

# Tetracenomycin F1 Monooxygenase: Oxidation of a Naphthacenone to a Naphthacenequinone in the Biosynthesis of Tetracenomycin C in *Streptomyces glaucescens*<sup>†</sup>

Ben Shen<sup>†</sup> and C. Richard Hutchinson<sup>\*,‡,§</sup>

School of Pharmacy and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

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**ABSTRACT:** Tetracenomycin (Tcm) F1 monooxygenase, which catalyzes the oxidation of the naphthacenone Tcm F1 to the 5,12-naphthacenequinone Tcm D3 in the biosynthesis of the anthracycline antibiotic Tcm C in *Streptomyces glaucescens*, has been purified to homogeneity and characterized. Gel filtration chromatography yields a molecular weight of 37 500 whereas SDS–PAGE gives a single band with a molecular weight of 12 500, indicating that the Tcm F1 monooxygenase is a homotrimer in solution. The N-terminal sequence of the enzyme establishes that it is encoded by the *tcmH* gene. The monooxygenase displays an optimal pH of 7.5 and has a  $K_m$  of  $7.47 \pm 0.67 \mu\text{M}$  and  $V_{\max}$  of  $473 \pm 10 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ . Formally, the Tcm F1 monooxygenase can be classified as an internal monooxygenase that requires only  $\text{O}_2$  for the enzymatic oxidation. Yet, it apparently does not possess any of the prosthetic groups of known monooxygenases, such as flavin or heme groups, nor does it utilize metal ions. It is inactivated by *p*-chloromercuribenzoic acid, *N*-ethylmaleimide, and diethyl pyrocarbonate, suggesting that sulfhydryl groups and histidine residues are essential for the enzyme activity.

Tetracenomycin (Tcm)<sup>1</sup> C, **1**, is an antitumor anthracycline-like antibiotic produced by *Streptomyces glaucescens* GLA.0 (Weber et al., 1979). We have previously described the isolation of several groups of tetracenomycin C-nonproducing mutants (Motamedi et al., 1986), the structural elucidation of metabolites isolated from these mutants (Yue et al., 1986) and related recombinant strains (Shen et al., 1993), and the establishment of the biosynthetic pathway of **1** from acetate and malonate with all the biosynthetic intermediates identified (Shen et al., 1993; Yue et al., 1986). We have cloned the gene cluster for the biosynthesis of **1** (Motamedi & Hutchinson, 1987) and analyzed the nucleotide sequences of the biosynthetic genes (Bibb et al., 1989; H. Decker and C. R. Hutchinson, unpublished data; Guilfoile & Hutchinson, 1992; Summers et al., 1992; ref 2) to provide support for the proposed biosynthetic pathway to **1**. From this information, we believe that the 5,12-naphthacenequinone Tcm D3, **2** (Figure 1A), results from oxidation of the naphthacenone Tcm F1, **3**, by a monooxygenase that requires molecular oxygen. Although until recently, a naphthacenone like **3** had not been identified, nor had an enzyme catalyzing such a conversion been reported (Shen et al., 1993), it is known that in the biosynthesis of anthraquinones via a polyketide pathway one of the two oxygens of the anthraquinone moiety is derived from molecular oxygen (Mann, 1987; O'Hagan, 1992; Simpson, 1991) while

the other comes from the carbonyl group of a fatty acid precursor. This has also been assumed to occur in the biosynthesis of many anthracyclines of polyketide origin, and the incorporation of molecular oxygen into the quinone moiety has been confirmed by in vivo  $^{18}\text{O}_2$  feeding experiments in a few cases (Anderson et al., 1989; Fujii et al., 1991; Vederas & Nakashima, 1980).

In order to clarify the biosynthetic events in the biosynthesis of anthracyclines and to gain some insight into the mechanism for the formation of the quinone functionality, we studied the Tcm F1 to Tcm D3 conversion in vitro and report here the isolation and characterization of the Tcm F1 monooxygenase from *S. glaucescens* WMH1068 (formerly GLA.11-47) (Motamedi et al., 1986). This enzyme is encoded by the *tcmH* gene<sup>2</sup> and catalyzes the oxidation of **3** to **2**. It requires only molecular oxygen and contains neither heme nor flavin as a prosthetic group and does not require a metal ion for activity. However, sulfhydryl groups and histidine residues appear to be essential for the enzyme activity.

## EXPERIMENTAL PROCEDURES

**General.** UV/vis spectra were recorded on a Cary 14/Olis UV/vis spectrophotometer. Metal analysis was performed on a Perkin-Elmer atomic absorption spectrophotometer 3030 with a HGA 300 graphite furnace. Refrigerated centrifugation was done in a Sorvall RC-5B superspeed centrifuge. A Pharmacia fast protein liquid chromatography (FPLC) system was used for enzyme purification, and all FPLC columns were purchased from Pharmacia LKB. High-performance liquid chromatography (HPLC) was done with a Waters Model 501 pump system and a Waters 484 variable-wavelength absorbance detector. Enzyme incubations were performed in a HAAKE A81 heating/cooling fluid circulator ( $\pm 0.1^\circ\text{C}$ ). Fermentations were carried out in a rotary shaker-incubator (New Brunswick Model 25). Analytical thin-layer chromatography (TLC) was done on precoated Keisegel 60 F254 glass plates (0.25 mm) and was visualized by long- and/or short-wave UV light. Unless specified, common chemicals,

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<sup>\*</sup> Address correspondence to this author at the School of Pharmacy. Tel: (608)262-7582, Fax: (608)262-3134.

<sup>‡</sup> School of Pharmacy.

<sup>§</sup> Department of Bacteriology.

<sup>1</sup> Abbreviations: Tcm, tetracenomycin; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography;  $\epsilon$ , molar absorbance index; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis-(oxyethylenetriamino)tetraacetic acid; PCMB, *p*-chloromercuribenzoic acid; DEPC, diethyl pyrocarbonate.

<sup>2</sup> R. G. Summers, H. Motamedi, E. Wendt-Pienkowski, and C. R. Hutchinson, unpublished data.

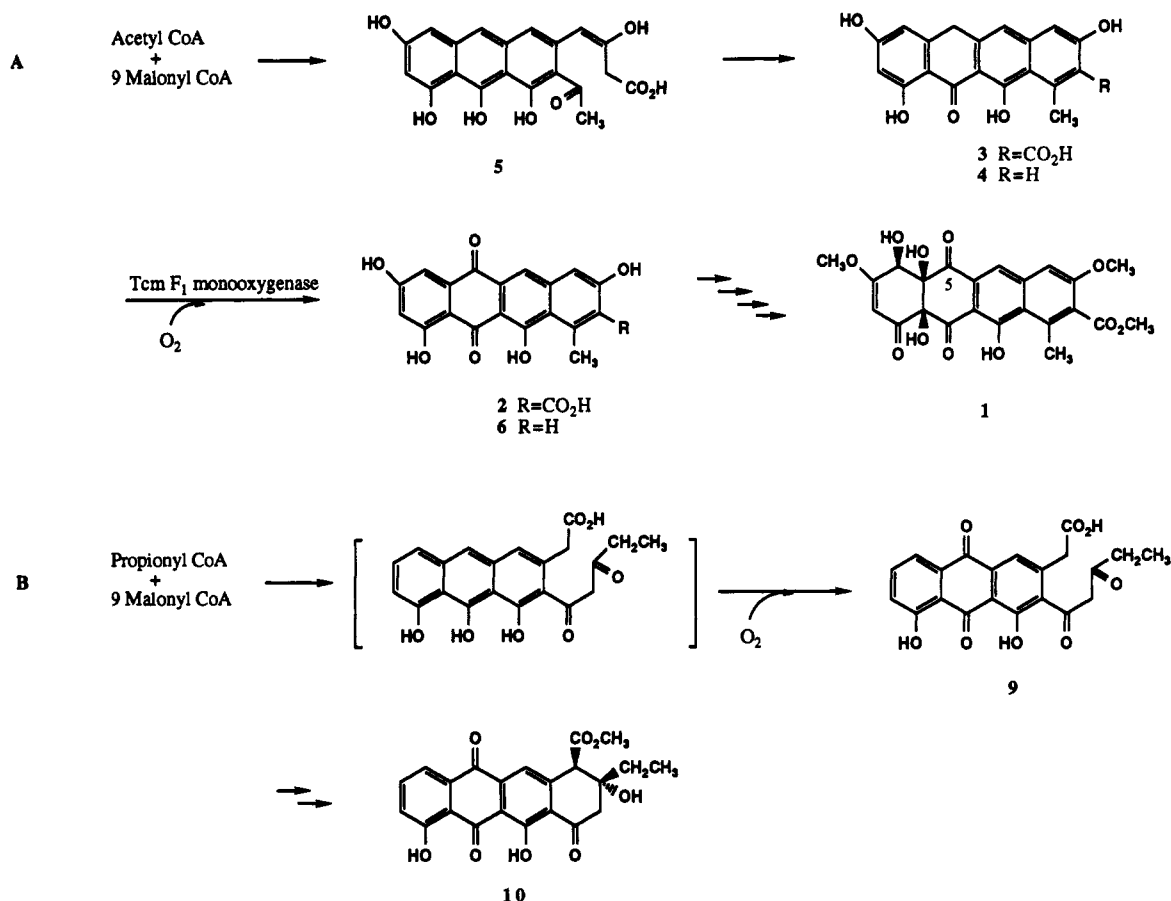


FIGURE 1: A. Biosynthesis of Tcm C in *S. glaucescens*. B. Biosynthesis of anthracyclines in *S. peucetius*.

biochemicals, and reagents were from commercial sources and used without further purification.

**Protein Analysis.** Protein concentrations were determined by the Bradford method with bovine serum albumin as the calibration standard (Spector, 1978). Pure Tcm F<sub>1</sub> monooxygenase also was quantified by UV absorption at 280 nm where the molar absorbance index ( $\epsilon_{280 \text{ nm}}$ ) is 8370 M<sup>-1</sup> cm<sup>-1</sup> (this value is calculated from the amino acid sequence deduced from the *tcmH* gene). The molecular weight of the enzyme subunit was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using the low molecular weight standards of Bio-Rad (phosphorylase B 97 400, bovine serum albumin 66 200, ovalbumin 45 000, carbonic anhydrase 31 000, trypsin inhibitor 21 500, lysozyme 14 400). SDS–PAGE was performed on the PhastSystem (Pharmacia LKB) as described by the manufacturer, and gels were stained with Coomassie blue (Heukeshoven & Dernick, 1988). The molecular weight of the native enzyme was determined by gel filtration chromatography on a Superose 6 HR 10/30 column in 20 mM sodium phosphate (pH 7.2)–1 mM DTT–150 mM NaCl with a flow rate of 0.5 mL/min, and the column was calibrated with bovine serum albumin (66 000), carbonic anhydrase (29 000), cytochrome C (12 400), and aprotinin (6500) as standards (Sigma).

**Enzyme Assays.** The preparation of 3, 9-decarboxy-Tcm F<sub>1</sub>, 4, and Tcm F<sub>2</sub>, 5, and the characterization of 2 and Tcm D, 6, are described elsewhere (Rohr et al., 1988; Shen et al., 1993; Yue et al., 1986).

**TLC Method.** This method assays the consumption of 3 and the formation of 2 simultaneously. Typically, 500  $\mu$ L of assay solution, consisting of 80  $\mu$ M 3 and 1 mM dithiothreitol (DTT) in 0.1 M sodium phosphate buffer, pH 7.5, in the presence of enzyme (10–50  $\mu$ L), was incubated at 30 °C. The

assay was initiated by addition of 3 and terminated by addition of solid NaH<sub>2</sub>PO<sub>4</sub> to saturation and extraction with EtOAc (2  $\times$  250  $\mu$ L). The EtOAc extracts were collected and concentrated in vacuo to dryness, and then the residue was dissolved in 50  $\mu$ L of methanol and analyzed by TLC. The TLC plates were developed in CHCl<sub>3</sub>/MeOH/AcOH (85/15/0.25, v/v); under these conditions, 3 and 2 have an *R<sub>f</sub>* of 0.38 and 0.29 and, under UV light, display a characteristic yellow and red fluorescence, respectively. This method was used throughout the enzyme purification and was also used to monitor the enzymic oxidation of 4 to 6, which under the conditions described above have an *R<sub>f</sub>* of 0.86 and 0.80, respectively.

**Spectrophotometric Method.** The production of 2 can be continuously monitored by measuring the absorbance at 490 nm, at which  $\epsilon_{490 \text{ nm}}$  for 3 and 2 are approximately 0 and 10 960 M<sup>-1</sup> cm<sup>-1</sup>, respectively (Figure 2). This particular wavelength was chosen because very little change of  $\epsilon_{490 \text{ nm}}$  was observed when the spectra were recorded in 0.1 M sodium phosphate buffer, at pH's ranging from 4.0 to 8.0. Typically, 1.0 mL of assay solution, consisting of 80  $\mu$ M 3 and 1 mM DTT in 0.1 M sodium phosphate buffer, pH 7.5, in the presence of enzyme (10–50  $\mu$ L), was incubated at 30 °C. The assay solution was preincubated in the cuvette for 10 min, and the reaction was initiated by addition of 3. The production of 2 was continuously monitored by measuring the increase of the absorbance at 490 nm, from which the initial velocity of the reaction was determined. Similar analysis was also applied to the studies of the enzymatic conversion of 4 to 6, whose  $\epsilon_{490 \text{ nm}}$  are approximately 0 and 12 210 M<sup>-1</sup> cm<sup>-1</sup>, respectively.

This standard assay method was used in all studies with the following modifications. For the pH dependence study, the assays were performed in 0.1 M sodium phosphate buffer, pH

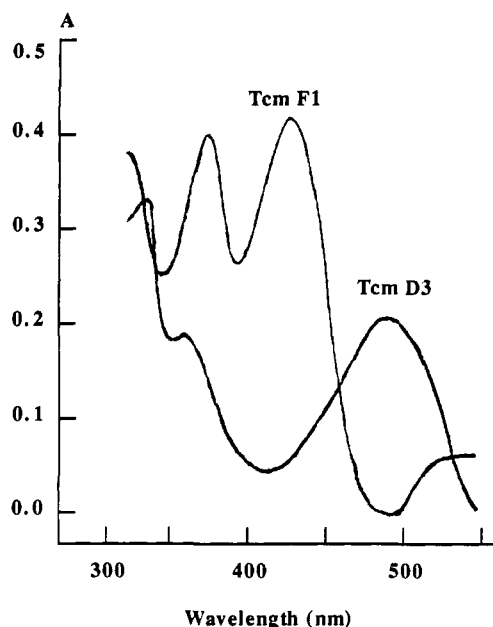


FIGURE 2: Absorption spectrum of 3 and 2 in methanol.

4.0–8.0. For prosthetic group investigations and inhibition studies, the assays were carried out in the presence of the indicated amount of reagent unless specified otherwise. For determination of the kinetic parameters, the assays were done with the concentration of 3 varied from 6.25 to 100  $\mu$ M in the presence of 6.8  $\mu$ g of enzyme.

**Enzyme Purification.** All steps were carried out at 4 °C except for the brief time when the enzyme was on the FPLC columns that were at room temperature.

**Step 1. Preparation of Cell-Free Extract.** Cultures of *S. glaucescens* WMH1068 were grown in R2YENG media (Motamedi et al., 1986) in a 2-L baffled Erlenmeyer flask. After incubation at 30 °C and 300 rpm for 28 h, cells were harvested by centrifugation (13600g, 20 min, 4 °C) and washed sequentially with 0.5 M NaCl and 0.1 M sodium phosphate buffer, pH 7.2, with centrifugation as necessary. The washed cells were suspended in 100 mM sodium phosphate buffer (pH 7.2)–2 mM DTT–0.1 mM (phenylmethyl)sulfonyl fluoride–1 mM EDTA–10% glycerol (10 mL/g cells). Lysozyme (1 mg/mL) was added, and the mixture was left to incubate at room temperature for 2 h. To this viscous slurry were added solid  $MgCl_2$  (5 mg/mL) and DNase (1  $\mu$ g/mL). The resulting slurry was incubated on ice for 1 h, and cell debris were removed by centrifugation (27500g, 20 min, 4 °C) to yield a cell-free extract.

**Step 2. Ammonium Sulfate Fractionation.** The cell-free extract was brought to 41% saturation (234 g/L) by addition of solid ammonium sulfate. The suspension was stirred for 1 h and centrifuged as above to remove the precipitate. The resulting supernatant was brought to 72% saturation (453 g/L) with solid ammonium sulfate and was stirred for an additional hour. Centrifugation as above afforded a pellet that had the enzyme activity.

**Step 3. Sephacryl S-200 Column.** The ammonium sulfate pellet was dissolved in a minimum volume of 0.1 M sodium phosphate buffer (pH 7.2)–1 mM DTT–1 mM EDTA–10% glycerol and applied to a Sephacryl S-200 HR column (2.6  $\times$  60 cm). Fractions (2.0 mL/min, 5-mL fractions) were collected after elution with 20 mM sodium phosphate (pH 7.2)–1 mM DTT–150 mM NaCl. The enzyme activity in fractions from this and succeeding purification steps were assayed by the TLC method.

**Step 4. Mono Q HR 10/10 Column.** Fractions containing the enzyme activity after gel filtration chromatography were dialyzed against 25 mM Tris–HCl (pH 8.0)–1 mM DTT and applied to a Mono Q HR 10/10 column. The column was washed with 25 mM Tris–HCl buffer (pH 8.0)–1 mM DTT and developed with a linear 60-mL gradient from 0 to 0.3 M NaCl in the same buffer (2 mL/min, 2-mL fractions).

**Step 5. Phenyl Superose HR 5/5 Column.** The active fractions after anion-exchange chromatography were brought to 1.0 M ammonium sulfate by addition of solid ammonium sulfate and were applied to a Phenyl Superose HR 5/5 column. The column was washed with 50 mM sodium phosphate (pH 7.2)–1 mM DTT–1.0 M  $(NH_4)_2SO_4$  and developed with a linear 15-mL gradient from 1.0 to 0 M ammonium sulfate in the same buffer (0.5 mL/min, 0.5-mL fractions). This final preparation of active enzyme was stored at –80 °C, and no significant loss of enzyme activity was observed over a 4-week period.

**N-Terminal Sequence Determination.** The amino-terminal sequence of the purified Tcm F1 monooxygenase was determined by automated Edman degradation after desalting by passage through a Vydac protein C<sub>4</sub> reversed-phase HPLC column developed with a linear 50-mL gradient from 5 to 100% acetonitrile in 0.1% (v/v) trifluoroacetic acid (1.0 mL/min, 1.0-mL fractions).

**Metal Ion Analysis.** The metal content of Tcm F1 monooxygenase was determined by atomic absorption spectroscopy using a graphite furnace. Samples (5–10  $\mu$ L) were preheated at 160 °C, ashed at 1500 °C, and atomized at 2200 °C for Mn and 2400 °C for both Fe and Cu. The spectrophotometer was calibrated with 2.5, 5.0, and 10.0 ng/mL of  $Fe^{2+}$ ,  $Cu^{2+}$  and  $Mn^{2+}$ , respectively. Neutron activation analysis was also used for detecting metal ions since this method allows multielement screening over all biological metals.

## RESULTS

**Purification of the Tcm F1 Monooxygenase from *S. glaucescens* WMH1068.** From a cell-free extract of *S. glaucescens* WMH1068, the Tcm F1 monooxygenase activity was precipitated with  $(NH_4)_2SO_4$  at 41% to 72% saturation. The active pellet was then subjected sequentially to size exclusion, anion exchange, and hydrophobic interaction chromatography. As summarized in Table I, these procedures gave Tcm F1 monooxygenase with an overall 503-fold purification.

The purified enzyme was homogeneous when examined by SDS–PAGE where it migrated as a single band (Figure 3) with a relative subunit molecular weight of 12 500.

**N-Terminal Sequence Determination.** The purified Tcm F1 monooxygenase (100 pmol) was subjected to amino-terminal sequencing, and the first 18 residues were determined as A-T-I-S-P-S-P-D-L-F-T-L-V-N-V-F-G-V-. These data establish that the Tcm F1 monooxygenase is encoded by the *tcmH* gene that has been sequenced recently in our lab.<sup>2</sup> Figure 4 shows the deduced sequence of the *tcmH* gene product. We presume that the fMet is cleaved posttranslationally since the amino terminus of the isolated enzyme was found to be Ala. From the DNA sequence, it can be calculated that the Tcm F1 monooxygenase has a molecular weight of 12 554.

**Molecular Weight Determination.** As determined by chromatography on a Superose 6 HR 10/30 column, the native form of Tcm F1 monooxygenase has a molecular weight of 37 500.

Table I: Purification of Tcm F1 Monooxygenase from *S. glaucescens* WMH1068

step	protein (mg)	activity (nmol·min <sup>-1</sup> )	yield (%)	specific activity (nmol·min <sup>-1</sup> ·mg <sup>-1</sup> )	purification (fold)
cell-free extract	768	918	100	1.19	1.00
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	420	925	101	2.20	1.85
Sephacryl S-200	86.6	549	59.8	6.34	5.33
Mono Q HR 10/10	5.82	535	58.3	91.9	77.3
Phenyl Superose HR 5/5	0.760	455	49.6	599	503

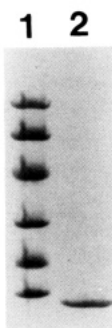


FIGURE 3: SDS-PAGE analysis of Tcm F1 monooxygenase on a 8–25% gradient Phast Gel (lane 1, Bio-Rad low molecular weight standards described under Experimental Procedures; Lane 2, Tcm F1 monooxygenase).

1 MATISPSPL FTLVNVFGVA PEKQRELRDH LVQVTEDLIR HMPGFVSATF  
 51 HLSRDGEQVV NYAQWRSEAD FRAMHAPRL QPHFDYCRSV SRPKPIFCEV  
 101 THSFGATSPE GA\*

FIGURE 4: Amino acid sequence of the Tcm F1 monooxygenase deduced from the *tcmH* gene.

Table II: Effect of Potential Oxygenase Components on Tcm F1 Monooxygenase Activity

entry	addition	concn (μM)	rel activity <sup>a</sup> (%)
1	none		100
2	– enzyme		0
3	boiled enzyme		0
4	N <sub>2</sub>		0
5	NAD <sup>+</sup>	50	96
6	NADH	50	97
7	NADP <sup>+</sup>	50	94
8	NADPH	50	94
9	FMN	50	82
10	FAD	50	83

<sup>a</sup> Assay conditions are described in Experimental Procedures.

**Prosthetic Group Investigation.** All known monooxygenases have either a flavin or heme as their prosthetic group or require a metal ion for activity, and many of them require reduced nicotinamide cofactors. Since flavin or heme groups have characteristic absorption maxima at 380 and 450 nm or 350–650 nm, respectively (Hayaishi, 1974), we determined the absorption spectrum of the purified Tcm F1 monooxygenase. No absorption above 300 nm was observed. To examine whether loosely bound cofactors are required for enzyme activity, we added NAD(P)<sup>+</sup>, NAD(P)H, FMN, and FAD separately to the assay solution; however, none of these compounds significantly stimulated or inhibited the enzyme activity (Table II). As expected, removing O<sub>2</sub> by exchange with nitrogen inhibited the oxidation completely, as did heat denaturation.

Since the Tcm F1 monooxygenase does not appear to contain a heme or flavin prosthetic group, nor does it appear to require loosely bound redox cofactors, we examined the effect of metal ion on the enzymic oxidation of 3. No activation was observed upon addition of a 100 μM concentration of any metal ion

Table III: Effect of Metal Ions on Tcm F1 Monooxygenase Activity

entry	addition	concn (μM)	rel activity <sup>a</sup> (%)
1	none		100
2	FeSO <sub>4</sub>	100	95
3	FeCl <sub>3</sub>	100	99
4	CuSO <sub>4</sub>	100	98
5	CuCl	100	69
6	MgSO <sub>4</sub>	100	100
7	ZnSO <sub>4</sub>	100	99
8	CdCl <sub>2</sub>	100	101
10	MnSO <sub>4</sub>	100	97
11	CaCl <sub>2</sub>	100	97
12	–DTT		103
13	–DTT		
	NiCl <sub>2</sub>	100	99
14	–DTT		
	CoSO <sub>4</sub>	100	95
15	–DTT		
	HgCl <sub>2</sub>	100	0

<sup>a</sup> Assay conditions are described in Experimental Procedures.

Table IV: Effect of Metal Ion Chelating Reagents on Tcm F1 Monooxygenase Activity

entry	addition	concn (μM)	rel activity <sup>a</sup> (%)
1	none		100
2	EDTA	100	98
3	EGTA	100	100
4	8-hydroxyquinoline	100	101
5	dipicolinic acid	100	101
6	Tiron	100	95
7	Tiron	500	99 <sup>b</sup>
8	Chelex 100	1 (v/v) <sup>c</sup>	100
9	1,10-phenanthroline	100	95
10	1,10-phenanthroline	500	91 <sup>b</sup>
11	3,4-dimercaptotoluene	100	89
12	3,4-dimercaptotoluene	500	73 <sup>b</sup>
13	3,4-dimercaptotoluene	500	75 <sup>d</sup>
14	1,10-phenanthroline + ascorbic acid	500 each	94 <sup>b</sup>
15	1,10-phenanthroline + Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub>	500 each	91 <sup>b</sup>

<sup>a</sup> Assay conditions are described in Experimental Procedures. <sup>b</sup> Preincubation at 4 °C for 20 h before assaying the residual enzyme activity.<sup>c</sup> Equal volume of enzyme was added to Chelex 100 and the resulting suspension was incubated on ice for 10 min. After centrifugation in a Eppendorf centrifuge, the supernatant was assayed for enzyme activity.<sup>d</sup> Preincubation at 4 °C for 48 h before assaying the residual enzyme activity.

tested (Table III), but significant inhibition by Cu<sup>+</sup> (entry 5) and total inhibition by Hg<sup>2+</sup> (entry 15) were observed. No significant inhibition was observed upon addition of 100–500 μM of several different chelating reagents either alone (Table IV; entries 2 to 10) or with typical reductants (entries 14 and 15), except for 3,4-dimercaptotoluene, which displayed some inhibitory effect if it was incubated with the enzyme for a prolonged time (entries 11 and 13). These results suggest that loosely bound metal ions are not important for enzyme activity. To determine whether the Tcm F1 monooxygenase contains tightly bound metal ions, pure samples of the enzyme

Table V: Atomic Absorption Analysis of Tcm F1 Monooxygenase<sup>a</sup>

entry	sample	ng/mL		
		Fe	Mn	Cu
1	Tcm F1 monooxygenase (25 µg/mL)	0.0	0.0	0.0
2	Tcm F1 monooxygenase (85 µg/mL)	0.0	0.0	0.0
3	Tcm F1 monooxygenase (25 µg/mL) + Fe <sup>2+</sup> (5 ng/mL)	4.2		
4	Tcm F1 monooxygenase (25 µg/mL) + Mn <sup>2+</sup> (10 ng/mL)		9.7	
5	Tcm F1 monooxygenase (85 µg/mL) + Cu <sup>2+</sup> (5 ng/mL)			4.8

<sup>a</sup> Conditions for atomic absorption analysis are described in Experimental Procedures.

Table VI: Effect of Potential Inhibitors on Tcm F1 Monooxygenase Activity

entry	addition	concn (µM)	rel activity <sup>a</sup> (%)
1	none		100
2	DTT	1000	102
3	iodoacetamide	100	96
4	PCMBA	100	0
5	PCMBA	25	0
6	<i>N</i> -ethylmaleimide	100	0
7	<i>N</i> -ethylmaleimide	25	0
8	cerulenin	100	100
9	catalase	60 units	101
10	catalase	600 units	99
11	glutathione reduced	200	99
12	glutathione peroxidase	10 units	98
13	glutathione peroxidase	50 units	100
14	DEPC	100	0
15	DEPC	25	10
16	-3, +4	80	<2
17	4	80	73
18	4	160	72
19	5	80	100

<sup>a</sup> Assay conditions are described in Experimental Procedures.

were subjected to metal analysis either by atomic absorption spectroscopy or by neutron activation analysis, and in both cases neither Fe, Cu, Mn (Table V), nor any other biological metals were detected.

**Inhibition Studies.** Potential inhibitors were examined for their effect on enzyme activity (Table VI). While sulfhydryl inhibitors like iodoacetamide and cerulenin had no effect (entries 3 and 8), the reaction was completely inhibited by the addition of as little as 25 µM *p*-chloromercuribenzoic acid (PCMBA) (entries 4 and 5) or *N*-ethylmaleimide (entries 6 and 7). Addition of catalase, a hydrogen peroxide scavenger, did not inhibit the oxidation (entries 9 and 10), neither did addition of glutathione peroxidase, which should reduce organic peroxide intermediates (entries 12 and 13) (Burk et al., 1978; Little & O'Brien, 1968; Larson & Suttie, 1978; Smith & Lands, 1972). Finally, the reaction was completely inhibited by addition of diethyl pyrocarbonate (DEPC) (Dominici et al., 1985; Lundblad, 1991), which is assumed to modify histidine residues selectively (entries 14 and 15).

**pH Dependence.** Figure 5 shows the pH dependence of the enzymic reaction with the provision that both the substrate 3 and the product 2 start to decompose significantly at a pH > 8.0. The activity of Tcm F1 monooxygenase displayed an optimal pH of 7.5 in 0.1 M sodium phosphate buffer. An increase of a half unit above the optimal pH resulted in a very small change (10%) of the reaction rate, but a decrease of a half unit below the optimal pH caused a 60% loss of the specific

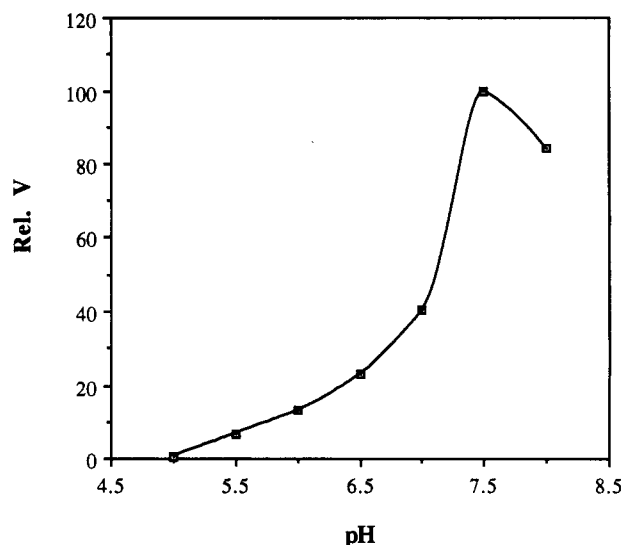


FIGURE 5: pH dependence of the Tcm F1 monooxygenase in 0.1 M sodium phosphate buffer.

activity, and the enzyme was totally inactive when the pH was further decreased to 5.0.

**Kinetics.** Assuming that the O<sub>2</sub> concentration was constant in the assay solution, a kinetic analysis was carried out on the basis of a pseudo-first-order treatment with a steady-state approach. The effect of the initial concentration of 3 on the formation of 2 indicated that the Tcm F1 monooxygenase follows the Michaelis–Menten kinetics. From a Lineweaver–Burk plot, the apparent *K<sub>m</sub>* and *V<sub>max</sub>* were determined to be  $7.47 \pm 0.67$  µM and  $473 \pm 10$  nmol·min<sup>-1</sup>·mg<sup>-1</sup>, respectively.

**Substrate Specificity.** The natural substrate for the Tcm F1 monooxygenase is 3. Two analogs of 3, 4, and 5 (Figure 1A) were also examined as potential substrates for this enzyme. Like 3, 4 is a naphthacenequinone but was oxidized to the corresponding naphthacenequinone, 6, at a much slower rate than 3 (Table VI, entry 16); the presence of 4 in the assay solution inhibited the enzymic oxidation of 3 significantly (Table VI, entries 17 and 18). On the other hand, 5, which has an anthrone instead of naphthacenequinone structure, was not oxidized, nor did the presence of 5 in the assay solution have any effect on the oxidation of 3 (Table VI, entry 19).

## DISCUSSION

The Tcm F1 monooxygenase from *S. glaucescens* WMH1068 has been purified to homogeneity, and its N-terminal sequence establishes that it is encoded by the *tcmH* gene. The latter finding supports the prediction that the *S. coelicolor actVa-orf6* gene encodes a hydroxylase (Caballero et al., 1991) since its deduced product is the only known protein similar to the TcmH protein. SDS–PAGE analysis shows that the purified enzyme displays a single band with a molecular weight of 12 500, in good agreement with the molecular weight of 12 554 (excluding the fMet) predicted from translation of the *tcmH* gene. The molecular weight of the native enzyme, 37 500, suggests that the Tcm F1 monooxygenase is a homotrimer in solution.

A key structural feature of anthracycline metabolites is their naphthacenequinone skeleton. Although the biosynthesis of anthracyclines has been extensively studied, very little is known about the mechanism of the formation of the quinone moiety. For anthracyclines of a polyketide origin, the oxidation of a naphthacenequinone to the corresponding naphthacenequinone is assumed to occur, and in a few cases, the incorporation of molecular oxygen into the quinone has

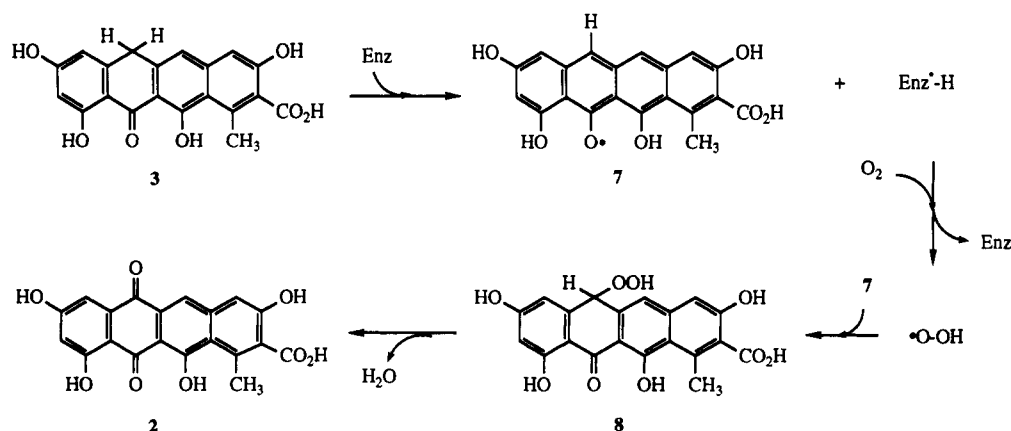


FIGURE 6: Proposed mechanism for the Tcm F1 monooxygenase catalyzed oxidation of 3 to 2.

been established by in vivo  $^{18}\text{O}_2$  feeding experiments (Anderson et al., 1989; Fujii et al., 1991; Vederas & Nakashima, 1980). The properties of the Tcm F1 monooxygenase reported here unambiguously establish that the naphthacene is oxidized directly to form a naphthacenequinone and only molecular oxygen is required for this process. Tcm F1 monooxygenase is the first example of an unrecognized class of enzymes that we now refer to as "semiquinone monooxygenases".

Monooxygenases generally use either a flavin or heme prosthetic group, or employ a metal ion, to activate molecular oxygen or the substrate and to mediate the electron transfer during the oxidation (Hayaishi, 1974; Ortiz de Motellano 1986; Walsh 1979). Since the flavin and heme groups each have a characteristic absorption with maxima at about 380 and 450 nm or 350–650 nm, respectively (Hayaishi, 1974), the method of choice to detect the presence of such groups is the absorption spectrum of the purified enzyme. Tcm F1 monooxygenase displays no absorption above 300 nm, eliminating the presence of a flavin or heme group bound to the enzyme. This is further supported by the fact that addition of FMN or FAD neither stimulates nor significantly inhibits the enzymic reaction. Unlike many other monooxygenases that require a reduced cofactor such as NAD(P)H, Tcm F1 monooxygenase requires only molecular oxygen to catalyze the oxidation of a naphthacene. Addition of cofactors such as NAD(P)H and NAD(P) $^{+}$  has no effect on the reaction. Thus, the substrate, 3, apparently serves as the reducing equivalent. This has been observed with amino acid oxidases (Worthington, 1988), and this type of enzyme has been named an internal monooxygenase. Since D- and L-amino acid oxidases are flavoproteins (Worthington, 1988), we believe that the reaction mechanism of Tcm F1 monooxygenase is different from that of a normal internal monooxygenase. However, two hydroquinone monooxygenases (epoxidizing) from *Streptomyces* LL-C10037 and MPP 3051 (Shen & Gould, 1991), the mammalian dihydrovitamin K-dependent carboxylase (Dowd et al., 1992; Kuliopolos et al., 1992), and a particulate preparation from the fungus *Penicillium patulum* (Priest & Light, 1989), all of which are reported not to have a flavin or heme as a prosthetic group, display the same type of stoichiometry: epoxidation of a hydroquinone requires only molecular oxygen with the substrate hydroquinone itself serving as the reducing equivalent. More recently, emodinanthrone oxygenase from the fungus *Aspergillus terreus* (Chen, 1991; Fujii et al., 1991) has been shown to catalyze the oxidation of an anthrone to an anthraquinone. However, in all these cases the enzymic oxidation either requires (Chen, 1991; Priest & Light, 1989; Shen & Gould, 1991) or can be activated by (Suttie et al., 1981) metal ions.

The possibility of a metal ion requirement of the Tcm F1 monooxygenase was examined by three different approaches. (1) Since it is possible that during the purification the enzyme could lose a loosely bound metal ion, different metal ions were added back to the purified enzyme, but no activation was observed. This suggests that either no metal ion is required or the active site of the enzyme is saturated with a tightly bound metal ion. (2) Metals bound to an enzyme often can be removed by complexation with chelating reagents. It is known that  $\text{Fe}^{3+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ , and in a few cases  $\text{Mn}^{2+}$  are the most common metal ions associated with monooxygenases (Hayaishi, 1974; Ortiz de Motellano, 1986; Walsh, 1979) and these metal ions form tight complexes with selected chelating reagents. This method is risky because it is also known that some enzymes bind metal ions so tightly that chelating reagents have no effect on them unless the enzymes are denatured (Pistorius & Axelrod, 1974; Roza & Francke, 1973). Nevertheless, addition of chelating reagents with either oxygen, nitrogen, or sulfur ligands showed no significant inhibitory effect on the enzymic reaction, suggesting that Tcm F1 monooxygenase does not contain a metal ion. The same negative results were seen with a combination of reducing reagents such as  $\text{Na}_2\text{S}_2\text{O}_3$  or ascorbic acid and chelating reagents like 1,10-phenanthroline that have been proven to be very effective in removing tightly bound  $\text{Fe}^{3+}$  from enzymes (Chen, 1991; Pistorius & Axelrod, 1974; Roza & Francke, 1973). (3) Finally, to examine directly whether the Tcm F1 monooxygenase contains bound metal ions, samples of the enzyme (0.66 and 2.26 nmol/mL) were subjected to atomic absorption spectroscopy and the Fe, Cu and Mn content of different samples was analyzed. Neither Fe, Cu, nor Mn was found in the Tcm F1 monooxygenase, although an externally added amount of each metal exhibited the expected metal concentration as positive controls for the given experimental conditions. The same negative results were obtained from neutron activation analysis that allows multielement screening over all biological metals. We therefore conclude that Tcm F1 monooxygenase is the first member of a new class of monooxygenases that do not possess any of the known prosthetic groups or require a metal ion.

The exact mechanism of the Tcm F1 monooxygenase catalyzed oxidation of 3 to 2 is not clear yet. Since the enzyme does not have any of the prosthetic groups or metal ion requirements of known monooxygenases and direct reaction of triplet oxygen with 3 in the absence of these groups or ions would be a spin-forbidden process, we believe that this reaction could be a radical process. As proposed in Figure 6, 3 could be activated by the enzyme to generate a phenolic radical intermediate, 7, and a radical species at some residue of the

enzyme. The latter then could activate O<sub>2</sub> to an <sup>•</sup>OOH species, which couples with 7 to yield the semiquinone peroxide, 8, with dehydration of 8 finally affording 2. This mechanism is consistent with the finding that catalase has no effect on the enzymic oxidation, ruling out the possibility of free hydrogen peroxide as an intermediate. We therefore prefer that the enzyme activates 3 to give 7, which could be coupled with O<sub>2</sub> to yield 8. An intermediate similar to 8 has been postulated (Dowd et al., 1992; Kuliopulos et al., 1992) in the dihydrovitamin K-dependent carboxylase. Unlike the latter case in which the presence of an organic peroxide intermediate is supported by the inhibitory effect of glutathione peroxidase (Larson & Suttie, 1978; Smith & Lands, 1972), Tcm F1 monooxygenase is not inhibited by this enzyme. This could indicate the absence of an organic peroxide intermediate or, alternatively, the presence of an enzyme-bound peroxide intermediate that is inert to externally added glutathione peroxidase.

The formal dehydration of 8 to 2 may be facilitated by a basic residue of the enzyme. This would be consistent with the pH dependence of the enzyme since decreasing the pH from 7.5 to 5 results in a total loss of the activity. The most likely residue with a pK<sub>a</sub> between 5 and 7.5 would be histidine. The Tcm F1 monooxygenase has six histidine residues per subunit (Figure 4), and addition of the histidine-specific blocking reagent DEPC causes a total loss of the enzyme activity, which suggests that histidine is essential for the enzyme activity (Dominici et al., 1985; Lundblad, 1991).

The role of a sulfhydryl group in the enzyme reaction was examined because Tcm F1 monooxygenase has two cysteine residues per subunit (Figure 4), i.e., a potential total of six sulfhydryl groups in the homotrimer. Although it is not known yet if these sulfhydryl groups are part of the active site or involved in the stabilization of the enzyme conformation, addition of PCMB and *N*-ethylmaleimide results in the complete loss of the activity. This supports the idea that free sulfhydryl groups are required for the activity of Tcm F1 monooxygenase and may also explain the inhibitory effect of Cu<sup>+</sup> and Hg<sup>2+</sup>, both of which could complex with sulfhydryl groups.

It is interesting to point out the substrate specificity of the Tcm F1 monooxygenase. One would predict that the carboxyl group of 3, which at the optimal pH for enzyme activity is ionized, is important for substrate recognition or binding to the enzyme. The compound without this carboxyl, 4, is oxidized to 6 at less than 2% of the relative rate of 3 → 2, proving that the presence of the carboxyl group is essential. In contrast, 5, which differs from 3 by having an anthrone instead of a naphthacenone structure, cannot be oxidized by the enzyme. The latter result indicates that the naphthacenone structure is the signature moiety for substrate recognition. This conclusion is further supported by the fact that 5 also displays no inhibitory effect on the oxidation of 3, in contrast to a significant inhibitory effect observed with 4, which presumably results from its competitive binding to the enzyme active site.

The high specificity of the Tcm F1 monooxygenase unambiguously establishes the biosynthetic sequence shown in Figure 1A: anthrone → naphthacenone → naphthacenequinone. The same biosynthetic sequence has also been proposed in the biosynthesis of tetracycline in *Streptomyces aureofaciens* (McCormick et al., 1968a, 1968b). However, in a parallel biosynthetic pathway to daunorubicin and related compounds in *Streptomyces peucetius* and *Streptomyces galilaeus*, the earliest intermediate identified is the an-

thraquinone aklanoic acid, 9 (Eckardt et al., 1985), which is cyclized to the tetracyclic aklaviketone, 10 (Figure 1B), suggesting that an enzyme similar to Tcm F1 monooxygenase recognizes an anthrone as its substrate (Connors et al., 1990; Fujii et al., 1991). It will be very interesting to compare the sequence similarity between our naphthacenone and the latter anthrone monooxygenase once the gene for the *S. peucetius* enzyme is available and to determine the nature of the substrate specificity. It will be even more interesting to examine if the substrate specificity of the two enzymes could be altered by a few changes in their amino acid sequences.

#### NOTE ADDED IN PROOF

After helpful discussions with Paul Dowd and Larry Que, we feel it is important to note that an alternative mechanism from that shown in Figure 6, involving a direct electron transfer between the substrate 3 and molecular oxygen followed by bond formation between radical intermediate 7 and the resulting superoxide radical anion or molecular oxygen to give 8, cannot be excluded by the available data. This idea is consistent with the fact that 3 slowly converts to 2 spontaneously upon exposure to air in solution or on the surface of TLC plates. In this case, the role of the enzyme could be to lower the energy for the initial electron transfer in addition to catalyzing the conversion of 8 to 2.

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