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Determination of the Stereochemistry of Hydride Transfer from NADPH to FAD Catalyzed by VlmR, a Flavin Reductase from the Valanimycin Biosynthetic Pathway

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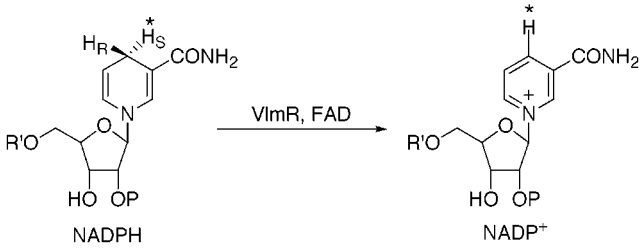
ABSTRACT



The stereospecificity of hydride transfer from NADPH to FAD catalyzed by VlmR, a flavin reductase from the valanimycin biosynthetic pathway has been determined. By using stereospecifically deuterated NADPH, it has been shown that the 4-pro *R* hydrogen of NADPH is transferred.

The antibiotic valanimycin is a naturally occurring azoxy compound produced by *Streptomyces viridifaciens* MG456-hF10. In addition to antibacterial activity, valanimycin exhibits potent cytotoxic activity against in vitro cell cultures of mouse leukemia L1210, P388/S (doxorubicin sensitive), and P388/ADR (doxorubicin resistant).[1] Valanimycin is a member of a growing family of naturally occurring azoxy compounds, which now includes the cycad toxins macrozamin and cycasin,[2−5] the carcinogen elaiomycin,[6−8] the antifungal agents maniwamycins A and B,[9] the nematocidal compounds jietacins A and B,[10] and the antifungal agent azoxybacilin.[11]

Previous investigations of valanimycin biosynthesis have established that the antibiotic is derived from L-valine and L-serine and that valine is converted into valanimycin via the intermediacy of isobutylamine and isobutylhydroxylamine (Scheme 1).[12] In addition, the conversion of isobutylamine to isobutylhydroxylamine was found to be catalyzed by a two-component flavin monooxygenase. The genes

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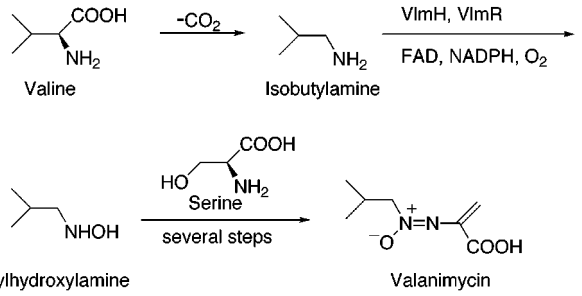
Scheme 1. Valanimycin Biosynthetic Pathway



(*vlmH*, *vlmR*) encoding the two components of this flavin monooxygenase have both been cloned from *S. viridifaciens* and overexpressed in *Escherichia coli*.[13,14] VlmR was found to be an NADPH:FAD oxidoreductase that catalyzes the transfer of hydrogen from NADPH to the FAD, yielding reduced FAD and $NADP^+$. Under aerobic conditions, the reduced FAD rapidly reacts nonenzymatically with molecular oxygen to produce hydrogen peroxide and the oxidized flavin.[14]

A BLAST analysis of the amino acid sequence of VlmR indicates that this protein is closely related to a small group of flavin reductases that appear to constitute a new flavin: NAD(P)H reductase subfamily.[14,15] Members of this family include ActVB, a flavin reductase from the actinorhodin biosynthetic pathway of *Streptomyces coelicolor*, HpaC, a flavin reductase component of the 4-hydroxyphenyl acetate 3-monooxygenase system of *E. coli*, and NtaB, the flavin reductase component of the nitrilotriacetate monooxygenase system from *Chelatobacter heintzii*. No three-dimensional structure is known for any of the proteins in this subfamily, and their evolutionary relationship to other families of flavin: NAD(P)H oxidoreductases is unclear. Furthermore, critical arginine residues that are involved in NADPH recognition and binding have been identified for other flavin oxidoreductases,[16,17] but these residues do not appear to be present in the VlmR subfamily.[14,15] To gain information concerning the active site of the VlmR subfamily of enzymes, we have investigated the stereospecificity of hydride transfer from NADPH to FAD catalyzed by VlmR. The results of these studies are described here.

(4*R*)-(4-$^2H_1$)-NADPH and (4*S*)-(4-$^2H_1$)-NADPH were synthesized enzymatically from [2-$^2H_1$]-2-propanol (98% D) and [1-$^2H_1$]-D-glucose (98% D), respectively, using the methodology of Ottolina et al.[18] Integration of the ^1H NMR spectra of the labeled cofactors revealed that the ratio of the 4-pro *S* hydrogen to the 4-pro *R* hydrogen in the (4*R*)-(4-$^2H_1$)-NADPH was ca. 76:24, while the ratio of 4-pro *S* hydrogen to the 4-pro *R* hydrogen in the (4*S*)-(4-$^2H_1$)-NADPH at C-4 was ca. 8:92.[19] VlmR was overproduced with an N-terminal His_6-tag in *E. coli* BL21(DE3) from plasmid pET28-(vlmRN1RC1).[14] The crude protein was purified to homogeneity by affinity chromatography on a TALON metal affinity column (CLONTECH).[14] The stereochemistry of the hydride transfer reaction was elucidated by incubation of purified VlmR (0.92 mg) in 400 mM ammonium bicarbonate buffer, pH 7.7, with 0.5 mM FAD, 1 U of catalase, and (4*R*)-(4-$^2H_1$)- or (4*S*)-(4-$^2H_1$)-NADPH at concentrations of 2.3 and 4.3 mM, respectively, at 24° C. The incubations were monitored by measurement of the decrease in NADPH absorption at 340 nm. Earlier studies had shown that VlmR catalyzes the complete reduction of FAD to $FADH_2$ in the presence of excess NADPH.[14] When there was no further decrease in NADPH absorption (ca. 10 min), each reaction mixture was evaporated to dryness below 40 °C, the residue was dissolved in 1 mL of D_2O, and the labeling pattern in the $NADP^+$ produced by the reaction was determined by ^1H NMR analysis. Table 1 summarizes the results of these analyses. The Table compares the relative areas of H-2 and H-6 in the pyridinium ring of the produced $NADP^+$ with that of H-4. It can be seen that the $NADP^+$ formed from (4*R*)-(4-$^2H_1$)-NADPH shows a preponderance of ^1H at the 4-position, while the $NADP^+$ derived from (4*S*)-(4-$^2H_1$)-NADPH shows a preponderance of ^2H. The reason for the apparent lack of complete stereospecificity in the hydride transfer reaction is not entirely clear, but it is worth noting that similar behavior has previously been observed in the reduction of FMN and FAD by the NAD(P)H:FMN reductase from the luminescent bacterium *Vibrio (Photobacterium) fischeri*.[20] The behavior of both VlmR and the *Vibrio* reductase might be a consequence of the fact that each of these proteins normally functions as part of a two-component system in which the aerobically unstable reduced flavin cofactor may be transferred directly from the reductase to the oxidase.[21] Thus, the formation of a complex between the reductase and oxidase components might be required for optimal binding of NADPH by the reductase. Our results demonstrate that the reduction of FAD by NADPH catalyzed

Table 1. Stereospecificity of the VlmR-Catalyzed Transfer of Hydride from (4*R*)-(4-$^2H_1$)- and (4*S*)-(4-$^2H_1$)-NADPH to FAD

substrate	% H at the C-4 position[a]
$NADP^+$ from (4*R*)-(4-$^2H_1$)-NADPH	91[b]
$NADP^+$ from (4*S*)-(4-$^2H_1$)-NADPH	25

[a] The %H at the C-4 position of $NADP^+$ was calculated by comparing the integrated peak area of the C-4 hydrogen atom with the averaged integrated peak area of the C-2 and C-6 hydrogen atoms. [b] The %H values have been corrected for the diastereotopic purity of the (4*R*)- and (4*S*)-(4-$^2H_1$)-NADPH. The measured values were 69% and 27%, respectively.

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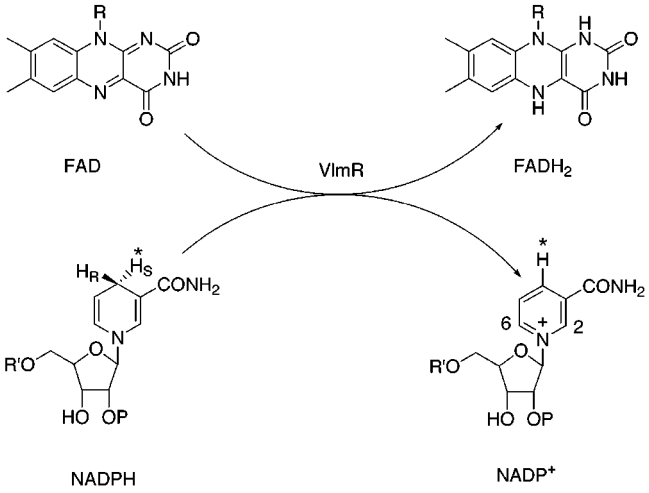
Scheme 2. Stereochemistry of Hydride Transfer from NADPH to FAD Catalyzed by VlmR



by VlmR proceeds with the transfer of the 4-pro *R* hydrogen atom (Scheme 2). The same absolute chemistry of hydride transfer has been observed for the *V. fischeri* reductase[20] and for several flavin monooxygenases that catalyze the hydroxylation of aromatic rings.[22] These observations suggest that the spacial relationships between the nicotinamide cofactor and the flavin cofactor are conserved in the active sites of all of these enzymes. This finding is of considerable interest since the VlmR subfamily of reductases does not exhibit significant sequence similarity to either the *Vibrio* flavin reductases or the flavoproteins that hydroxylate aromatic substrates.

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