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To cite this version:
Julien Valton, Carole Mathevon, Marc Fontecave, Vincent Nivière, David P Ballou. Mechanism and regulation of the Two-component FMN-dependent monooxygenase ActVA-ActVB from Streptomyces coelicolor. Journal of Biological Chemistry, American Society for Biochemistry and Molecular Biology, 2008, 283, pp.10287-96. <10.1074/jbc.M709730200>. <hal-01075299>

HAL Id: hal-01075299
https://hal.archives-ouvertes.fr/hal-01075299
Submitted on 7 Jan 2015
THE TWO-COMPONENT FMN-DEPENDENT MONOOXYGENASE ACTVA-ACTVB FROM STREPTOMYCES COELICOLOR: MECHANISM AND REGULATION*

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Running title: Mechanism and regulation of ActVA-ActVB monooxygenase system

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The ActVA-ActVB system from Streptomyces coelicolor is a two-component flavin-dependent monooxygenase involved in the antibiotic actinorhodin biosynthesis. ActVB is a NADH:flavin oxidoreductase that provides a reduced FMN to ActVA, the monooxygenase that catalyzes the hydroxylation of dihydrokalafungin, the precursor of actinorhodin. In this work, using stopped-flow spectrophotometry, we investigated the mechanism of hydroxylation of dihydrokalafungin catalyzed by ActVA and that of the reduced FMN transfer from ActVB to ActVA. Our results show that the hydroxylation mechanism proceeds with the participation of two different reaction intermediates in ActVA active site. First, a C(4a)-FMN-hydroperoxide species is formed after binding of reduced FMN to ActVA and reaction with O₂. This intermediate hydroxylates the substrate and is transformed to a second reaction intermediate, a C(4a)-FMN-hydroxy species. In addition, we demonstrate that reduced FMN can be transferred efficiently from the reductase to the monooxygenase without involving any protein-protein complexes. The rate of transfer of reduced FMN from ActVB to ActVA was found to be controlled by the release of NAD⁺ from ActVB and was strongly affected by NAD⁺ concentration, with an IC₅₀ of 40 μM. This control of reduced FMN transfer by NAD⁺ was associated with the formation of a strong charge-transfer complex between NAD⁺ and reduced FMN in the active site of ActVB. These results suggest that in Streptomyces coelicolor, the reductase component ActVB can act as a regulatory component of the monooxygenase activity by controlling the transfer of reduced FMN to the monooxygenase.

The flavin dependent monooxygenases are important enzymes that are involved in a wide variety of biological reactions. One of their fundamental functions uses their reduced flavins to activate molecular oxygen by generating flavin-hydroperoxide intermediates, the reactive species that oxygenate the substrate. Two different classes of flavin-dependent monooxygenases have been described. The first class comprises the well-known single-component flavoprotein monooxygenases, in which within a single polypeptide chain, the flavin cofactor is reduced by NAD(P)H and reacts with O₂ to generate the flavin-hydroperoxide species. Some of these flavoproteins have been investigated in great detail, p-hydroxybenzoate hydroxylase being the prototype of this class of flavoprotein monooxygenase (1). More recently, several two-component flavin-dependent monooxygenases, present in prokaryotic cells and involved in a broad range of oxygenation reactions, have been reported. These systems use two different proteins, a reductase for reducing the flavin and an oxygenase for binding the reduced flavin and...
catalyzing the reaction with O$_2$. Examples of two-component monooxygenases that use reduced FAD (FAD$_{red}$) as a cofactor are 4-hydroxyphenylacetate monooxygenase (HpaB) from *Escherichia coli* (2), phenol hydroxylase (PheA1) from *Bacillus thermoglucosidasefluorescens* (3) and styrene monooxygenase (StyA) from *Pseudomonas fluorescens* (4, 5). Two-component flavin-dependent monooxygenases that use reduced FMN (FMN$_{red}$) include enzymes involved in the biosynthesis of the antibiotic pristinamycin in *Streptomyces pristinaespiralis* (6, 7), in the utilization of sulfur from aliphatic sulfonates in *Escherichia coli* (8) and in the desulfurization of fossil fuels by *Rhodococcus* species (9), as well as the hydroxylation of 4-hydroxyphenylacetate in *Acinetobacter baumannii* (10). We have been investigating the mechanism of the FMN-dependent two-component enzyme system, ActVA-ActVB, which participates in the last steps of the biosynthesis of the antibiotic actinorhodin in *Streptomyces coelicolor* (11-13) (Scheme 1).

The first enzyme of the two-component flavin-dependent oxygenases is a NAD(P)H:flavin oxidoreductase that catalyzes the reduction of free oxidized flavins (FAD and/or FMN) by the reduced pyridine nucleotides, NADPH or NADH (3, 14-16). The second enzyme is an oxygenase that binds the resulting free reduced flavin and promotes its reaction with O$_2$ to hydroxylate the substrate (2, 3, 5-10, 17). Therefore, in contrast to the single-component flavin hydroxylases, the two-component systems catalyze the reduction of the flavin and the hydroxylation of the substrate on separate polypeptides. In the biosynthesis of actinorhodin the hydroxylase system consists of the reductase, ActVB, and the oxygenase, ActVA (14,15,17). There is now general agreement that the hydroxylation steps of flavin-dependent hydroxylases proceed through the reaction of the reduced flavin with molecular oxygen to generate a flavin C(4a)-hydroperoxide intermediate (18). This is followed by the transfer of an oxygen atom from the peroxide to the substrate.

Catalysis by the two-component flavin-dependent monooxygenases requires that the reduced flavin be transferred from the reductase to the monooxygenase without being oxidized by molecular O$_2$. This would appear to be a very challenging process because free reduced flavin becomes oxidized quite rapidly in the presence of O$_2$. Such an oxidation must be avoided or the reduction of the flavin and the ensuing hydroxylation reaction would effectively be uncoupled and, instead, NAD(P)H would be consumed to produce H$_2$O$_2$ and superoxide. We have previously demonstrated that with the ActVA-ActVB system from *S. coelicolor* the transfer of FMN$_{red}$ from the reductase to the oxygenase is thermodynamically favorable (17). Indeed the monooxygenase ActVA binds FMN$_{red}$ with a $K_d$ value of 0.39 µM and FMN$_{ox}$ with a $K_d$ value of about 20 µM, while the reductase ActVB binds FMN$_{ox}$ more tightly than FMN$_{red}$. There is also no evidence for specific binding interactions between the two components that might facilitate a channeling mechanism allowing the flavin to travel from one protein to another within a protein complex. These data suggest that in spite of the drawbacks associated with the simultaneous presence of free FMN$_{red}$ and O$_2$, FMN$_{red}$ is probably transferred between the two components by diffusion and rapid binding to the oxygenase during the hydroxylation reaction.

In this work, we have determined that FMN$_{red}$ can be transferred very efficiently from the reductase to the monooxygenase without the participation of any protein-protein complexes. In addition, our data show that in the *S. coelicolor* system the rate of transfer of FMN$_{red}$ from the reductase, ActVB to the hydroxylase, ActVA, is controlled by the release of NAD$^+$ from ActVB, and that the concentration of NAD$^+$ strongly affects this rate. This is manifest by the formation of a strong charge-transfer complex between NAD$^+$ and FMN$_{red}$ in the active site of ActVB.

**EXPERIMENTAL PROCEDURES**

**Materials.** NAD$,^+$, NADH, and FAD were from Sigma. FMN was prepared by conversion of FAD to FMN with snake venom from *Crotalus adamanteus* (19). DCPIP and menadione were from Sigma. 5-Deazaflavin (5-deaza-5-carba-riboflavin) was a gift from Dr. Philippe Simon (Grenoble, France). Enantiopure (+)-Kalafungin was synthesized by Dr. Rodney Fernandes and provided by Prof. Dr. Reinhard Brückner (Freiburg, Germany) (20). DHK (dihydrokalafungin), the non-lactonic analogue of (+)-kalafungin, was obtained from the reductive treatment of (+)-kalafungin by NaBH$_4$ as described in (21). A
solution containing 40 µM (+)-kalafungin in anaerobic 50 mM Tris-HCl buffer, pH 7.4, was mixed with ~7 equivalents of NaBH₄. The reaction was allowed to proceed for a few seconds under anaerobic conditions and the mixture was then opened to air. The formation of DHK was verified by UV-visible spectrophotometry and LC-MS/MS. Both techniques showed that (+)-kalafungin was efficiently transformed to DHK with a yield of ~100 % (data not shown). HPAH-C₂ from A. baumanii was provided by Dr. Jeerus Sucharitakul (Mahidol University, Thailand). Recombinant ActVA-Orf5 and His-tagged ActVB from S. coelicolor were overexpressed in E. coli and purified as described previously (14, 17).

Anaerobic procedures and rapid reaction experiments. All reactions were performed in 50 mM Tris-HCl buffer, pH 7.4, at 4°C. Solutions were made anaerobic in glass tonometers by ~20 cycles of vacuum and equilibration with Ar₂ gas that was depleted from oxygen contamination with an Oxiclear oxygen removal column (Labclear) as described previously (22). To minimize oxygen contamination of reaction mixtures due to diffusion through valves, etc, all anaerobic solutions contained an oxygen-scrubbing solution consisting in 12.5 to 25 µM protocatechuate (PCA, final concentrations) and ~0.06 units/ml of protocatechuate dioxygenase (PCD, final concentration). This mixture has been shown to maintain anaerobic conditions efficiently (23).

Rapid kinetics reactions were performed with a Hi-Tech Scientific stopped-flow Model SF-61DX instrument operating either in single or double mixing mode, and using either a diode array spectrophotometer or a photomultiplier detector. Kinetic traces were analysed and fitted with KinetAsyst 3 software (Hi-Tech Scientific, Salisbury, UK). Before each experiment, the stopped-flow instrument flow system was made anaerobic by incubating the flow system overnight with an oxygen scrubbing solution consisting of 100 mM PCA and 0.06 units/ml PCD in potassium phosphate buffer, pH 7.

Reaction of ActVA-FMN_red with O₂ in the presence or in the absence of the pyronaphthoquinone substrate DHK_red. A solution containing ActVA (50 µM), FMNox (20 µM), deazaflavin (60 nM), EDTA (10 mM) and catalase (50 nM) in 50 mM Tris-HCl buffer, pH 7.4, was made anaerobic in a glass tonometer as described above. FMNox was then photoreduced for 5 to 10 sec using a commercial 1000 W tungsten-halogen lamp placed ~10 cm away from the solution. The redox status of FMN was monitored by UV-visible spectrophotometry. When experiments were performed in the presence of DHK, the same reaction mixture as indicated above was used, but included the presence of 80 µM DHK. Both FMNox and oxidized DHK (DHK_red) were photoreduced together by the procedure described above. This solution was then mixed in the stopped-flow instrument with an equal volume of a solution containing various concentrations of oxygen, and the reaction was followed spectrophotometrically at various wavelengths, or by spectrofluorimetry with excitation at 390 nm and emission at wavelengths > 530 nm. Oxygenated solutions were prepared by equilibrating 50 mM Tris-HCl buffer for 30 min with air (21 % oxygen) or with certified oxygen/nitrogen gas mixtures (5, 10, 50, 100 % oxygen).

Investigation of the kinetics of FMN_red transfer. To study the transfer of FMN_red from ActVB to the monoxygenase (ActVA or HPAH-C₂), the ActVB-FMN_red complex was prepared. A solution containing ActVB (85 µM) and FMNox (20 µM) in Tris-HCl buffer, pH 7.4, in a glass tonometer equipped with a cuvette was made anaerobic as described above. The excess ActVB was to assure that most of the FMN was bound. The complex was then reduced by titration with an anaerobic solution of NADH delivered by a gastight Hamilton syringe connected to the tonometer via an Airless Ware fitting. Approximately 20 µM of NADH was sufficient to fully reduce the ActVB-FMN complex. This complex was then mixed in the stopped-flow instrument with an equal volume of Tris-HCl buffer containing ActVA (45 µM) and O₂ (600 µM), HPAH-C₂ from A. baumanii (45 µM) and O₂ (600 µM), oxidized DCPIP (2,6-dichlorophenolindophenol, 20 µM), or oxidized menadione (40 µM). The concentrations given are those in the syringes before mixing. The inhibition of FMN_red transfer from ActVB to HPAH-C₂ by NAD⁺ was investigated by a double mixing stopped-flow technique. The ActVB-FMN_red:NAD⁺ complex prepared as described above (235, 40
and 40 µM of each component respectively), was first mixed into an aging chamber with a solution containing various concentrations of NAD⁺ (from 0 to 5 mM). After a 5 s delay to assure that the binding of NAD⁺ had come to equilibrium, the solution from the first mix was mixed with an equal volume of 50 mM Tris-HCl buffer containing HPAH-C₂ (50 µM) and O₂ (255 µM), and the FMN_red transfer to HPAH-C₂ was followed spectrophotometrically at 386 and 700 nm to observe the formation of the C(4a)-FMN-OOH intermediate and the disappearance of the FMN_red–to–NAD⁺ charge-transfer complex.

RESULTS

Reaction of ActVA-FMN_red with O₂ in the absence of the pyronaphthoquinone substrate. Formation and decay of the C(4a)-FMN-OOH intermediate. It has been previously shown that ActVA is able to stabilize a C(4a)-FMN-OOH species within its active site, but the kinetics of its formation and decay were not determined (17, 24). Here, we have investigated the kinetics of the formation and decay of the C(4a)-FMN-OOH intermediate by stopped-flow spectrophotometry at 4°C, as described in Materials and Methods. An anaerobic solution of FMN_red (20 µM), containing an excess of ActVA (50 µM) to promote full complex formation (Kₕ_FMNRed = 0.39 µM, (17)), was mixed with equal volumes of Tris-HCl buffer solution containing various concentrations of O₂. The reaction was monitored by UV-visible spectrophotometry. As shown in Fig. 1A (thick line) the first spectrum recorded at 5 s ([O₂] = 595 µM after mixing) has a main absorption band at 386 nm. This spectrum is characteristic of a C(4a)-FMN-OOH species, such as those found in other single- and two-component flavin-dependent hydroxylases, including 4-hydroxyphenylacetate 3-hydroxylase (HPAH-C₂) from A. baumannii (22) and p-hydroxybenzoate hydroxylase (PHBH) from Pseudomonas aeruginosa (25, 26). Reaction traces monitored at 386 nm were fit to a single exponential model and the rate constants for formation of the absorbing species were found to be proportional to the O₂ concentration (Fig. 1B). The linear plot of k₁/₂ at 386 nm versus oxygen concentration yielded a second-order rate constant of k = 2.97 ± 0.06 x 10⁵ M⁻¹ s⁻¹ (Fig. 1C). These data are consistent with a bimolecular reaction between the reduced flavin bound to ActVA and oxygen to form the C(4a)-FMN-OOH intermediate.

The C(4a)-FMN-OOH then slowly converted to FMNₒₓ with a half-life of about 1400 s and was complete by ~1 hour, as seen from the appearance of the two characteristic bands of the oxidized flavin at 370 and 445 nm (Fig. 1A). The absorbance changes at 445 nm (Fig. 1B) were found to be independent of oxygen concentration. These data, which are consistent with our previous results from experiments at 25 °C, clearly show that ActVA strongly stabilizes the C(4a)-FMN-OOH intermediate (17, 24). At the end of this slow decay of the intermediate, 10 µM FMNₒₓ was detected (ε₄₄₅nm = 13 mM⁻¹ cm⁻¹), indicating that all of the initial FMN_red was quantitatively oxidized to FMNₒₓ by O₂. An ε value at 386 nm of 9.6 mM⁻¹ cm⁻¹ for the C(4a)-FMN-OOH species could then be calculated.

Interestingly, when free FMN_red was mixed with an air-saturated ActVA solution in the stopped-flow apparatus, kinetic traces were the same as those obtained when a preformed ActVA-FMN_red complex was mixed with the same concentration of oxygen (data not shown). This implies that almost no spontaneous oxidation of free FMN_red by O₂ occurred, even though autoxidation of free FMN_red by O₂ is a reasonably fast process. For example, 40 µM FMN_red is oxidized by 240 µM O₂, final concentration, in an autocatalytic reaction with a t₁/₂ ~0.5 s (27, unpublished data), and no detectable C(4a)-FMN-OOH species is produced (28). This result implies that binding of FMN_red to ActVA preceding the formation of the C(4a)-FMN-OOH species is fast and precludes any significant oxidation of free FMN_red by oxygen. These results explain and are in full agreement with our previous steady-state kinetics experiments (24).

Reaction of ActVA-FMN_red with O₂ and pyronaphthoquinone substrate. The reaction of O₂ with FMN_red bound to ActVA in the presence of its substrate, reduced dihydrokalafungin (DHKₒₓ), was investigated by stopped-flow spectrophotometry. An anaerobic solution containing 80 µM of DHK_red, 20 µM of FMN_red and an excess of ActVA (50 µM, to ensure that most of the FMN_red was in complex with ActVA) was mixed with equal volumes of oxygenated Tris-HCl buffer solution containing various amounts of O₂. In Figure 2, the reported data were observed with a final O₂ concentration of
595 µM. The reaction was followed at: (i) 365 nm, a wavelength that is useful for monitoring the formation of the C(4a)-FMN-OOH intermediate species (22, 29, 30) and at which DHK_{red} contributes to the absorbance minimally; (ii) 445 nm to monitor the formation of FMN_{ox}; (iii) 520 nm to monitor the formation of the hydroxylated DHK_{ox} product (DHK_{red}-OH) (24). In addition, fluorescence emission at 530 nm (excitation at 390 nm) was recorded in order to follow the formation of C(4a)-FMN-OH (hydroxy-FMN) and FMN_{ox} species. For many flavin-dependent hydroxylases, the C(4a)-FMN-OOH intermediate does not exhibit significant fluorescence emission upon excitation at 390 nm, whereas the C(4a)-FMN-OH species is often highly fluorescent (22, 29, 31). The absorbance values at the above mentioned wavelengths are plotted as a function of time in Fig. 2A. These data indicate that the reaction proceeds through at least four successive phases (Fig. 2A, Table 1). Each trace could be fitted by the sum of four exponential processes using a single set of rate constants for data at all wavelengths.

The rate constant $k_1$ for phase 1 was directly dependent on O$_2$ concentration (data not shown), indicating a bimolecular reaction with O$_2$. No FMN$_{ox}$ (445 nm trace) and almost no C(4a)-FMN-OH (fluorescence at 530 nm) were observed during this first phase. Thus, the bimolecular rate constant ($3.96 \pm 0.05 \times 10^4$ M$^{-1}$ s$^{-1}$) determined from the increase in absorbance at 365 nm is clearly associated with the formation of the C(4a)-FMN-OOH intermediate. This rate constant is only slightly different from that determined in the absence of the DHK$_{red}$ substrate (Fig. 1C, $2.97 \pm 0.06 \times 10^4$ M$^{-1}$ s$^{-1}$). In the subsequent phases, $k_2$, $k_3$, and $k_4$ were found to be independent of O$_2$ concentration (data not shown), indicating that they did not involve direct reactions with O$_2$.

In phase 2, the appearance of fluorescence at 530 nm (with excitation at 390 nm) reached a maximum at ~2 sec reaction time, with a rate constant of $1.48 \pm 0.15$ s$^{-1}$. Although there is only a small decrease in absorbance at 365 nm in this phase, the fluorescence data clearly reflect the hydroxylation reaction. Therefore, the above data are consistent with the formation of the C(4a)-FMN-OH intermediate species in phase 2, implying that hydroxylation of the DHK$_{red}$ substrate occurred during that phase. The trace at 520 nm does not show any increase in absorbance during this phase as might be expected for DHK$_{ox}$-OH. The immediate product is therefore most likely DHK$_{red}$-OH, which has very little absorbance or fluorescence (unpublished data), and is not expected to contribute significantly to the absorption and fluorescence data during phase 2.

Phase 3 is best indicated by the strong increase in absorbance at 445 nm characterized by a rate constant of $0.16 \pm 0.02$ s$^{-1}$. This was due to formation of FMN$_{ox}$, which is expected to arise from dehydration of the C(4a)-FMN-OH intermediate. The small decrease of fluorescence with an identical rate constant ($0.16 \pm 0.02$ s$^{-1}$) likely reflects the smaller fluorescence quantum efficiency of FMN$_{ox}$ (with excitation at 390 nm) as compared to that of C(4a)-FMN-OH, as has been shown for other flavin-dependent hydroxylases (29, 31).

Finally, phase 4 was mainly characterized by a slow increase of absorbance at 520 and 450 nm ($0.013 \pm 0.002$ s$^{-1}$), which most likely represents the oxidation by O$_2$ of the hydroquinone, DHK$_{red}$-OH, into the quinone, DHK$_{ox}$-OH. This conclusion was also consistent with the spectrum recorded 200 s after mixing (Fig. 2B) that has an additional band extending to 600 nm. This spectrum is similar to that of a mixture of ~10 µM FMN$_{ox}$ and ~10 µM DHK$_{ox}$-OH; the latter spectrum was presented in our previous work (24). Oxidation of hydroquinones and their derivatives such as DHK$_{red}$ by O$_2$ appears to proceed through an autocatalytic mechanism with phases that are only partially dependent on the concentration of O$_2$ (28, 32, 33). This is also the case for the reaction of O$_2$ with DHK$_{red}$, which becomes oxidized in an autocatalytic process with very little O$_2$-dependence (data not shown).

The kinetic path of hydroxylation of DHK$_{red}$ catalyzed by ActVA is summarized in Scheme 2. One interesting feature of this reaction is that the formation of the C(4a)- FMN-OOH intermediate, which in the presence of O$_2$ forms quickly after FMN$_{red}$ is transferred to the monoxygenase, has very little dependence on the presence of the DHK$_{red}$ substrate. This indicates that the substrate may bind after the hydroperoxide intermediate is formed, as observed with HPAH-C$_2$ (22), cyclohexanone monooxygenase (34), and microsomal flavin monooxygenase (35).
**FMN\textsubscript{red} transfer from ActVB to ActVA.** Our previous investigations have shown that transfer of FMN\textsubscript{red} from the reductase ActVB to the monooxygenase ActVA is thermodynamically favorable (17). To study the kinetics of this transfer reaction, we took advantage of the UV-visible signature of the ActVB-FMN\textsubscript{red}·NAD\textsuperscript{+} complex, which has a broad charge-transfer (C–T) band between 550 and 800 nm, as well as the formation of the C(a)-FMN-OOH intermediate that can be monitored at 380 nm. The C–T band originates from the interaction between NAD\textsuperscript{+} and FMN\textsubscript{red} in the ActVB active site (14). Our previous studies showed that, under anaerobic conditions, the addition of 1 equivalent of ActVA to the ActVB-FMN\textsubscript{red}·NAD\textsuperscript{+} complex resulted in total disappearance of the C-T band, implying that FMN\textsubscript{red} was transferred from ActVB to ActVA (17).

In Fig. 3, an ActVB-FMN\textsubscript{red}·NAD\textsuperscript{+} complex (85, 20, and 20 µM, of each component, respectively) was anaerobically prepared by adding one equivalent of NADH to FMN\textsubscript{red} in the presence of ActVB. Then, the complex was mixed at 4°C with an equal volume of solution containing 45 µM ActVA and 600 µM O\textsubscript{2} in a stopped-flow spectrophotometer. The UV-visible difference spectra (spectra at various times minus the initial spectrum of the ActVB-FMN\textsubscript{red}·NAD\textsuperscript{+} complex; this mode of display makes it easier to view the overall changes) were recorded as a function of time from 7 to 150 ms after mixing. Addition of the aerobic solution of ActVA to the anaerobic solution containing ActVB-FMN\textsubscript{red}·NAD\textsuperscript{+} resulted in total disappearance of the broad C–T band within ~1 s, shown in Fig. 3A by the loss of absorbance between 440 and 700 nm. Simultaneously, an absorbance band centered at ~386 nm appeared, due to formation of the C(4a)-FMN-OOH species. The presence of an isobestic point at about 435 nm indicated an apparent direct conversion of the ActVB-FMN\textsubscript{red}·NAD\textsuperscript{+} complex to the C(4a)-FMN-OOH-ActVA complex.

Fig. 3B shows the kinetics of the absorbance changes at 386 nm (formation of C(4a)-FMN-OOH) and at 700 nm (disappearance of the C–T complex ActVB-NAD\textsuperscript{+}·FMN\textsubscript{red}). Both traces could be fitted to a single exponential model with almost identical rate constants ($k_{386\text{nm}} = 4.6 \pm 0.1$ s$^{-1}$ and $k_{700\text{nm}} = 5.0 \pm 0.4$ s$^{-1}$). It can be noted that this rate constant is smaller than that for the formation of the C(4a)-FMN-OOH-ActVA complex from the reaction of free FMN\textsubscript{red} with O\textsubscript{2} in the presence of ActVA ($k_{386\text{nm}} = 10.6 \pm 0.1$ s$^{-1}$ for the reaction of 10 µM free FMN\textsubscript{red} mixed with 22.5 µM ActVA and 300 µM O\textsubscript{2} final concentrations, Fig. 1 and data not shown). Although these results suggest that under the experimental conditions of Fig. 3 formation of the C(4a)-FMN-OOH intermediate is rate-limited by the release of FMN\textsubscript{red} from ActVB, the relatively small difference in rates of these two processes tempers confidence in this conclusion. In order to further clarify the kinetics of dissociation of FMN\textsubscript{red} from ActVB, we performed the experiments described below.

The HPAH-C\textsubscript{2} monooxygenase from *A. baumanii* is part of a two-component FMN-dependent monooxygenase similar to ActVA. Recently, it has been shown that under similar conditions to those used for ActVA in the experiment shown in Fig. 1, HPAH-C\textsubscript{2} monooxygenase binds free FMN\textsubscript{red} and reacts with O\textsubscript{2} to form a C(4a)-FMN-OOH adduct very rapidly. The rate constant for binding FMN\textsubscript{red} to HPAH-C\textsubscript{2} has been estimated to be $> 10^7$ M$^{-1}$ s$^{-1}$ and the ensuing reaction with O\textsubscript{2} occurs with a second-order rate constant of 1.1 $10^6$ M$^{-1}$ s$^{-1}$ (22). Thus, if HPAH-C\textsubscript{2} is reacted with free FMN\textsubscript{red} in the presence of 300 µM O\textsubscript{2}, the formation of the C(4a)-FMN-OOH intermediate will occur with a rate constant of ~330 s$^{-1}$, a value about 30 times greater than that reported for ActVA. Substitution of HPAH-C\textsubscript{2} for ActVA in an experiment such as that in Fig. 3 would clearly distinguish whether the release of FMN\textsubscript{red} from ActVB is rate limiting. The experiment shown in Fig. 3 was repeated in the presence of HPAH-C\textsubscript{2} monooxygenase (25 µM, final concentration) in place of ActVA. As reported for the experiment with ActVA (Fig. 3), the ActVB-FMN\textsubscript{red}·NAD\textsuperscript{+} complex disappeared in parallel with the formation of the C(4a)-FMN-OOH intermediate (data not shown). The kinetics of the absorbance changes at 386 nm (formation of the C(4a)-FMN-OOH species) and at 700 nm (disappearance of the C–T complex ActVB-NAD\textsuperscript{+}·FMN\textsubscript{red}) could be fitted to a single exponential model, with rate constants (Table 2, $k_{386\text{nm}} = 4.7 \pm 0.1$ s$^{-1}$ and $k_{700\text{nm}} = 3.8 \pm 0.1$ s$^{-1}$) nearly identical to those obtained with ActVA (Table 2). These data are consistent with the limiting step for the transfer
of FMN\textsubscript{red} from ActVB to the monooxygenase component being the release of FMN\textsubscript{red} from the ActVB-NAD\textsuperscript{+}-FMN\textsubscript{red} complex.

Menadione and DCPIP are two redox dyes known to react very rapidly with reduced free flavin (36). When free FMN\textsubscript{red} is mixed with two equivalents of either one of these oxidized dyes, the FMN\textsubscript{red} becomes nearly fully oxidized during the dead time of the stopped-flow experiment (implying that $k_{\text{on}} > 300$ s\textsuperscript{-1}, Table 2). However, when ActVB-FMN\textsubscript{red}-NAD\textsuperscript{+} was mixed with either DCPIP or menadione under anaerobic conditions, the FMN\textsubscript{red} was oxidized with rate constants of $6.2 \pm 0.1$ s\textsuperscript{-1} and $6.1 \pm 0.1$ s\textsuperscript{-1} for DCPIP and menadione, respectively (Table 2). In addition, the C–T complex (measured at 700 nm) disappeared with rate constants (6.5 $\pm 0.1$ s\textsuperscript{-1} and 6.3 $\pm 0.1$ s\textsuperscript{-1} for DCPIP and menadione respectively, Table 2). These rate constants are more than 50-fold smaller than those obtained when free FMN\textsubscript{red} reacted with these dyes (Table 2). As shown in Table 2, the rates of disruption of the charge-transfer complex obtained in the presence of the monooxygenases ActVA or HPAH-C\textsubscript{2} and with the two dyes were almost identical. These results suggest that the rate of oxidation of the ActVB-NAD\textsuperscript{+}-FMN\textsubscript{red} complex by the dyes as well as by the oxygenases is likely to be primarily limited by the release of FMN\textsubscript{red} from the complex. The slightly greater rates obtained with the dyes compared to those obtained with the two oxygenases can be explained by the dyes reacting with the FMN\textsubscript{red} before it is fully released from ActVB, whereas the C(4a)-FMN-OOH species can only form after FMN\textsubscript{red} is completely released and transferred to the oxygenases.

Altogether, these data demonstrate that the formation of the C(4a)-FMN-OOH intermediate with the monooxygenases ActVA and HPAH-C\textsubscript{2} is limited by the release of FMN\textsubscript{red} from the ActVB-NAD\textsuperscript{+}-FMN\textsubscript{red} complex followed by its transfer to the monooxygenase.

\textit{Inhibition of FMN\textsubscript{red} transfer by NAD\textsuperscript{+}.}

Previous steady-state kinetics studies had shown that during the ActVB-catalyzed reaction (FMN\textsubscript{ox} + NADH $\rightarrow$ FMN\textsubscript{red} + NAD\textsuperscript{+}), the release of the NAD\textsuperscript{+} product occurs before that of FMN\textsubscript{red} (14). We tested whether the presence of NAD\textsuperscript{+} influences the transfer of FMN\textsubscript{red} from the ActVB-NAD\textsuperscript{+}-FMN\textsubscript{red} complex to the monooxygenase by carrying out experiments similar to those described in the previous section with various concentrations of NAD\textsuperscript{+}. In these experiments HPAH-C\textsubscript{2} was used in place of ActVA because, as mentioned earlier, HPAH-C\textsubscript{2} forms C(4a)-FMN-OOH very rapidly (22). Therefore, under these conditions, the formation of the C(4a)-FMN-OOH species provides a convenient and reliable coupled assay for monitoring the release of FMN\textsubscript{red} from ActVB.

As shown in Fig. 4, the ActVB-FMN\textsubscript{red}-NAD\textsuperscript{+} complex (235, 40, and 40 $\mu$M of the respective components), was anaerobically mixed with equal volumes of solutions containing various amounts of NAD\textsuperscript{+}. After 5 s (to allow the NAD\textsuperscript{+} binding to come to equilibrium), this solution was mixed with an equal volume of an aerobic solution of HPAH-C\textsubscript{2} (50 $\mu$M HPAH-C\textsubscript{2} and 255 $\mu$M O\textsubscript{2}), and the absorbance values at 386 and 700 nm of the final mixture were followed as a function of time (Figs. 4A and 4B respectively). Addition of increasing amounts of NAD\textsuperscript{+} resulted in decreases of the rates of both the loss of the C–T complex (700 nm) and the formation of C(4a)-FMN-OOH (386 nm). For a given NAD\textsuperscript{+} concentration, traces at both 386 and 700 nm could be fitted with a single exponential function with almost identical rate constants (Figs. 4A and 4B). As shown in Fig. 4C, a plot of the rate constants obtained from the traces at 386 and 700 nm versus NAD\textsuperscript{+} concentration indicated that the presence of an excess of NAD\textsuperscript{+} could totally inhibit the transfer of FMN\textsubscript{red} from ActVB to HPAH-C\textsubscript{2} as well as the formation of the C(4a)-FMN-OOH species. The IC\textsubscript{50} concentration for inhibition by NAD\textsuperscript{+} was determined to be $\sim$40 $\mu$M.

\textbf{DISCUSSION}

In this work, we have carried out a detailed kinetic study of the reaction of the two-component FMN-dependent monooxygenase, ActVA-ActVB from \textit{Streptomyces coelicolor}, by stopped-flow spectrophotometric methods in order to test our earlier propositions on the reaction mechanism of ActVA-ActVB system (17, 24) and to gain insights into its mechanism of regulation. Recent characterization of several members of the two-component flavin-dependent monooxygenase family has revealed similarities to but also some specific
differences from the well-studied single component flavoprotein monooxygenase family, such as \( p \)-hydroxybenzoate hydroxylase. In both types of systems, the monooxygenase activates the oxygen molecule by forming a \( C(4a) \)-flavin hydroperoxide intermediate that delivers an oxygen atom to the substrate. In contrast to the single component hydroxylases, the reduction of the flavin in the two-component flavin-dependent monooxygenase systems is catalyzed by a separate NADH oxidoreductase and the resultant reduced flavin must then be delivered to the oxygenase. Thus, the mechanism by which the reduced flavin cofactor is transferred from the reductase to the monooxygenase in the presence of \( O_2 \) represents one of the most challenging aspects of these systems. Here we have presented several experiments that elucidate the essential features of the mechanism of transfer of \( \text{FMN}_{\text{red}} \) between ActVB and ActVA. Compared with the few studies recently published on other two-component FMN-dependent monooxygenase systems, our results highlight a unique property of the ActVA-ActVB system, namely that the overall hydroxylase reaction is partially limited by the release of \( \text{FMN}_{\text{red}} \) from the reductase, and this release is regulated by the dissociation rate of the charge transfer complex between \( \text{NAD}^+ \) and \( \text{FMN}_{\text{red}} \). Large concentrations of \( \text{NAD}^+ \), a product of the flavin reductase reaction, can greatly reduce the rate of \( \text{FMN}_{\text{red}} \) release. Moreover, we show that no complexes between ActVB and ActVA are required for efficient transfer of \( \text{FMN}_{\text{red}} \) between the two.

At first, we studied the monooxygenase-dependent reaction in the absence of the reductase component ActVB and under conditions where the ActVA-FMN\(_{\text{red}}\)-DHK\(_{\text{red}}\) complex was formed before it reacts with \( O_2 \). We showed that the hydroxylation mechanism proceeds with the participation of two different reaction intermediates, a \( C(4a) \)-FMN-OOH that precedes, and a \( C(4a) \)-FMN-OH species that accompanies the formation of the reaction product, DHK\(_{\text{red}}\)-OH (Fig. 2). The \( C(4a) \)-FMN-OOH intermediate, characterized by a typical single absorbance band centered at 386 nm, results from the bimolecular reaction of the ActVA-FMN\(_{\text{red}}\) complex with \( O_2 \). This step occurs with a rate constant of \( -4 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \), essentially independent of the presence of the DHK\(_{\text{red}}\) substrate. We note that in the monooxygenase HPAH-C\(_2\) component from \( A. baumannii \), when C\(_2\) in complex with \( \text{FMN}_{\text{red}} \) and its substrate, 4-hydroxyphenylacetate (at 2 mM), is mixed with \( O_2 \), the rate of formation of the \( C(4a) \)-FMN-OOH intermediate is significantly smaller than when the substrate is absent or when the HPAH-C\(_2\)-FMN\(_{\text{red}}\) complex is mixed with a solution containing 4-hydroxyphenylacetate and \( O_2 \). Thus, it could be concluded that normally \( \text{FMN}_{\text{red}} \) binds first, then \( O_2 \) reacts to form the \( C(4a) \)-FMN-OOH, and finally, 4-hydroxyphenylacetic acid binds before hydroxylation takes place (22). For ActVA, with the substrate concentrations used in this study (0, 10 and 40 \( \mu \text{M} \) DHK\(_{\text{red}}\), Fig. 1C, data not shown and Fig. 2A, respectively), no effect of DHK\(_{\text{red}}\) on the kinetics of the reaction could be observed. However, because of the limited availability of the DHK substrate (it is not commercially available and has limited solubility), we could not determine if such an effect occurs with higher concentrations of DHK\(_{\text{red}}\) and therefore we do not know the preferred order of binding.

In the presence of DHK\(_{\text{red}}\), the \( C(4a) \)-FMN-OOH intermediate in the ActVA active site converted to a \( C(4a) \)-FMN-OH species, with a rate constant of \( 1.48 \text{ s}^{-1} \). Formation of the \( C(4a) \)-FMN-OH species results from transfer of the distal oxygen of \( C(4a) \)-FMN-OOH to the substrate, so that hydroxylation of DHK\(_{\text{red}}\) to give DHK\(_{\text{red}}\)-OH almost certainly occurred during that phase. In the subsequent step, the dehydration of the \( C(4a) \)-FMN-OH intermediate to form FMN\(_{\text{ox}}\) was observed at a rate of \( 0.16 \text{ s}^{-1} \) (Fig. 2 and Scheme 2).

The mechanism of DHK\(_{\text{red}}\) hydroxylation catalyzed by ActVA with participation of the two reaction intermediates demonstrated in this study (Scheme 2) appears to be quite similar to that recently determined for the HPAH-C\(_2\) component from \( A. baumannii \) (22), as well as that proposed for several single component hydroxylases (1, 18, 25, 28, 34, 35). These results reveal that, despite a rather low sequence identity between ActVA and HPAH-C\(_2\) (23 % identities), the monooxygenase components in the two-component flavin-dependent monooxygenase family use similar chemical mechanisms of hydroxylation.

In the absence of the substrate, the \( C(4a) \)-FMN-OOH intermediate forms rapidly (\( -3 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \)) and is remarkably stable, as previously observed (17, 24), and thus slowly converts to FMN\(_{\text{ox}}\) (and probably \( \text{H}_2\text{O}_2 \)) with a
half-life of ~1400 s. Formation of the hydroperoxide intermediate in the absence of substrate has also been observed for other members of the two-component flavin-dependent monooxygenase family, such as styrene monooxygenase from Pseudomonas Putida S12 (37), the HPAH-C$_2$ monooxygenase from A. baumannii (22) and bacterial luciferase (38). The recently solved X-ray structure of the HPAH-C$_2$ monooxygenase from A. baumannii in complex with FMN$_{red}$ has revealed the presence of a cavity adjacent to the isoalloxazine ring of the flavin molecule that is appropriate for harbouring the oxygen atoms of the C(4a)-FMN-OOH intermediate (39). It was proposed that this cavity provides a solvent-free environment that prevents rapid breakdown of the peroxide intermediate, thus accounting for its stability. In ActVA, one might expect an even more sequestered environment around the C(4a)-position of the flavin, because the C(4a)-FMN-OOH intermediate is about 75-fold more stable than in HPAH-C$_2$ (half-life of 1400 s for ActVA and 18 s for HPAH-C$_2$, this work and (22)). A sequence alignment between these two proteins shows that in HPAH-C$_2$ the amino-acid residues involved in the formation of the cavity near the C(4a)-position of the flavin are not totally conserved in ActVA. Presumably, these differences are responsible for the variations in stability of the peroxide intermediates.

Because in two-component FMN-dependent monooxygenases the reduction of the flavin takes place in a protein separate from the monooxygenase, FMN$_{red}$ must be transferred from the reductase to the monooxygenase in the presence of O$_2$ without being oxidized unproductively. This represents a rather challenging process. Autooxidation of FMN$_{red}$ can be toxic to the cell because it produces the harmful O$_2^+$ and H$_2$O$_2$ species, and in addition, is wasteful of reducing equivalents by effectively decoupling the reduction of the flavin by NADH and the hydroxylation reaction. We have previously shown that in the case of the ActVA-ActVB system such decoupling does not occur to any measurable extent (17, 24), but the mechanism by which it is avoided was not documented. The results of the present study now provide some insight into this mechanism.

i) First, we showed that ActVA binds free FMN$_{red}$ rapidly enough to prevent autooxidation of free FMN$_{red}$. Bruce and colleagues have determined that the initial rate of reaction of O$_2$ with free FMN$_{red}$ occurs at 250 M$^{-1}$ s$^{-1}$ (40). Thus, in the presence of 200 µM O$_2$, this initial rate should be 0.05 s$^{-1}$ and, even considering the ensuing autocatalytic oxidation process known for such reactions (28, 32, 40), very little net oxidation of free FMN$_{red}$ will occur within the first 100 ms (27). The formation of the C(4a)-FMN-OOH intermediate (Fig. 1C) occurs at a rate of 5.9 s$^{-1}$ ($t_{1/2}$ ~100 ms) whether FMN$_{red}$ is premixed with ActVA before or after mixing with O$_2$. Because the binding of FMN$_{red}$ to ActVA does not limit the rate of formation of the rate of formation of the C(4a)-FMN-OOH, binding must occur at a rate ≥ 60 s$^{-1}$ ($t_{1/2}$ ≤ 10 ms), and thus will be complete before any appreciable autooxidation of flavin happens.

This fast binding of FMN$_{red}$ to ActVA represents one of the key catalytic features of these systems where reduction of the flavin and activation of O$_2$ occur in two different polypeptides. In addition to avoid uncoupling reactions during FMN$_{red}$ transfer, this fast binding of FMN$_{red}$ to ActVA also allows the rationalization of why the formation of a complex between the reductase and the monooxygenase is not required for efficient oxygenation reactions.

ii) Second, we demonstrated that dissociation of NAD$^+$ from ActVB limits the release and transfer of FMN$_{red}$ from the reductase ActVB to the monooxygenase ActVA. We showed that the dissociation of FMN$_{red}$ from ActVB is tightly regulated by NAD$^+$, which must dissociate before FMN$_{red}$ can be released in the rate limiting step. A stable C–T complex between FMN$_{red}$ and NAD$^+$ within the active site of ActVB limits the dissociation rate of NAD$^+$. Accordingly, when the kinetics of the FMN$_{red}$ transfer between ActVB and ActVA was studied in the presence of increasing amounts of NAD$^+$, a strong inhibition of the transfer of FMN$_{red}$ to ActVA was observed. An IC$_{50}$ for NAD$^+$ of 40 µM was determined, and the inhibition of the FMN$_{red}$ transfer was almost complete in the presence of 1 mM NAD$^+$. Therefore in S. coelicolor, ActVB, the reductase component can act as a regulatory component of the monooxygenase activity by controlling the transfer of the FMN$_{red}$ cofactor to the monooxygenase.
Earlier steady-state kinetics studies showing that during ActVB catalysis, after the reduction of FMN<sub>ox</sub> by NADH via hydride transfer, the NAD<sup>+</sup> product is released before FMN<sub>red</sub> from the active site, in an ordered sequential mechanism (14). This confirms the notion that FMN<sub>red</sub> cannot dissociate from the ActVB active site when NAD<sup>+</sup> is bound. Then, according to Le Chatelier’s principle, an increase of NAD<sup>+</sup> concentration is likely to promote the formation of ActVB-FMN<sub>red</sub>-NAD<sup>+</sup> complex and thereby prevent the release of free FMN<sub>red</sub>. This is consistent with the experiments presented above showing strong inhibition of the transfer of FMN<sub>red</sub> from ActVB to ActVA by NAD<sup>+</sup>.

Control of the monooxygenase activity by NAD<sup>+</sup> is unprecedented in the two-component flavin dependent monooxygenase family. However, in most studies, high concentrations of NAD<sup>+</sup> were not present in reaction mixtures. Furthermore, few systems share with ActVB the observable rather stable flavin<sub>red</sub>-NAD<sup>+</sup> charge-transfer complex. PheA2, the reductase of a two-component phenol hydroxylase from Bacillus thermoglucosidasius, is one such example (3), and it is possible that similar control of the activity by NAD<sup>+</sup> also occurs in that case. Crystal structures show that NAD<sup>+</sup> binds directly over the FAD<sub>red</sub> in a position that is ideal for charge-transfer interactions (41). Recently, it was proposed that the activity of the monooxygenase component in the A. baumannii system was limited by the transfer of FMN<sub>red</sub> from the reductase, HPAH-C<sub>red</sub>, to the monooxygenase, HPAH-C<sub>ox</sub> (27). Furthermore, HPA, the monooxygenase substrate, was shown to function as an effector of HPAH-C<sub>red</sub> for both FMN<sub>red</sub> reduction and FMN<sub>red</sub> release (19, 27). Thus, as in the case of the ActVA-ActVB system, HPAH-C<sub>red</sub>, the reductase component, is likely to regulate the overall monooxygenase activity. However, in that case the effector is the substrate of the monooxygenase, HPA, whereas in ActVA-ActVB system it is NAD<sup>+</sup>, the product of the reductase.

Interestingly, it was also previously suggested that the oxygenases of the ActVA-ActVB system (24) and the 4-hydroxyphenylacetate hydroxylase (42) systems could be endowed with a regulatory function with respect to their reductases. In the absence of substrate, ActVA, traps free FMN<sub>red</sub> and converts it to a stable C(4a)-FMN-OOH intermediate. The consequence is that the cellular content of free FMN can become depleted, resulting in a shut-down of the reductase component activity. As highlighted by this work, the regulatory role of ActVB that is dependent on the intracellular concentration of NAD<sup>+</sup> adds another important piece to the puzzle of the dynamic regulation of the ActVA-ActVB monooxygenase activity in a cellular context. It might allow coordination of the production of the DHK<sub>red</sub>-OH (and consequently the formation of the actinorhodin) to the energetic state of the cell, as determined by the NADH/NAD<sup>+</sup> ratio. When this ratio is low in cells depleted of NADH, the FMN<sub>red</sub> would remain bound to the reductase component, preventing further oxidation of NADH and leading to the arrest of the hydroxylase activity.

It therefore seems that for the two-component monooxygenase systems there is a mutual regulation mechanism allowing the interpretation of different cellular signals for rapidly tuning the activities of each partner according to the demand and the status of the cell. In the case of the ActVA-ActVB system, ActVA could be perceived as the sensor of the intracellular concentration of DHK, whereas ActVB could be a sensor of the cellular energetic level indicated the NADH/NAD<sup>+</sup> ratio.

Finally, the results on the two-component flavin-dependent systems from A. baumannii (27) and S. coelicolor (17, 24 and this work) reveal that free diffusion of FMN<sub>red</sub> between two components in the presence of O<sub>2</sub> can be very efficient for supporting monooxygenation reactions. It is clear that single component flavin-dependent monooxygenases can coordinate and carry out both reduction of the flavin and hydroxylation of substrates. Therefore, the advantage of a system using separate reductases and a monooxygenases might be questioned. Because flavin reduction and oxygen-dependent reactions have very different requirements that are difficult to fulfil with a single polypeptide, dividing the tasks by using two different enzymes is a reasonable strategy to alleviate this challenge. In the case of the single component flavoprotein hydroxylases, coordinated dynamics between two different conformations that effectively provides two “active sites” has been shown to be important for dealing with this challenge (1, 18). This coordination is usually linked to the
substrate of the enzyme, so that adaptation to new substrates to trigger this dynamic dance of catalysis can be quite involved and complicated. Therefore, the two-component monooxygenase organization might represent an evolutionary advantage for quickly adapting to the metabolism of new substrates. The reductase and the monooxygenase polypeptides can acquire new complementary properties individually without modifying the structural requirements of the other component. This strategy more readily allows for variations to adapt to the hydroxylation of a broad range of substrates, as well as to different regulatory profiles. As pointed out above, even though the hydroxylation reactions occur with similar reaction mechanisms, the regulatory processes of the monooxygenase activity at the level of the reductase component in the case of the A. baumannii and S. coelicolor appear to be very different. Although such regulatory processes have not yet been explored for other two-component flavin monooxygenase systems, one might expect to find similar as well as new regulatory schemes for other systems. Consistent with this notion is the realization that two-component systems are continually being shown to be involved in the oxidation of an ever broader range of substrates.

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**FOOTNOTES**

We thank Dr. Jeerus Sucharitakul for providing HPAH-C₂ from *Acinetobacter baumannii*, Prof. Dr. Reinhard Brückner and Dr. Rodney Fernandes for providing enantiopure (+)-Kalafungin, and L. David Arscott, Dr. Sumita Chakraborty and Dr. Mike Tarasev for fruitful discussions and technical assistance.

The abbreviations used are: C(4a)-FMN-OOH, FMN C(4a)-hydroperoxide; DHK<sub>ox</sub>, dihydrokalafungin quinone form; DHK<sub>red</sub>, dihydrokalafungin dihydroquinone form; DHK<sub>red</sub>-OH, hydroxylated dihydrokalafungin dihydroquinone; HPAH-C₂, 4-hydroxyphenylacetate 3-hydroxylase; HPAH-C₁, 4-hydroxyphenylacetate 3-hydroxylase reductase component; DCPIP, 2,6-dichlorophenolindophenol; PCA, protocatechuate; PCD, protocatechuate dioxygenase; C(4a)-FMN-OH, FMN C4(a)-hydroxy.
**FIGURE LEGENDS**

**Figure 1.** Reaction of ActVA-FMNRred with O₂ at pH 7.4, 4°C. A solution containing ActVA (50 µM) and FMNRred (20 µM) in anaerobic 50 mM Tris-HCl buffer, was mixed in the stopped-flow instrument with an equal volume of oxygenated 50 mM Tris-HCl buffer, (O₂ was 595 µM after mixing). A. UV-visible spectra of the mixture (from the bottom to the top at 450 nm) recorded before and 5, 62, 180, 300, 480, 660, 930, 1200, 1860, 2400, and 4320 s after mixing. B. Same protocol, but performed in the presence of various concentrations of O₂ (60, 125, 299 and 595 µM after mixing). The absorbance traces at 386 nm as a function of time (dotted lines) are shown for each O₂ concentration. Solid lines are fits to a single exponential model. (♦) is the absorbance trace at 445 nm in the presence of 595 µM O₂, indicating the appearance of oxidized FMN. C. Plot of the pseudo first-order rate constants determined from the traces recorded at 386 nm shown in Fig. 1B as a function of O₂ concentration. A second-order rate constant of 2.97 ± 0.06 10³ M⁻¹s⁻¹ for the formation of the ActVA-C(4a)-FMN-OOH species was calculated from the slope of the straight line.

**Figure 2.** Reaction of ActVA-FMNRred complex with DHKred and O₂. An anaerobic solution containing ActVA (50 µM), FMNRred (20 µM), DHKred (80 µM), in 50 mM Tris-HCl buffer, pH 7.4, containing 10 % glycerol, was incubated for 20 minutes in the dark in the presence of PCA (50 µM), PCD (0.12 unit.mL⁻¹), EDTA (10 mM), and 5-deazaflavin (60 nM). The mixture was illuminated by white light until the FMN and DHK were fully reduced (5-10 s), and then was mixed at 4°C in the stopped-flow instrument with an equal volume of the same buffer containing oxygen (595 µM O₂). A. Traces of fluorescence (λexc=390 nm and λem= 530 nm) (□) and of absorbance at 365 (◇), 445 (○) and 520 nm (V) vs. time (dotted lines). The four reaction phases are demarcated by dashed vertical lines. The dependence of each individual phase on oxygen is indicated at the top of the figure. Solid lines are fits for multi-exponential models, with $k_1 = 3.96 ± 0.05$ x 10⁵ M⁻¹s⁻¹, $k_2 = 1.48 ± 0.15$ s⁻¹, $k_3 = 0.25 ± 0.03$ s⁻¹ for the 365 nm trace, $k_j = 0.16 ± 0.02$ s⁻¹ and $k_j = 0.013 ± 0.002$ s⁻¹ for the 445 and 520 nm traces and $k_2 = 1.48 ± 0.15$ s⁻¹, $k_3 = 0.16 ± 0.02$ s⁻¹, and $k_4 = 0.013 ± 0.002$ s⁻¹ for the fluorescence trace. For the sake of clarity, all the traces are shifted to be zero intensity at 5 ms. B. Same protocol, but monitored with a diode array spectrophotometer. Spectra shown are recorded vs. anaerobic buffer (○), and at 150 ms (△), 2 s (□), 200 s (◇) after the addition of O₂.

**Figure 3.** FMNRred transfer from ActVB to ActVA and formation of C(4a)-FMN-OOH. An anaerobic solution containing ActVB (85 µM), NAD⁺ (20 µM), FMNRred (20 µM), PCA (50 µM), and PCD (0.12 unit.mL⁻¹) was mixed in the stopped-flow instrument with an equal volume of oxygenated buffer containing ActVA (45 µM) and O₂ (600 µM) at 4°C. Both solutions were in 50 mM Tris-HCl buffer, pH 7.4, containing 10 % glycerol. Concentrations given are those before mixing. A. UV-visible difference spectra of the mixture (spectrum at a given time minus the initial spectrum) recorded from 7 ms to 1.5 s with a diode array spectrophotometer. The arrows indicate the direction of spectral evolution as a function of time. B. Absorbance traces (solid line) at 386 nm and 700 nm as a function of time (dotted lines). The four reaction phases are demarcated by dashed vertical lines. The dependence of each individual phase on oxygen is indicated at the top of the figure. Solid lines are fits for multi-exponential models, with $k_1 = 3.96 ± 0.05$ x 10⁵ M⁻¹s⁻¹, $k_2 = 1.48 ± 0.15$ s⁻¹, $k_3 = 0.25 ± 0.03$ s⁻¹ for the 365 nm trace, $k_j = 0.16 ± 0.02$ s⁻¹ and $k_j = 0.013 ± 0.002$ s⁻¹ for the 445 and 520 nm traces and $k_2 = 1.48 ± 0.15$ s⁻¹, $k_3 = 0.16 ± 0.02$ s⁻¹, and $k_4 = 0.013 ± 0.002$ s⁻¹ for the fluorescence trace. For the sake of clarity, all the traces are shifted to be zero intensity at 5 ms. B. Same protocol, but monitored with a diode array spectrophotometer. Spectra shown are recorded vs. anaerobic buffer (○), and at 150 ms (△), 2 s (□), 200 s (◇) after the addition of O₂.

**Figure 4.** Inhibition of FMNRred transfer from ActVB to HPAH-C₂ by NAD⁺. An anaerobic buffered solution containing ActVB (235 µM), NAD⁺ (40 µM), FMNRred (40 µM), PCA (50 µM), PCD (0.12 unit.mL⁻¹), was first mixed with an equal volume of the same buffer containing various concentrations of free NAD⁺ (0 to 5000 µM). After 5 s this solution was mixed with an equal volume of the same buffer solution containing HPAH-C₂ (50 µM) and O₂ (255 µM). All concentrations given are those before mixing. The buffer used was 50 mM Tris-HCl, pH 7.4, containing 10 % glycerol, 4°C. The absorbance traces at 700 nm (A) and 386 nm (B), for each concentration of NAD⁺ (10, 40, 125, 325, 625 and 1250 µM, concentrations after the second mix) are plotted as a function of time. The solid and smooth lines are fits for a single exponential model. C. The rate constants $k_\text{obs} 386 \text{ nm} (○)$ and $k_\text{obs} 700 \text{ nm} (▵)$ obtained from Figs. 4A and 4B are plotted as a function of NAD⁺ concentration. The concentration of NAD⁺ required to inhibit the rate of FMNRred transfer from ActVB to HPAH-C₂ by 50 % is 40 µM.
Table 1. Rate constants determined from multi-exponential fits for the 365, 445, and 520 nm absorbance and fluorescence traces of Fig. 2A. The $k_1$ (M$^{-1}$ s$^{-1}$) value is directly proportional to O$_2$ concentration, whereas $k_2$, $k_3$, and $k_4$ (s$^{-1}$) values are independent of O$_2$ concentration.

| Trace            | $k_1$ (M$^{-1}$ s$^{-1}$) | $k_2$ (s$^{-1}$) | $k_3$ (s$^{-1}$) | $k_4$ (s$^{-1}$) |
|------------------|---------------------------|------------------|------------------|------------------|
| 365 nm           | 3.96 ± 0.05 x 10$^3$      | 1.48 ± 0.15 s$^{-1}$ | 0.25 ± 0.03 s$^{-1}$ | /                |
| 445 nm           | /                         | /                | 0.16 ± 0.02 s$^{-1}$ | 0.013 ± 0.002 s$^{-1}$ |
| 520 nm           | /                         | /                | 0.16 ± 0.02 s$^{-1}$ | 0.013 ± 0.002 s$^{-1}$ |
| Fluorescence at 530 nm | /                         | 1.48 ± 0.15 s$^{-1}$ | 0.16 ± 0.02 s$^{-1}$ | 0.013 ± 0.002 s$^{-1}$ |

Table 2. Reactions of free FMN$_{\text{red}}$ and ActVB·FMN$_{\text{red}}$·NAD$^{+}$ with ActVA, HPAH-C$_2$, DCPIP, and menadione. Anaerobic solutions containing either ActVB (85 µM), NAD$^{+}$ (20 µM) and FMN$_{\text{red}}$ (20 µM) or solely FMN$_{\text{red}}$ (20 µM), as well as PCA (25 µM), and PCD (0.12 unit mL$^{-1}$) in 50 mM Tris-HCl buffer, pH 7.4, containing 10 % glycerol, were mixed with an equal volume of the same buffer containing either ActVA (45 µM) and O$_2$ (600 µM), HPAH-C$_2$ from Acinetobacter baumannii (45 µM), and O$_2$ (600 µM), oxidized DCPIP (20 µM), or menadione (40 µM). All reactions were carried out at 4°C and the concentrations given are those after mixing. The absorbance traces at 386 and 700 nm were recorded as a function of time (as in Fig. 3) and were fit with a single exponential model. The rate constants (s$^{-1}$) obtained from the traces at 386 (a), 450 (b) and 700 nm (c) are given below.

|                      | ActVA + O$_2$ | HPAH-C$_2$ + O$_2$ | ActVA, no O$_2$ | DCPIP | Menadione |
|----------------------|---------------|--------------------|-----------------|-------|-----------|
| Free FMN$_{\text{red}}$ | 10.6 ± 0.1$^a$ | 330$^a$ (22)       | /               | > 300$^b$ | > 300$^b$ |
| ActVB·FMN$_{\text{red}}$·NAD$^+$ | 4.6 ± 0.1$^a$ | 4.7 ± 0.1$^b$     | 6.6 ± 0.9$^c$  | 6.2 ± 0.1$^b$ | 6.1 ± 0.1$^b$ |
|                      | 5.0 ± 0.4$^c$ | 3.8 ± 0.1$^c$     | 6.5 ± 0.1$^c$  | 6.3 ± 0.1$^c$ |           |
Scheme 1. Hydroxylation reaction catalyzed by the two-component ActVA-ActVB system from *Streptomyces coelicolor*. The reductase ActVB provides FMN\textsuperscript{red} to the monooxygenase ActVA. The ActVA substrate (DHK\textsuperscript{red}), is the dihydroquinone form of dihydrokalafungin (DHK\textsuperscript{ox}). Formation of Actinorhodin requires an additional DHK\textsuperscript{red}−OH dimerization step that has not been characterized (24).

Scheme 2. Minimal catalytic scheme of the ActVA-catalyzed hydroxylation of DHK\textsuperscript{red}.

Scheme 3. Scheme of FMN\textsuperscript{red} transfer from reductase, ActVB, to the hydroxylase, ActVA. NAD\textsuperscript{+} controls the release of FMN\textsuperscript{red} from ActVB and therefore acts as a regulator of the hydroxylase activity.
Figure 1
Figure 3
Figure 4