

# Identification of a Monooxygenase from *Streptomyces coelicolor* A3(2) Involved in Biosynthesis of Actinorhodin: Purification and Characterization of the Recombinant Enzyme

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**The oxidation of phenols to quinones is an important reaction in the oxidative tailoring of many aromatic polyketides from bacterial and fungal systems. Sequence similarity between ActVA-Orf6 protein from the actinorhodin biosynthetic cluster and the previously characterized TcmH protein that is involved in tetracenomycin biosynthesis suggested that ActVA-Orf6 might catalyze this transformation as a step in actinorhodin biosynthesis. To investigate the role of ActVA-Orf6 in this oxidation, we have expressed the *actVA-Orf6* gene in *Escherichia coli* and purified and characterized the recombinant protein. ActVA-Orf6 was shown to catalyze the monooxygenation of the tetracenomycin intermediate TcmF1 to TcmD3, strongly suggesting that it catalyzes oxidation of a similar intermediate in actinorhodin biosynthesis. The monooxygenase obeys simple reaction kinetics and has a  $K_m$  of  $4.8 \pm 0.9 \mu\text{M}$ , close to the figure reported for the homologous enzyme TcmH. The enzyme contains no prosthetic groups and requires only molecular oxygen to catalyze the oxidation. Site-directed mutagenesis was used to investigate the role of histidine residues thought to be important in the reaction; mutants lacking His-52 displayed much-reduced activity, consistent with the proposed mechanistic hypothesis that this histidine acts as a general base during catalysis.**

A plausible biosynthetic pathway to actinorhodin has been proposed based largely on the analysis of products secreted by mutants blocked at particular stages of biosynthesis (1, 4, 6, 7, 10, 21, 25, 32). Subsequent studies have probed the events that lead to the assembly of the actinorhodin polyketide chain, and those of related actinomycete antibiotics, by the iterative condensation of acetate units (11, 14, 20, 27) and have sought to elucidate the folding patterns and cyclizations that occur after polyketide assembly (13, 22). These molecular genetic studies have been facilitated by the cloning and sequence analysis of the actinorhodin biosynthetic gene cluster (3, 6–8, 10, 17, 18). This has allowed many of the proteins responsible for the construction of the carbon skeleton to be identified, based on sequence similarities to other polyketide synthases and to the well-characterized enzymes of fatty acid biosynthesis (7, 10, 14). In contrast, the identities and functions of the enzymes that perform the later steps in this pathway remain elusive.

These late-acting enzymes have been termed tailoring enzymes, and they catalyze a wide range of modifications to polyketide structures, such as hydroxylations, methylations, or glycosylations, important for the compounds' biological activity. Sequence similarities with genes in other polyketide biosynthetic clusters have allowed enzyme activities to be tentatively assigned to some of the actinorhodin gene products. However, the difficulty involved in purifying and assaying these enzymes, together with inadequate knowledge of the biosynthetic pathway, has meant that in general these assignments have not been confirmed by biochemical studies on the proteins.

We have chosen to investigate the enzymes involved in the

later oxidative steps of the biosynthesis of actinorhodin that are encoded within the *actVA* and *actVB* regions of the *act* biosynthetic cluster (3, 4, 7). Previously, we have overexpressed the gene product of the *actVB* region and shown it to be a flavin mononucleotide:NADH oxidoreductase (15). This enzyme is probably part of a two-component flavin-dependent oxidase that catalyzes C-8 hydroxylation and/or the dimerization of actinorhodin. Mutational studies have implicated the genes within the *actVA* region in the events leading to the hydroxylations at C-6 and C-8 of actinorhodin (3). The production of the mederrhodins (8-hydroxymedermycins) by genetically engineered medermycin-producing strains containing segments of the *actVA* region also supports the idea that this region is involved in the late oxidative steps (12, 24). However, the precise functions of the individual enzymes encoded by this region have not been established.

One of the open reading frames in the *actVA* region, Orf6, has sequence similarity (39% identity and 55% similarity) to TcmH protein from *Streptomyces glaucescens*. TcmH catalyzes oxidation of the naphthacenone TcmF1 to the 5,12-naphthacenequinone TcmD3 (28). TcmH has been shown to be a novel monooxygenase that appears to require only molecular oxygen for the oxidation of TcmF1 to TcmD3 and, intriguingly, appears to require no cofactors or metal ions for activity. By analogy to TcmH, it was thought that ActVA-Orf6 may catalyze oxygenation at C-6 of the actinorhodin precursor shown in Fig. 1 (6). To investigate this possibility, and to help clarify the late steps in actinorhodin biosynthesis, we have overexpressed, purified, and characterized the recombinant ActVA-Orf6 protein. We have also undertaken mutagenic studies that point to a histidine residue playing an important role in catalysis.

## MATERIALS AND METHODS

**Materials.** The sources of plasmids, restriction endonucleases, DNA-modifying enzymes, and biochemical reagents have been described previously (15).

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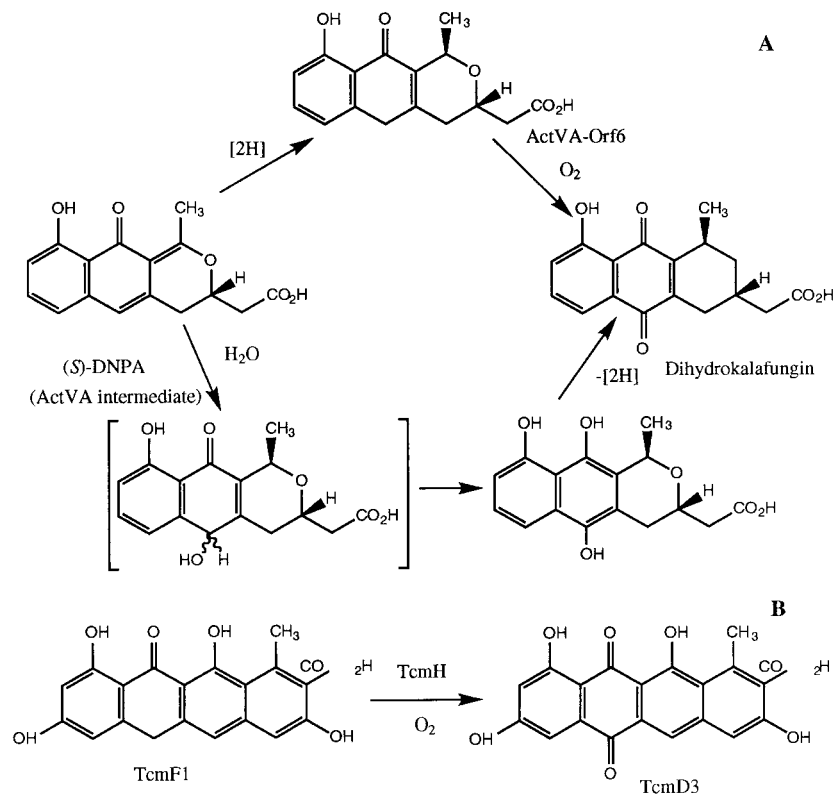


FIG. 1. Formation of quinones in polyketide biosynthesis. (A) Two potential biosynthetic pathways have been proposed for the formation of dihydrokalafungin from (S)-DNPA. The upper route proceeds by reduction of (S)-DNPA followed by ActVA-Orf6-catalyzed oxygenation (6). The lower route proceeds by a hydration, and presumably tautomerization, step that is followed by oxidation of the quinol (4). (B) The oxidation of TcmF1 to TcmD3, catalyzed by the monooxygenase TcmH (28), is analogous to the proposed biosynthetic role of ActVA-Orf6.

Tetracenomyacin F1 (TcmF1) (29) was the kind gift of C. R. Hutchinson (School of Pharmacy, University of Wisconsin—Madison).

**Construction of an expression vector for actVA-Orf6.** The actVA-Orf6 region was originally subcloned as a 11.7-kb *Bgl*III fragment (sites 6 to 15 [18]) in the vector pIJ280 to give plasmid pIJ2325. Two oligonucleotide primers were designed to amplify the coding region of actVA-Orf6 and to introduce a unique *Nde*I site at the presumptive ATG translational start codon and a *Hind*III site beyond the 3' end of the gene. The sequences were AGGAGCACATATGGC TGAAGTGAACGATCCC and GACGAAAGCTTCAGGACGGCAGATCGC CCC. A PCR was set up by using Vent polymerase in the manufacturer's buffer with pIJ2325 (40 ng) as the template and 100 pmol of each primer in a final volume of 100  $\mu$ l. Amplification was achieved with 40 cycles of 95°C for 1 min, 67°C for 1 min, and 72°C for 1 min. The amplified product was subcloned by standard methods (26) into the expression vector pT7-7 (31) and was maintained in *Escherichia coli* TG1 *recO* (23). The sequence of the entire actVA-Orf6 gene was redetermined by automated sequencing to confirm that no unintended mutations had been introduced. The plasmid containing the actVA-Orf6 gene under the control of the T7 promoter was designated pActVA-Orf6.

**Construction of pActVA-Orf6-H52Q.** The pActVA-Orf6-H52Q mutant was constructed by using the Promega Altered Sites II mutagenesis kit according to the manufacturer's protocol. An *Nde*I-*Hind*III fragment from pActVA-Orf6, encompassing the actVA-Orf6 gene, was subcloned into the mutagenesis vector pALTER-Ex1. A 20-base mutagenic oligonucleotide (CCACGTATCAGGCGA GCACG) encoding the His-26-Gln mutation was used to direct the synthesis of the mutated strand. To confirm that the actVA-Orf6 gene had been correctly mutated, plasmids were isolated from the transformants and the gene sequence was redetermined. To facilitate expression of actVA-Orf6-H52Q, it was resubcloned, as an *Nde*I-*Hind*III fragment, into pT7-7. This plasmid, containing the actVA-Orf6-H52Q gene under the control of the T7 promoter, was designated pActVA-Orf6-H52Q and was initially maintained in *E. coli* JM109.

**Construction of pActVA-Orf6-H26Q and pActVA-Orf6-H26Q-H52Q.** The ActVA-Orf6-H26Q mutation was introduced by PCR. A reverse oligonucleotide primer (59-mer; CTCGCGGATCCACTCTGCACCCCGCCGGTGGCCAGC TCCACCAGCTTCTGCTGGGTGG) incorporating the desired His-to-Gln mutation and spanning an internal *Bam*HI restriction site was used in a PCR with the ActVA-Orf6 N-terminal forward primer to amplify the region between the start codon and the internal *Bam*HI site. The resulting 120-bp DNA fragment,

comprising the 5' end of the actVA-Orf6 gene with the H26Q mutation, was digested with *Nde*I and *Bam*HI, and the *Nde*I-*Bam*HI fragment was isolated by agarose gel electrophoresis. This fragment was subcloned into *Nde*I- and *Bam*HI-digested, gel-isolated, and dephosphorylated pActVA-Orf6 or pActVA-Orf6-H52Q to form either the single mutant, actVA-Orf6-H26Q, or the double mutant, actVA-Orf6-H26Q-H52Q, respectively. To confirm the mutations, the nucleotide sequence of the entire actVA-Orf6 region was redetermined in each case. The resulting plasmids, containing the actVA-Orf6-H26Q or actVA-Orf6-H26Q-H52Q gene under the control of the T7 promoter, were designated pActVA-Orf6-H26Q and pActVA-Orf6-H26Q-H52Q, respectively. These plasmids were initially maintained in *E. coli* JM109.

**Overexpression of recombinant ActVA-Orf6.** To facilitate expression of ActVA-Orf6 and the ActVA-Orf6 mutant proteins, the expression constructs were introduced into *E. coli* BL21(DE3) (26). Cultures were grown in 2-liter flasks containing 1 liter of 2TY medium supplemented with 50 mM potassium phosphate (pH 6.9), 10% glycerol, and 100  $\mu$ g of ampicillin/ml at 37°C, with shaking at 200 rpm, until an  $A_{600}$  between 1.5 and 2.0 was reached. Expression of ActVA-Orf6 was induced by addition of 238 mg of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG)/liter, and the cells were grown for a further 2.5 h, by which time protein expression was maximal. Cells were harvested by centrifugation (at  $10,000 \times g$  for 20 min), washed with 50 mM Tris-HCl (pH 7.4), repelleted, and stored at -20°C.

**Purification of recombinant ActVA-Orf6.** All steps were performed on ice or at 4°C. In a typical purification, 16 g of cells was resuspended in 20 ml of buffer A (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM dithiothreitol [DTT], 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 10% glycerol). A crude protein extract was obtained by rupture of the cells with a French pressure cell, followed by precipitation of nucleic acids by dropwise addition of streptomycin sulfate (final concentration, 4%) and finally by ammonium sulfate fractionation (20 to 60%), as described previously (15). The ammonium sulfate pellet was dialyzed against buffer A, and any remaining insoluble material was removed by centrifugation. Proteins were gel filtered through a 2.5- by 100-cm Sephacryl S-300-HR column equilibrated in buffer A at a flow rate of 28 ml/h. Fractions (3.5 ml) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); ActVA-Orf6 (12 kDa) eluted in a broad peak after the majority of the *E. coli* proteins. The ActVA-Orf6-containing fractions were pooled, diluted with water to <40 mM Tris-HCl, and adsorbed onto a 2.5- by 20-cm Q-Sepharose Fast

Flow anion exchange column equilibrated in buffer B (40 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol). Loosely bound proteins were removed by washing with 200 ml of buffer B, and ActVA-Orf6 protein was eluted with a 600-ml linear gradient of 0 to 1 M KCl in the same buffer. Fractions containing ActVA-Orf6 were pooled and concentrated by ultrafiltration. The ActVA-Orf6 protein was stored at  $-20^{\circ}\text{C}$  in the presence of 20% glycerol.

**Purification of ActVA-Orf6 mutant proteins.** The purification of ActVA-Orf6-H26Q followed almost exactly the protocol devised for the purification of recombinant ActVA-Orf6. However, after ion exchange the protein was still not homogeneous. Therefore, 2 mg of protein was dialyzed against 20 mM Tris-HCl (pH 7.4) containing 10% glycerol and was adsorbed onto a Mono-Q fast protein liquid chromatography (FPLC) column equilibrated in the same buffer and running at 1 ml/min. The column was washed with 5 ml of buffer to remove loosely bound material, and protein was eluted with a 0 to 0.5 M KCl gradient over 20 min. Fractions containing ActVA-Orf6-H26Q were pooled, concentrated, and stored at  $-20^{\circ}\text{C}$  in the presence of 20% glycerol.

The proteins ActVA-Orf6-H52Q and ActVA-Orf6-H26Q-H52Q were purified and refolded from inclusion bodies. Cells (13 g) were resuspended in 22 ml of buffer A and lysed by two passes through a French pressure cell operating at an internal pressure of 15,000 lb/in<sup>2</sup>. The inclusion bodies and cell debris were pelleted by centrifugation (at 25,000  $\times g$  for 15 min), and the white inclusion body pellet was washed to remove the brown cellular debris lying on top of the pellet. The inclusion bodies were resuspended and washed twice in 10 ml of a solution containing 50 mM Tris-HCl (pH 7.4), 1 mM DTT, 1 mM EDTA, 0.1 mM PMSF, 0.5% Triton X-100, and 10% glycerol; after each wash the inclusion bodies were pelleted by centrifugation (at 25,000  $\times g$  for 15 min). It proved important to remove as much of the cellular debris as possible for successful purification. The inclusion body pellet was solubilized in 5 ml of the same buffer containing 6 M guanidinium hydrochloride and stirred overnight. Insoluble material was removed by centrifugation (at 25,000  $\times g$  for 30 min), and the supernatant containing the solubilized protein was added dropwise into 100 ml of a solution containing 100 mM Tris-HCl (pH 7.4), 1 mM DTT, 1 M guanidinium hydrochloride, and 10% glycerol. The remaining guanidinium hydrochloride was removed by dialysis overnight against two changes of 2 liters of buffer A, any insoluble material was removed by centrifugation (at 25,000  $\times g$  for 30 min), and the resulting solubilized protein was concentrated by ultrafiltration. Final purification was achieved by using a Mono-Q FPLC column as described above.

**Protein analysis.** SDS-PAGE was carried out by using the buffer system of Laemmli (16) in a 20% polyacrylamide gel, and protein bands were visualized by staining with Coomassie brilliant blue R250. Protein concentrations were determined by the method of Bradford (2); bovine serum albumin was used to construct standard curves. The identities of recombinant proteins were confirmed by N-terminal automated sequencing. Exact protein molecular weights were determined with an electrospray mass spectrometer. Analytical ultracentrifugation sedimentation equilibrium experiments were performed on a Beckman XLA machine by methods described previously (19). Circular dichroism (CD) spectroscopy was performed on a Jobin Yvon CD6 spectrometer at 25 $^{\circ}\text{C}$ . Samples were dialyzed against 25 mM potassium phosphate at a concentration of  $\sim 1$  mg/ml.

**Enzyme assay.** Monooxygenase activity was assayed with TcmF1 as a substrate by monitoring the increase in absorbance at 490 nm due to the production of TcmD3 ( $\epsilon = 10,960 \text{ M}^{-1} \text{ cm}^{-1}$ ) (28). Assays were performed in a total volume of 0.4 ml at 30 $^{\circ}\text{C}$  in 0.1 M sodium phosphate buffer, pH 7.5. The assay solution containing buffer and substrate was preincubated in the cuvette for 5 min prior to initiation by addition of enzyme.

## RESULTS

**Expression of ActVA-Orf6.** ActVA-Orf6 protein was overexpressed in *E. coli* by placing the gene under the control of the bacteriophage T7  $\phi 10$  promoter (30). This strategy has been used successfully to overexpress in *E. coli* a number of *Streptomyces* genes whose high GC content can otherwise lead to low levels of heterologous expression (9). No attempt was made to optimize codon usage at the N terminus of the gene, which has in some cases led to increased levels of *Streptomyces* protein overexpression in *E. coli* (5, 9). It was found that Vent polymerase was the only thermostable enzyme that could be used to amplify this gene; *tac* polymerase, *pfu* polymerase, and Vent *exo*<sup>-</sup> polymerase all failed to give any amplified product despite a number of attempts to optimize the amplification conditions. This observation confirms the previously noted superiority of Vent polymerase for primer extensions in which the template DNA has a high GC content or is significantly structured. The ActVA-Orf6 protein was expressed by a modification of the procedure used to express the ActVB protein in

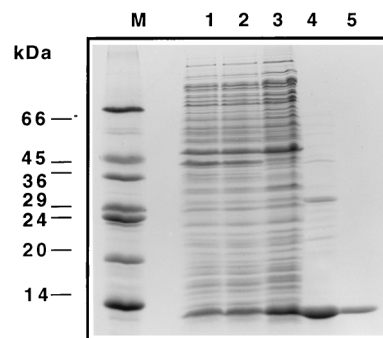


FIG. 2. Purification of recombinant ActVA-Orf6. Results of SDS-PAGE of samples taken after each step of the purification (gels stained with Coomassie brilliant blue) are shown. Lane M, molecular weight marker proteins; lane 1, total cellular protein after induction; lane 2, soluble protein fraction; lane 3, ammonium sulfate fractionation; lane 4, pooled fractions after gel filtration; lane 5, pooled fractions after ion exchange chromatography. The molecular masses of marker proteins are indicated at the side of the gel.

*E. coli* by growing the cells to mid-exponential phase and inducing with IPTG (15). In this case it was found unnecessary to grow the cells in the presence of glucose prior to induction; presumably the *actVA*-Orf6 gene product is less toxic to the host cells. Typically, 16 g of cells was produced from 3 liters of culture, and the ActVA-Orf6 protein comprised 5 to 10% of the total cell protein.

**Purification of ActVA-Orf6.** The majority of the recombinant protein was produced in soluble form, and the purification of ActVA-Orf6 proved straightforward. A purification scheme involving ammonium sulfate fractionation, gel filtration on a Sephacryl S-300-HR column, and ion exchange on a Q-Sepharose anion exchange column afforded protein that was homogeneous as judged by SDS-PAGE (Fig. 2). The purified protein was stable when stored at  $-20^{\circ}\text{C}$  in the presence of 20% glycerol. Typically, 1 liter of culture yielded 9 mg of purified protein; the overall purification was 20-fold, and the recovery (based on ActVA-Orf6 activity) was greater than 90%. The specific activity of the purified protein was 0.2  $\mu\text{mol}/\text{min}/\text{mg}$ .

**Physical characterization of the ActVA-Orf6 protein.** ActVA-Orf6 prepared from *E. coli* was colorless and, like TcmH, showed no evidence of any bound chromophoric cofactors, such as heme or flavin. The subunit molecular size determined by SDS-PAGE was approximately 12 kDa, close to the weight deduced from the DNA sequence. The identity of the protein was confirmed by automated N-terminal sequencing of the first 10 amino acid residues. Sequencing also showed that the N-terminal methionine residue had been posttranslationally cleaved. The relative molecular weight of the protein, determined by electrospray mass spectrometry, was  $11,968.88 \pm 2.1$ , in excellent agreement with the calculated  $M_r$  of 11,968.40 for the ActVA-Orf6 protein from which the N-terminal methionine has been removed.

Initial estimations of the native molecular weight were made by gel filtration on a calibrated Superose-12 FPLC column equilibrated with 50 mM Tris-HCl (pH 7.4 containing 1 mM EDTA and 10% glycerol and yielded an apparent  $M_r$  of  $\approx 35,000$  for ActVA-Orf6. This suggested a trimeric structure, consistent with the results obtained for the homologous protein TcmH. However, when the native molecular weight was measured by analytical ultracentrifugation, the weight-average molecular weight,  $M_{r,w}$ , was  $19,000 \pm 3,000$ . This suggests that ActVA-Orf6 is more likely to be a dimer, probably in rapid

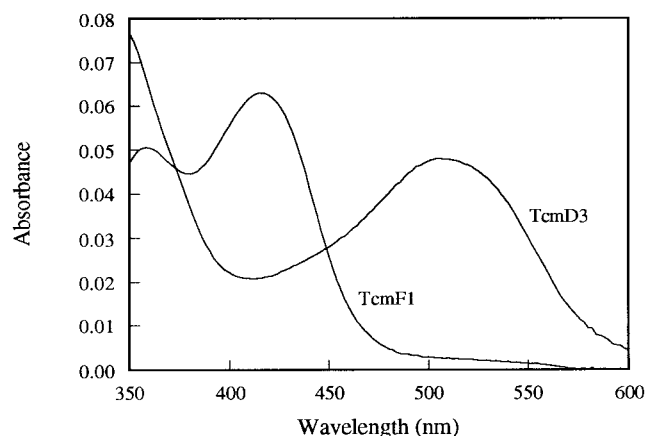


FIG. 3. UV-visible spectra of TcmF1 and TcmD3 in 0.1 M potassium phosphate buffer, pH 7.5. In oxygenated buffer, TcmF1 was converted to TcmD3 by incubation with ActVA-Orf6 for 10 min at 30°C.

equilibrium with the monomer form. We conclude that the discrepancy in the observed native molecular weights may be due to the protein running anomalously on the gel filtration column and that ActVA-Orf6 is most likely predominantly dimeric in the native state.

**Substrate specificity and kinetic analysis.** Purified ActVA-Orf6 catalyzed the oxidation of yellow TcmF1 to red TcmD3, whose formation could be followed by monitoring the increase in absorbance at 490 nm (Fig. 3). To confirm that the substrate TcmF1 was converted to TcmD3, the products were analyzed by thin-layer chromatography (CHCl<sub>3</sub>/methanol/acetic acid ratio, 85:15:0.25 [vol/vol/vol]); the observed  $R_f$  values for TcmF1 (0.37) and TcmD3 (0.28) are in close agreement with those reported previously (28). Monooxygenase activity depended solely on the presence of substrate TcmF1 and molecular oxygen; the enzyme did not require any exogenously added cofactors or metal ions.

The steady-state kinetic properties of ActVA-Orf6 were investigated. Assays were performed at 30°C in 0.1 M sodium phosphate buffer, pH 7.5. The oxidation of TcmF1 was well described by Michaelis-Menten kinetics; the  $K_m$  for TcmF1 was  $4.8 \pm 0.9 \mu\text{M}$ . The maximal velocity of ActVA-Orf6 was calculated as  $0.2 \mu\text{mol}/\text{min}/\text{mg}$ , which gives a value for  $k_{\text{cat}}$  of  $2.5 \text{ min}^{-1}$ . These values are close to those reported for oxidation of TcmF1 by TcmH (28) and indicate that the two enzymes have similar kinetic properties. The pH dependence of enzyme activity was investigated. Measurements were made in 50 mM potassium phosphate buffer at a range of pH values between 6 and 8. ActVA-Orf6 displayed optimal activity between pH 7.5 and 8.0, with a midpoint at about pH 7.0 (Fig. 4). The dependence of activity on pH suggests that an active-site base is important for catalysis and/or binding.

**The role of histidine residues in ActVA-Orf6.** Chemical modification studies on TcmH protein have previously indicated that a histidine or cysteine residue, or both, was important for catalysis (28). TcmH protein contains 6 histidine and 2 cysteine residues that could potentially be responsible for the loss of activity observed when the enzyme is treated with modifying reagents, whereas ActVA-Orf6 contains only 2 histidine residues and no cysteine residues. We therefore investigated the role of histidine in catalysis by site-directed mutagenesis. Both His-26 and His-52 were mutated separately to glutamine, and the double mutant, H26Q-H52Q, was also constructed. His-52 was mutated by using the "mismatched oligonucleotide" tech-

nique to direct second-strand synthesis on a single-stranded template according to standard protocols to give the construct pActVA-Orf6-H52Q. His-26 was mutated by a PCR-based strategy in which the 5' portion of the *actVA-Orf6* gene was amplified and the desired mutation was incorporated within the internal primer, as described in Materials and Methods. Subcloning of this fragment into pActVA-Orf6 gave the construct pActVA-Orf6-H26Q, whereas subcloning of the fragment into pActVA-Orf6-H52Q gave the double mutant ActVA-Orf6-H26Q-H52Q.

**Purification of mutant ActVA-Orf6 proteins.** ActVA-Orf6-H26Q was expressed as soluble protein, and its purification followed almost exactly the protocol devised for the purification of recombinant ActVA-Orf6. However, to obtain homogeneous protein, a further purification step using FPLC on a Mono-Q anion exchange column was necessary. In contrast, ActVA-Orf6-H52Q and ActVA-Orf6-H26Q-H52Q proteins were expressed predominantly as inclusion bodies; presumably, His-52 is important to the *in vivo* folding pathway. Lowering the temperature at which the protein was expressed and decreasing the concentration of the inducer, IPTG, failed to give soluble protein. Consequently, these proteins were purified as inclusion bodies, denatured in guanidinium hydrochloride, and refolded. The majority of the protein solubilized by guanidinium hydrochloride refolded as soluble protein when the denaturant was removed by dialysis. In both cases the mutant ActVA-Orf6 proteins comprised >90% of the total refolded protein and, once refolded, were stable and showed no tendency to precipitate. Final purification was achieved by FPLC on a Mono-Q anion exchange column and produced electrophoretically pure material.

CD spectroscopy was used to verify that, after refolding from inclusion bodies, ActVA-Orf6-H52Q and ActVA-Orf6-H26Q-H52Q were in a native-like conformation. The CD spectra of these proteins (Fig. 5) displayed minima at 208 and 222 nm, characteristic of folded proteins. The spectra of the mutant proteins are very similar to that of ActVA-Orf6 prepared from the soluble cell extract. Whereas small differences between the structures of the wild-type and refolded mutant proteins cannot be ruled out, the CD spectra are consistent with the mutant proteins having refolded correctly. This implies that production of these proteins as inclusion bodies is a kinetic effect caused by an alteration of the *in vivo* folding pathway by mutation of His-52.

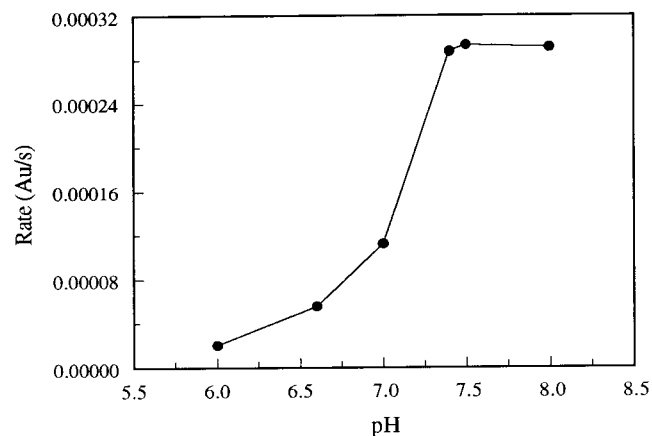


FIG. 4. pH dependence of ActVA-Orf6 monoxygenase activity measured in 50 mM potassium phosphate buffer at various pHs and 20  $\mu\text{M}$  TcmF1. Au, absorbance units.

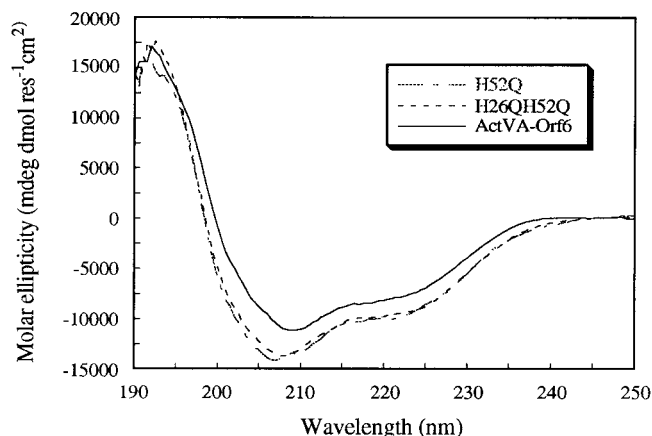


FIG. 5. CD spectra of wild-type and refolded mutant ActVA-Orf6 proteins. The spectra indicate that the mutant proteins have refolded correctly to a native-like structure. Molar ellipticity is expressed as millidegrees per decimals of residue per square centimeter.

**Activity of the mutant enzymes.** The mutant proteins were assayed for monooxygenase activity by using TcmF1 as the substrate. ActVA-Orf6-H26Q was active and exhibited kinetic properties comparable to those of the wild-type enzyme. The  $K_m$  for ActVA-Orf6-H26Q, at  $14.6 \pm 3.3 \mu\text{M}$ , was somewhat higher than that for the wild type, although  $k_{\text{cat}}$  was essentially unchanged at  $2.6 \text{ min}^{-1}$ . In contrast, ActVA-Orf6-H52Q and ActVA-Orf6-H26Q-H52Q showed less than 5% of the activity of the wild-type enzyme. This suggests that His-52, although not essential, plays an important role in catalysis. The low activity of these mutants precluded a detailed investigation of their kinetics. It was apparent, however, that there was an initial lag period, of 30 to 40 s, after addition of enzyme to the assay before any turnover was observed. This lag phase was not seen with the wild-type enzyme. Given the rather sluggish turnover of even the wild-type enzyme, this lag phase may be due to accumulation of an enzyme-bound intermediate.

## DISCUSSION

Our knowledge of the later, tailoring steps of actinorhodin biosynthesis, where comparisons with fatty acid biosynthesis cannot be drawn, remains rudimentary. Characterization of blocked mutants (4) has revealed two late intermediates, (*S*)-DNPA (Fig. 1) and dihydrokalafungin, that occur after the carbon skeleton has been formed. Two potential routes for conversion of (*S*)-DNPA to dihydrokalafungin have been proposed (Fig. 1) (4, 6). In the first, a 1,4 hydration of (*S*)-DNPA occurs, followed by dehydrogenation of the resulting semiquinone to give the naphthoquinone (4). Alternatively, reduction of the double-bond system to give the 1,8-dinaphthol compound shown in Fig. 1 (or its tautomer) may occur first, followed by oxidation to the naphthoquinone (6). In the absence of *act* mutant strains that secrete either putative intermediate, we used TcmF1, an established intermediate in the tetracenomycin biosynthetic pathway that is a substrate for the TcmH enzyme, to demonstrate the monooxygenase activity of ActVA-Orf6. Our results suggest that the biosynthesis of actinorhodin proceeds by the monooxygenation of a 1,8-dinaphthol intermediate, catalyzed by ActVA-Orf6. This would require that the pathway furnish an enzyme capable of reducing (*S*)-DNPA. ActVI-Orf2 and ActVI-Orf4 gene products, which show sequence similarity to enoyl reductases from bacteria, animals, and plants (6), are candidates for this activity.

ActVA-Orf6 protein displayed physical and kinetic properties very similar to those of the homologous TcmH enzyme. Preliminary results indicate that, in addition to catalyzing the oxidation of TcmF1, ActVA-Orf6 can catalyze the oxidation of anthracenones, such as 1,8-dihydroxy-3-carboxymethylanthrone, that incorporate minimal functionality (15a). This suggests a broad substrate specificity for these enzymes that should facilitate mechanistic studies of this unusual oxidation. Thermodynamically, oxygenation of aromatic rings is very favorable because it is coupled to reduction of the second oxygen atom to water. Kinetically, though, oxidation of TcmF1 is expected to be more facile than oxidation of the corresponding *act* intermediate because of the presence of the additional electron-rich phenolic ring system, and indeed TcmF1 undergoes a slow rate of spontaneous oxidation.

In the apparent absence of any cofactors (28) associated with biological oxidations, the mechanism for the oxygenation reactions catalyzed by TcmH and ActVA-Orf6 is unclear. It has been proposed (28) that TcmF1 is activated by TcmH to produce a phenolic radical intermediate species and a radical species on the enzyme. The latter would then react with molecular oxygen to form a peroxide radical that couples with the phenolic radical species to form an organic peroxide intermediate that is subsequently dehydrated. Alternatively, direct electron transfer from the substrate to molecular oxygen could occur, followed by bond formation between the radical intermediate and the superoxide radical anion.

TcmH is inactivated by reagents that react with cysteine and histidine (28). ActVA-Orf6 contains no cysteine residues, making it unlikely that thiols play a role in the mechanism. However, our experiments do support the idea that a histidine residue, His-52, plays an important mechanistic role, because mutants lacking this residue catalyze the reaction at only 5% of the rate of wild-type enzyme. Significantly, this is the only histidine residue that is conserved between ActVA-Orf6 and TcmH. One possibility is that this histidine residue may act as a general base and catalyze the dehydration of the putative peroxide intermediate in the second step of the reaction. The lag phase observed with the His-52 mutants may reflect accumulation of the peroxide intermediate on the enzyme.

In conclusion, we have demonstrated that the ActVA-Orf6 protein encodes the second example of an unusual class of monooxygenases that convert phenolic compounds, or their keto tautomers, to quinones. The relaxed substrate specificity of this enzyme suggests that potentially such tailoring enzymes could be used to further modify products from the polyketide synthetic or other biosynthetic pathways to generate "unnatural products." The mechanism of this oxidation is most intriguing because the enzyme appears to require no cofactors or metal ions; this will be the focus of future investigations.

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