+[f]	++
pWHM864 (Cys→Ser)	−	−	+	++
pWHM865 (His-Ser→His-Ala)	++	++	++[g]	++
pWHM866 (His-Ser→Leu-Ala)	−	−	+	++
tcmJKLMN				
pWHM862 (Wild type)	++[h]	++[h]	++	++
pWHM875 (Cys→Ala)	−	−	+	++
pWHM876 (Cys→Ser)	−	−	+	++
pWHM877 (His-Ser→His-Ala)	++[h]	++[h]	++[g]	++
pWHM878 (His-Ser→Leu-Ala)	−	−	+	++
Vector				
pWHM860 (pWHM3 derivative)	−	−	−	−

[a] *S. glaucescens* WMH1077 is a *tcmGHIJKLMNO* null mutant (5, 23).
[b] *S. glaucescens* WMH1061 is a *tcmK* mutant (32).
[c] *S. glaucescens* WMH1068 is a *tcmL* deletion mutant (32, 34).
[d] ++, TCM F2 production in the 1326 and WMH1077 strains or TCM C production in the WMH1061 and WMH1068 strains.
[e] −, lack of TCM F2 or TCM C production.
[f] +, low level of TCM C production.
[g] Known intermediates of TCM C biosynthesis accumulated.
[h] Unidentified shunt products (→) were formed as shown in Fig. 2.

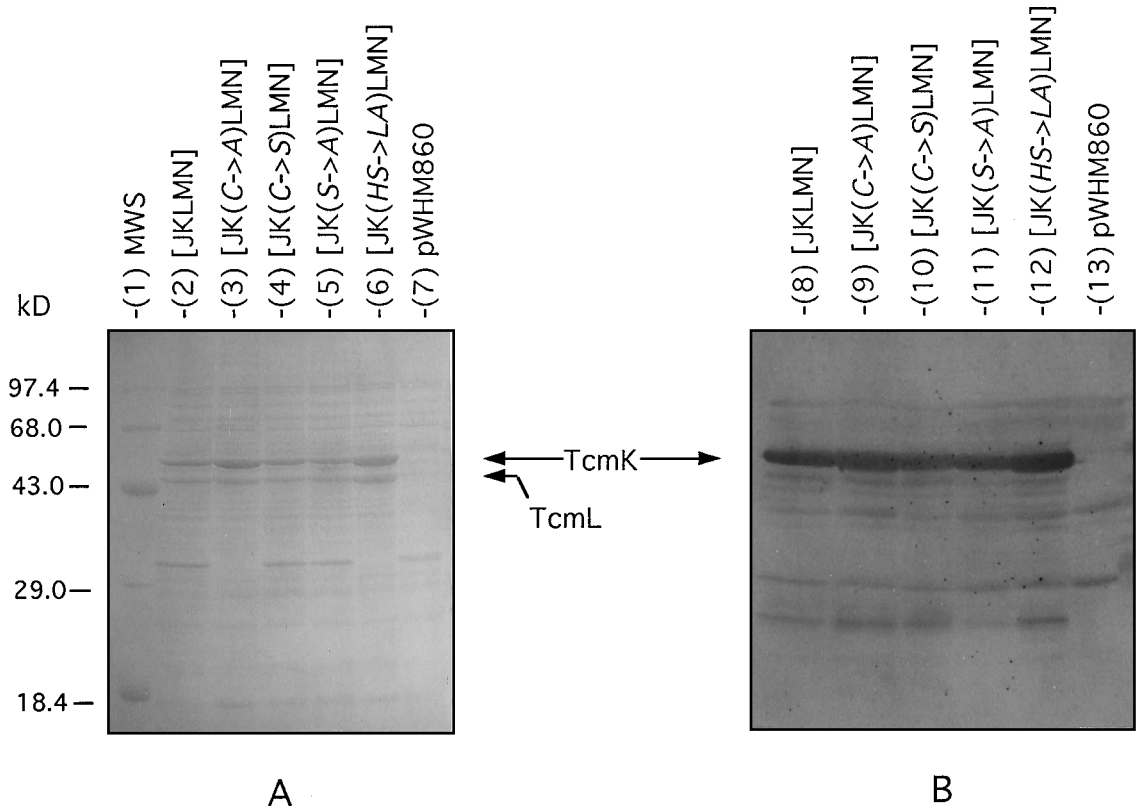

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot) analysis of expression of mutant TcmK proteins from *S. lividans* 1326 carrying *tcmJKLMN*-based plasmids (Table 1). (A) Samples (100 μg) of total protein from cell extracts were separated by SDS–10% PAGE. The overexpressed TcmK and TcmL proteins are indicated by arrows. (B) Identical SDS–10% PAGE run in which the separated proteins were transferred to an Immobilon P membrane and subjected to immunoprecipitation using anti-TcmK antibodies (9). The sizes of molecular weight standards (MWS) are shown on left.

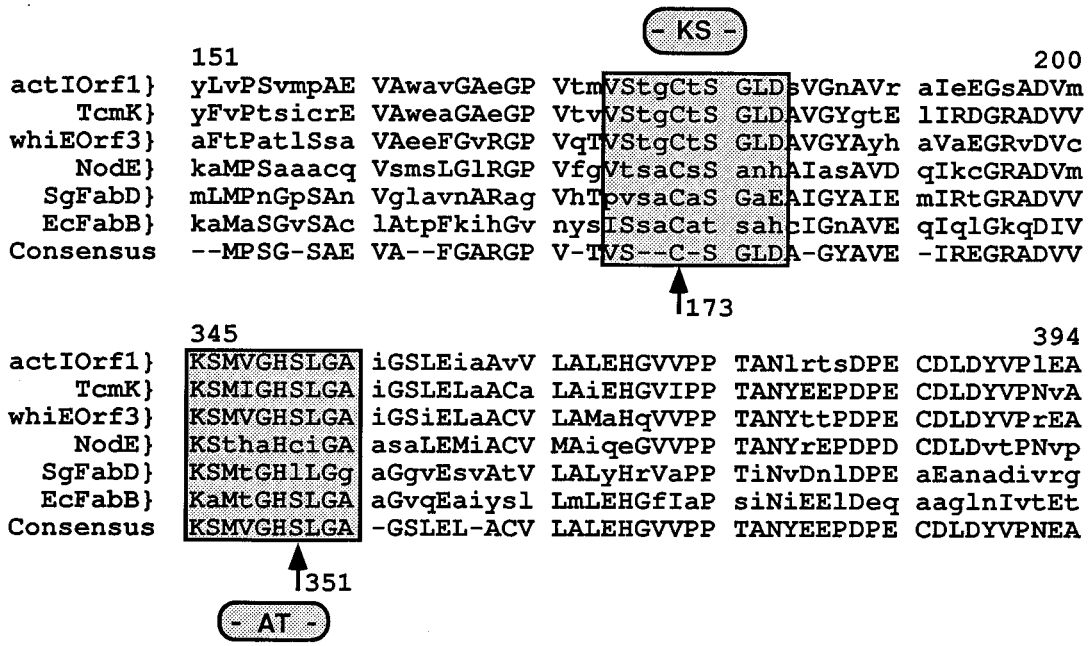

FIG. 3. Comparison of the KS and AT regions of selected type II KS proteins around the proposed active site Cys-173 and Ser-351 residues. actI Orf1 is used for actinorhodin biosynthesis in *Streptomyces coelicolor* (7), whiE Orf3 is used for spore pigment biosynthesis in *S. coelicolor* (4), NodE is an *R. meliloti* nodulation factor (8), SgFabD is a putative type II FAS enzyme of *S. glaucescens* (31), and EcFabB is the KS I enzyme of *E. coli* FAS (14).

settes in the *tcmK* background. *S. glaucescens* WHM1061 carries a mutation within the chromosomal *tcmK* gene (32), and as expected, pWHM865 and pWHM877 restored high-level TCM C production upon introduction into *S. glaucescens* WMH1061 (data not shown). Unexpectedly, all of the other *tcmK* mutations in both the *tcmKLMN*^{177} and *tcmJKLMN* cassettes also complemented the *tcmK* mutation in *S. glaucescens* WMH1061 to a small degree (Table 2), allowing low levels of TCM C biosynthesis. These results suggest that heterogeneous PKS complexes were formed containing both kinds of mutant TcmK proteins and that these mixed complexes were able to restore a low level of TCM PKS activity by some type of intramolecular interaction between the enzyme subunits. This could mean that TcmK has more than one activity.

Malfunctional TCM PKS complexes are not due either to an inability to produce mutant TcmK enzymes or to polar effects of the mutant *tcmK* genes on the expression of *tcmLMN*. To make sure that the observed effects on the activity of TcmK were direct results of the introduced mutations and not to polar effects of the mutations on the expression of the downstream *tcmLMN* genes, the plasmid-borne *tcmKLMN*^{177} and *tcmJKLMN* cassettes were introduced into *S. glaucescens* WMH1068, a *tcmL* deletion mutant (32, 34). The results of chromatographic analysis (not shown) established that all the constructs yielded similar levels of *tcmL* complementation, as determined by TCM C production, although the extracts from unmutated controls (pWHM861 and pWHM862) and from the S351A mutant constructs (pWHM865 and pWHM877) showed high amounts of accumulated intermediates (Table 2). In these cases active PKS complexes can be formed entirely by plasmid-encoded gene expression to yield high enzyme concentrations, whereas levels of expression of the single-copy genes on the chromosome, whose products act on TCM F2, create a bottleneck for processing TCM F2 and the subsequent biosynthetic intermediates. Although the other mutant constructs are also able to provide high amounts of active TcmLMN proteins, the cells do not accumulate intermediates because the number of active PKS complexes formed is limited by the expression level of the chromosomal *tcmK* gene.

Immunoblotting of cell extracts with anti-TcmK antibody (9, 17) showed clearly that all mutant TcmK proteins were produced at similarly high levels (Fig. 2). It is therefore evident that none of the amino acid changes introduced affected the expression of the mutant *tcmK* gene or the stability of the TcmK protein.

Concluding remarks. While our results clearly demonstrate the importance of Cys-173 as the presumed attachment site for the polyketide intermediates in the condensation (KS) reaction, the role of the AT motif around Ser-351 is still uncertain. In theory a PKS could require acyltransferases for any of the following catalytic reactions: (i) acetyl-CoA:ACP or acetyl-CoA:KS acetyl transfer, (ii) malonyl-CoA:ACP malonyl transfer, (iii) transfer of the nascent acyl chain from ACP to KS prior to further condensations, or (4) hydrolysis of the polyketide-*COS*-PKS thioester to release the final product. All of these reactions could occur without a dedicated AT, however, or some could be catalyzed by the *S. glaucescens* FabA malonyl-CoA:ACP transacylase presumed to be part of its FAS (31). Thus, either Ser-351 is not an essential component of the AT activity of TcmK or there is no such activity. If the latter is true, the H350L-S351A TcmK mutant protein might be aberrantly folded and totally dysfunctional. On the other hand, His-350 could be important for the overall TcmK activity. The His-350 residue is conserved throughout all KS enzymes listed (Fig. 3), but Ser-351 is substituted for by Cys in the NodE protein of *Rhizobium meliloti* (8) and by Leu in the FabD protein of a putative *S. glaucescens* FAS (31). Moreover, acetyl-CoA:ACP and malonyl-CoA:ACP AT activities distinct from the *E. coli* FabB (KS I) protein (Fig. 3) are reportedly involved in *E. coli* fatty acid biosynthesis (18–20, 27), and the *E. coli* FabH (KS III) protein even lacks the conserved GH SXG motif yet exhibits both acetyl-CoA:ACP acetyltransferase

activity and KS activity to form acetoacetyl*S*-ACP from acetyl-CoA and malonyl-CoA in vitro (13, 38).

The idea that His-350 but not Ser-351 may be critical for a second TcmK activity is supported by recent work on *E. coli* thioesterase II (24), which highlights the importance of His-58 instead of the classical catalytic triad Ser-His-Asp that has been found in a number of hydrolases (16) and in rat mammary gland thioesterase II (35). Moreover, the classical GXSXG motif, thought to form the so-called "nucleophilic elbow" (25), has been shown not to be a necessary requirement for thioesterase activity in two cases (3, 16).

Thus, although His-350 still might be an essential part of a second TcmK activity (AT and/or thioesterase), the precise identification of this activity will have to await purification of the mutant TcmK protein and the establishment of an in vitro system to study its role in TCM F2 formation.

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