Mechanism and Substrate Specificity of the Flavin Reductase ActVB from Streptomyces coelicolor*  

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ActVB is the NADH:flavin oxidoreductase participating in the last step of actinorhodin synthesis in Streptomyces coelicolor. It is the prototype of a whole class of flavin reductases with both sequence and functional similarities. The mechanism of reduction of free flavins by ActVB has been studied. Although ActVB was isolated with FMN bound, we have demonstrated that it is not a flavoprotein. Instead, ActVB contains only one flavin binding site, suitable for the flavin reductase activity and with a high affinity for FMN. In addition, ActVB proceeds by an ordered sequential mechanism, where NADH is the first substrate. Whereas ActVB is highly specific for NADH, it is able to catalyze the reduction of a great variety of natural and synthetic flavins, but with K_m values ranging from 1 μM (FMN) to 69 μM (lumiflavin). We show that both the ribitol-phosphate chain and the isoalloxazine ring contribute to the protein-flavin interaction. Such properties are unique and set the ActVB family apart from the well characterized Fre flavin reductase family.

NAD(P)H:flavin oxidoreductases or flavin reductases are enzymes defined by their ability to catalyze the reduction of free flavins, riboflavin, FMN, or FAD, by reduced pyridine nucleotides, NADPH, or NADH (1). Since flavins do not bind tightly to them, flavin reductases should not be classified as flavoproteins. What the enzyme does is to provide an active site that transiently accommodates both the reduced pyridine nucleotide and the flavin, close to each other, in such a relative orientation that the direct hydride transfer can be enormously accelerated (2, 3). The real biological function of the reduced flavins, the released products of the catalyzed reaction, is still not well understood. Free reduced flavins have been suggested to play an important role as redox mediators in iron uptake and metabolism in prokaryotes (4) or in light emission in bioluminescent bacteria (5, 6). More recently, a group of flavin reductases has been found to be essential in combination with flavin-dependent oxygenases (7–13), such as those involved in antibiotic biosynthesis, as discussed below (7–9).

Organisms have evolved a great variety of such enzymes, which can thus be classified within several families or subfamilies according to their sequence similarities and biochemical properties. Because of their simplicity and their variety, flavin reductases provide a unique tool to understand how a polypeptide chain deals with both the isoalloxazine ring and the ribitol chain of a flavin molecule to modulate its binding constant, to accelerate its reduction by reduced pyridine nucleotides, and to use it for a diversity of functions. Surprisingly, our knowledge of this class of enzymes is very limited so far, and this is the reason why flavin reductases have been the subject of intensive investigations in our laboratory in recent years (3, 14–17).

The prototype of one group of flavin reductases is the Fre enzyme found in Escherichia coli (18) and also in luminescent bacteria (19). The enzyme from E. coli consists of a single polypeptide chain with a molecular mass of 26 kDa. It uses both NADPH and NADH as the electron donor and a great variety of flavin analogues as electron acceptors (14, 17). This clearly demonstrates that the recognition of the flavin by the polypeptide chain occurs exclusively through the isoalloxazine ring, with very limited contribution of the ribitol side chain (3, 14). The crystal structure of Fre (3) reveals that the general enzyme structure is, despite very low sequence similarities, similar to the structures of a large family of flavoenzymes, with spinach ferredoxin-NADP^+ reductase as the prototype (20). It provides insights to the understanding of the structural basis for the difference in flavin recognition between a flavoprotein and a flavin reductase.

A second group of flavin reductases, different from the Fre family and the flavin reductases purified from bioluminescent bacteria, has recently emerged (7–13). However, very few members of this group were purified to homogeneity and carefully characterized. These enzymes are defined on the basis of their amino acid sequence similarities and their role during biological oxidation reactions. Indeed, some monooxygenase systems depend on the presence of a reduced flavin, mainly FMNH_2, as a co-substrate rather than a prosthetic group. The flavin is supposed to react with molecular oxygen in the active site of the monooxygenase component in order to generate a flavin hydroperoxide intermediate that serves as the active oxidant for substrate oxidation. A separate flavin reductase is thus absolutely required to supply the reduced flavins (with NADPH or NADH as the reductant) that diffuse to the oxygenase component. In recent years, the following flavin reductases have been shown to belong to this family: ActVB (7), SnaC (8), and VlmR (9) for the biosynthesis of the antibiotics actinorhodin in Streptomyces coelicolor, pristinamycin in Streptomyces pristinaespiralis, and valaninicin in Streptomyces viridifaciens; HpaC (10) for the oxidation of 4-hydroxyphenylacetate in E. coli; DszD (11) for the conversion of sulfides to sulfoxides and sulfones in Rhodococcus sp., allowing the utilization of these microorganisms in fossil fuel desulfurization biotechnological processes; and cB (12) for the degradation of nitroliotriacetate in Chelatobacter heintzii. It should be noted that a flavin reductase called FeR (21, 22), with some homology to ActVB and SnaC, found as a ferric reductase in the hyperthermophilic
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archaea Archaeoglobus fulgidus, has been structurally characterized in complex with FMN (22).

Considering the biological importance of this group of flavin reductases and the very limited amount of available information regarding their substrate specificity, reaction mechanisms, and three-dimensional structure, we found it worth characterizing these enzymes in more detail in order to compare them with the Fre enzyme and get new insights into the protein-flavin interaction. We have chosen ActVB as a representative of this group of flavin reductase and report original data showing that ActVB has a unique mode of flavin binding and operates by a sequential mechanism.

EXPERIMENTAL PROCEDURES

Enzyme Assay—In the standard aerobic assay, flavin reductase activities were carried out under aerobic conditions allowing continuous reoxidation of reduced flavin by oxygen. Flavin reductase activity was determined at 25 °C from the decrease of the absorbance at 340 nm (ε184 nm = 6.22 mm−1 cm−1) due to the oxidation of NADH, using a Varian Cary 1 Bio spectrophotometer. Under standard conditions, the spectroscopic cuvette contained, in a final volume of 500 µl, 50 mM Tris/HCl, pH 7.6, 100 µM NADH, and 50 µM FMN. The reaction was initiated by adding 0.5–1 µg of enzyme. Enzyme activities were determined for the linear part of the progress curve, with less than 10% of reduced pyridine nucleotide utilized over the time course of the reaction. One unit of activity is defined as the amount of protein catalyzing the oxidation of 1 µmol of NADH per min. When high concentrations of NADPH were investigated, a 0.1-cm path length cuvette was used (final volume 0.3 ml). The hydrophobic flavin analogues lumichrome, lumiflavin, and lumichrome were dissolved in 100% Me2SO, and the enzymatic assays thus contained 90 µM Me2SO, final concentration. Me2SO solutions up to 500 mM had no measurable effect on Km and Vmax values.

NAD(P) analogs and flavin concentrations were determined spectrophotometrically using the following extinction coefficients: AMP and ADP, ε260 nm = 15.4 mm−1 cm−1; (NAD)P, ε260 nm = 17.8 mm−1 cm−1; NADH, ε260 nm = 6.0 mm−1 cm−1.

ActVB Expression Plasmids—For the production of wild-type ActVB, pACTVB plasmid was used (7), where the actVB structural gene was placed under the control of the T7 polymerase promoter, in the pT7-7 plasmid. For the production of ActVB-His as C-terminal histidine-tagged protein, the actVB gene was amplified by PCR from the plasmid pACTVB (7) with the oligonucleotide primers GGGATCCCAATGCTGCGTCGACGAG and CGGGATCCCTCAATTGCTGGATGGTGATGGTACCGCGCCGTCGAC in order to introduce EcoRI, NdeI, and BamHI restriction sites (underlined) and the six histidine codons (in italic type). The 549-base pair PCR product was digested with EcoRI, NdeI, and BamHI, and the resulting fragment was ligated in pUC18 resulting in the plasmid pUC18-ActVB. This plasmid was sequenced to confirm that no changes had been introduced during PCR amplification. The NdeI-BamHI fragment from pUC18-ActVB was subsequently cloned in pT7-7, resulting in the plasmid pACTVB-His tag.

Preparation of Soluble Extracts—E. coli B384(DE3) pLyS8 transformed with the appropriate plasmid (pACTVB or pACTVB His tag) was grown at 37 °C and 220 rpm in a 3-liter Erlenmeyers flask containing 1 liter of Luria-Bertani medium in the presence of 200 µg/ml ampicillin and 34 µg/ml chloramphenicol. Growth was monitored by following the absorbance at 600 nm. Expression of ActVB and ActVB-His recombinant proteins was induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 250 µM when the optical density of the culture was about 0.3. To minimize the formation of insoluble protein aggregates, cultures were cooled to 25 °C after the addition of isopropyl-1-thio-β-D-galactopyranoside and then further grown at 4 °C. The cells were collected by centrifugation for 10 min at 15,400 × g at 4 °C. Extraction of soluble proteins was performed by lysozyme digestion and freeze-thawing cycles, in the presence of anti-protease Complete™ buffer. All of the following operations were performed at 4 °C. After ultracentrifugation at 45,000 rpm during 90 min in a Beckman 60 Ti rotor, the supernatant was used as soluble extracts for purification.

Purification of ActVB—The soluble extracts (130 mg) were loaded onto an ACA54 column (360 ml) previously equilibrated with 10 mM Tris/HCl, pH 7.6, 10% glycerol, 10 mM EDTA. Proteins were eluted with a flow rate of 0.3 ml/min. Fractions containing flavin reductase activity were pooled and concentrated to 2 ml using a Diaflow cell equipped with YM 10 membrane. The concentrated enzyme solution was loaded onto a Superdex 75 column (120 ml from Amersham Biosciences) equilibrated with 25 mM Tris/HCl, pH 7.6, 10% glycerol, 10 mM EDTA (buffer A). Proteins were eluted with the same buffer at a flow rate of 0.8 ml/min. Fractions containing flavin reductase activity were pooled and loaded onto a UNO Q column (6 ml, Bio-Rad), equilibrated with buffer A. A linear 0–500 mM NaCl gradient in buffer A was applied for 60 ml. ActVB was eluted with 100 mM NaCl.

Purification of ActVB-His—The soluble extracts (180 mg) were loaded at 0.5 ml/min onto a 25-ml Ni2+-nitrilotriacetic acid column (Qiagen) equilibrated with 50 mM Tris/HCl, pH 7.6 (buffer B). Then the column was washed with 100 ml of buffer B, and elution was achieved with the same imidazole in the same buffer at 1 ml/min. The proteins were then immediately loaded onto a UNO Q column (6 ml, Bio-Rad), and further eluted with a linear 0–500 mM NaCl gradient in buffer B for 60 ml at 1 ml/min.

Analytical Determination—SDS-PAGE polyacrylamide gels (15% polyacrylamide) were done according to Laemml (23). The gels were calibrated with the Amersham Biosciences low molecular weight markers. The native molecular mass of the protein was determined with a Superdex 75 gel filtration column (120 ml; Amersham Biosciences) equilibrated with 25 mM Tris/HCl, pH 7.6, and 150 mM NaCl using a flow rate of 0.4 ml/min. Bovine serum albumin (66 kDa), ovalbumin (45 kDa), trypsin inhibitor (20.1 kDa), and cytochrome c (12.4 kDa) were used as the markers for molecular mass. The void volume was determined with ferritin (450 kDa). Protein concentration was determined using the Bio-Rad protein assay reagent (24) with bovine serum albumin as a standard. Analytical experiments were carried out in a Jacomex glove box equipped with an HP 8453 diode array spectrophotometer coupled to the measurement cell by optical fibers (Photonics system).

Cofactor Analysis—A sample of pure ActVB or ActVB-His protein was boiled for 10 min in the dark, chilled on ice, and then centrifuged for 10 min at 10,000 × g in order to pellet the denatured protein. An aliquot of the supernatant was analyzed both by UV-visible spectrophotometry and by thin layer chromatography on silica gel 60 F254 (Merck) plate (15×10 cm) with Me2SO/H2O/CH3COOH/water (15/60/20/5) as the eluent. As a control, pure FMN, FAD, and riboflavin were run separately or as a mixture under the same conditions.

Kinetic Analysis—The molar concentration of ActVB was calculated assuming a molecular mass value of the polyepptide chain of 18,280 Da (7). Reciprocal initial velocities (1/V) were plotted against reciprocal substrate concentrations (1/[S]) and fitted with a straight line determined by a linear regression program. In some cases, kinetic parameters (Vmax, Km, Ki, Kcapp) were determined from saturation curves, fitted with the following equation: v = (Vmax[S]/(Km + [S])), using a Levenberg-Marquardt algorithm. Inhibition constants (K) for competitive inhibitors were determined using a secondary plot of the reciprocal slopes against the concentrations of the inhibitor (I), corresponding to the following equation: y = (K/Vmax) + (1 + ([I]/(K))25). In the cases of noncompetitive and uncompetitive inhibitors, the inhibition constant (K) was determined using a secondary plot of the intercepts from the double reciprocal plot against the concentration of the inhibitor (I), corresponding to the following equation: y = ([[I]/(K/Vmax)] + 1)/(1 /Vmax) (25). When applicable, values are shown ± S.D.

RESULTS

Purification of ActVB—In a first set of experiments, ActVB was overexpressed in E. coli using the pACTVB plasmid (7). Using the purification procedure described under “Experimental Procedures,” a low yield (6–7%) of purified ActVB could be obtained (Table I, top). This was then explained by the great instability of the flavin reductase activity in the soluble extracts. The activity, routinely assayed from the oxidation of NADH by an excess of FMN monitored spectrophotometrically, was found to decrease by 50% when the protein was left in buffer for 3 h at 4 °C. The addition of 10% glycerol, 10 mM EDTA, and Complete™ buffer solution to the soluble extracts provided a significant stabilization of the activity (data not shown). However, even under these conditions, more than 90% of the flavin reductase activity was lost during the first two chromatographic steps (Table I, top). After the UNO Q column, activity remained stable, suggesting that instability of ActVB activity in soluble extracts arose from reactions with some...
cellular components. SDS-PAGE analysis after the UNO Q purification step revealed the presence of two polypeptide bands at 18,000 and 17,000 Da (data not shown), with the same AADQGMLRDA N-terminal sequence corresponding to the ActVB protein (7). This suggested a partial C-terminal proteolysis of ActVB when expressed in E. coli, as described previously (7). In contrast, overexpression of ActVB as a C-terminal His-tagged fusion protein (ActVB-His) allowed a more efficient purification of the enzyme (Table I, bottom). SDS-PAGE analysis after the UNO Q column revealed the presence of only one polypeptide chain at 18,000, without evidence for partial proteolysis (data not shown).

Gel filtration experiments on a Superdex 75 column with ActVB and ActVB-His gave an apparent molecular mass of 36,000 Da for both proteins, confirming the homodimeric structure for ActVB (7).

Flavin reductase specific activities of both ActVB and ActVB-His proteins were found to be comparable (Table I), indicating that the C-terminal part of the protein lost during proteolysis was not important for activity and that the presence of the His tag was neutral with regard to the enzyme activity. However, it should be noted that from one preparation to another, we obtained purified proteins with slightly different specific flavin reductase activities.

In the following experiments presented here, we report data obtained with ActVB. In the case of the kinetic experiments, the same enzyme preparation was used, allowing a direct comparison of the parameters obtained under different kinetic conditions.

Flavin Content of ActVB—Purified ActVB was yellow, with absorption spectra typical for a flavin-containing protein, with maxima at 378 and 455 nm (Fig. 1). After denaturation of the protein by boiling for 10 min and centrifugation, the chromophore contained in the supernatant was analyzed by thin layer chromatography with FMN, riboflavin, and FAD as standards. The chromatophore was identified as FMN (data not shown). Quantification of the free FMN released in the supernatant by UV-visible spectroscopy demonstrated that the amount of FMN in purified ActVB varied from one preparation to another from 0.1 to 0.6 mol of FMN/mol of polypeptide chain. An extinction coefficient of 13,640 M⁻¹ cm⁻¹ at 455 nm, as a function of the [NADH]/[FMN] ratio.

Reconstitution experiments of ActVB with FMN or riboflavin gave the following results. A preparation of ActVB (50 μM) containing little FMN (0.1 mol of FMN/mol of protein) was incubated with 1 mM FMN or 400 μM riboflavin for 1 h in 50 mM Tris/HCl buffer, pH 7.6, and then chromatographed on an NAP10™ column (Amersham Biosciences) in order to remove unbound flavin. The protein was then analyzed both by UV-visible spectroscopy and thin layer chromatography, as previously described, in order to identify and quantify the bound flavin in the reconstituted ActVB. Reconstitution with FMN resulted in a 6–7-fold increase of the amount of protein-bound FMN and a protein containing 0.6–0.7 mol of FMN/mol of protein (data not shown). In contrast, reconstitution with riboflavin failed to increase the amount of protein-bound flavin and did not result in the removal of FMN initially bound to ActVB (data not shown).

Titration of the ActVB FMN Bound by NADH—In order to verify that the FMN bound to the isolated ActVB protein was correctly located at the flavin reductase active site, reductive titration of FMN was carried out with NADH, under anaerobic conditions. A preparation of ActVB containing 0.5 mol of FMN/
Flavin reductase activity of different ActVB preparations, containing various amounts of bound FMN, measured in the presence of NADH (200 μM) and FMN (100 μM) or riboflavin (100 μM) as substrates, in 50 mM Tris/HCl, pH 7.6

| Protein-bound FMN | Specific activity | \( \text{mol FMN/mol ActVB} \) | \( \text{μmol min}^{-1} \text{mg}^{-1} \) |
|-------------------|------------------|--------------------------|-----------------|
| 0.1               | 42               | 42                       |
| 0.3               | 39               | 45                       |
| 0.5               | 40               | 39                       |
| 0.6               | 42               | 41                       |

The dependence of the reaction catalyzed by ActVB on the concentration of both substrates, flavin and reduced pyridine nucleotide, was investigated by kinetic experiments under steady-state conditions. For all of the following kinetic experiments, the same homogeneous enzyme preparation was used. For such a bisubstrate-biproduct reaction, kinetic analysis provides insights into the mechanism. Double reciprocal plots of initial velocities \( v_i \) as a function of NADH concentration in the presence of 11 (○), 3.3 (△), 2.2 (●), 1.1 (□), 0.6 (▲), or 0.22 μM (▲) FMN. B, flavin reductase initial velocity \( v_i \) as a function of FMN concentration in the presence of 11 (○), 8.2 (△), 6.6 (●), 4 (□), 2 (▲), or 1.3 μM (●) NADH. The flavin reductase concentration \( e \) used in these assays was 0.0545 μM. The results are presented in the form of double reciprocal plots.

There were no significant changes in absorbance at 340 nm during reaction of ActVB-bound FMN with NADH. In addition, no significant increase in absorbance at 340 nm was observed, indicating that no NADH was formed during incubation of reduced FMN with NAD\(^+\). This shows that the reduction of FMN by NADH at the active site of ActVB is irreversible.

Flavin Reductase Activity Is Not Dependent on the Bound FMN—The previous experiments have shown that the purified protein contains various amounts of FMN bound at its active site, depending on the enzyme preparation. In order to investigate the dependence of the flavin reductase activity of ActVB on the amount of protein-bound FMN, different preparations containing various amounts of FMN were assayed either with FMN or riboflavin as a substrate and with NADH as the electron donor (Table II). In the absence of exogenous flavins, only a very weak activity could be detected (data not shown), excluding an NADH oxidase function for ActVB. With either FMN or riboflavin as a substrate, the flavin reductase activity of ActVB was found to be independent on the FMN content of the polypeptide chain (Table II). Since riboflavin does not bind to ActVB (see above), reconstitution for a flavin site during the enzymatic test is excluded. These observations thus rule out the possibility that the protein-bound FMN was involved in the flavin reductase activity and are consistent with ActVB being a flavin reductase accepting both FMN and riboflavin as substrates. It is likely that, under the assay conditions, the protein-bound FMN can be displaced by the large excess of riboflavin.

Kinetic Analysis of ActVB—The dependence of the reaction catalyzed by ActVB on the concentration of both substrates, flavin and reduced pyridine nucleotide, was investigated by kinetic experiments under steady-state conditions. For all of the following kinetic experiments, the same homogeneous enzyme preparation was used. For such a bisubstrate-biproduct reaction, kinetic analysis provides insights into the mechanism. Double reciprocal plots of initial velocities versus substrate concentrations show intersecting patterns in the case of a sequential mechanism and parallel patterns for a ping-pong
mechanism (25). Flavin reductase activity was determined as a function of NADH concentration at several levels of FMN (Fig. 4A) and as a function of FMN concentration at several levels of NADH (Fig. 4B). Initial velocities followed typical Michaelis-Menten kinetics, since double reciprocal plots of the data showed a series of lines. Moreover, a ping-pong mechanism could be excluded, since the lines were intersecting each other at the same point at the left of the vertical axis. A sequential mechanism for ActVB is thus indicated (25). However, whether it is an ordered or a random sequential mechanism cannot be concluded from such an experiment. It may be experimentally deduced from the inhibition studies described below (28).

When the enzyme activity was determined as a function of NADH concentration in the absence or in the presence of three concentrations of AMP (Scheme 1), a dead end inhibitor, double reciprocal plots revealed typical competitive inhibition kinetics (Fig. 5A). A $K_i$ value of $3 \pm 0.5$ mM was obtained for AMP. On the other hand, a pattern of noncompetitive inhibition was observed for AMP with respect to the FMN substrate, with a $K_i$ value of $7.7 \pm 0.4$ mM (Fig. 5B). Furthermore, lumichrome, a flavin analogue (Scheme 1) with no redox activity, was a competitive inhibitor with respect to FMN with a $K_i$ value of $104 \pm 17$ $\mu$M (Fig. 6A) and an uncompetitive inhibitor with respect to NADH, with a $K_i$ value of $91 \pm 1$ $\mu$M (Fig. 6B). All of these data support the conclusion that the flavin reductase has an ordered mechanism with NADH binding first and FMN being the second substrate (28).

The kinetic mechanism of product release has been determined by studying inhibition by products (25). When NADH concentration was varied with a fixed concentration of FMN, inhibition by NAD$^+$ was found to be noncompetitive with respect to NADH (data not shown). When FMN was varied at a fixed NADH concentration, NAD$^+$ appeared to inhibit noncompetitively (data not shown). This now suggests that the first product to be released is NADH, followed by the reduced flavin.

**Flavin Substrate Specificity**—Scheme 1 shows the structures of the various flavin analogs used as substrates or inhibitors. Table III reports the kinetic parameters for each substrate obtained during enzymatic reduction by NADH under steady-state conditions. The apparent $K_m$ values were determined in experiments where flavin reductase activities were measured as a function of the concentration of a given substrate in the presence of saturating concentrations of the other substrate. In addition, the $K_m$ and $k_{cat}$ values for the FMN substrate were determined from the data shown in Fig. 4, A and B, using the Dalziel treatment, which is well adapted for a sequential bireactant mechanism (16, 17, 29). The latter were found to be $0.78 \pm 0.10$ $\mu$M and $8.9 \pm 0.3$ s$^{-1}$, respectively, which are comparable with those reported in Table III.

As shown in Table III, $V_{max}$ values (reported as $k_{cat}$ values) were only slightly sensitive to modifications of the flavin moiety both at the isoalloxazine ring and the ribityl side chain. On the contrary, $K_m$ values for the flavin substrate strongly depended on the nature of the substituent at the N-10 position of the isoalloxazine ring. As previously reported in (7), FMN was found to be the best substrate, with a $K_m$ value of 1 $\mu$M. Riboflavin and FAD exhibited a 8-fold larger $K_m$ value, suggesting that the terminal phosphate group on the ribityl chain of the FMN molecule provided significant stabilization of the

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1 For a two-substrate enzyme, the $K_i$ values for an inhibitor with respect to each substrate are not necessarily equal.
flavin-protein complex. Lumiflavin, with a methyl group at the N-10 position, was also a substrate of ActVB, although its $K_m$ value was much larger than that for FMN, FAD, or riboflavin. This shows the importance of the isoalloxazine ring for the flavin-protein recognition, in agreement with lumichrome and alloxazine being competitive inhibitors of FMN, with $K_i$ values of 104 and 93 $\mu$M, respectively, could be obtained. The $K_m$ value for lumichrome and alloxazine was in the following order: derivative 1 > derivative 3 > riboflavin. Furthermore, in the case of compound 2, the $k_{cat}$ value was significantly decreased with regard to $k_{cat}$ values with derivative 3 and riboflavin. Those data suggest that the 2'-OH contributes to the protein-flavin recognition, whereas the 3'-OH plays a minor role. It should be noted that ribitol, up to 10 mM, is not an inhibitor (data not shown).

Reduced Pyridine Nucleotide Substrate Specificity—Table IV shows the kinetic and thermodynamic parameters for various NAD(P)H analogs (Scheme 1) obtained during enzyme reaction under steady-state conditions. The $K_m$ value for the NADH substrate was also determined from the data shown in Fig. 4, A ...
and B, using the Dalziel treatment (16, 17, 29), and found to be 9.7 ± 0.4 μM, which is comparable with the value reported in Table IV. As previously reported in Ref. 7, ActVB is strictly specific for NADH. NADPH cannot be used as a substrate, even at very high concentrations (up to 0.8 mM). The \( K_m \) value for NADH (6.6 ± 0.5 μM) (Table IV) was comparable with its \( K_d \) value (7.8 ± 0.7 μM) determined from the Dalziel mathematical treatment of the initial velocities data reported in Fig. 4, A and B (16, 17, 29). \( K_m \) values for AMP and ADP-ribose, which are competitive inhibitors with respect to NADH (Fig. 5A and data not shown, respectively), were very large (3 and 17.4 mM, respectively) (Table IV). In the case of a sequential ordered mechanism, the inhibition constant \( K_i \) for a competitive inhibitor with respect to the first substrate is equal to the dissociation constant of the enzyme-inhibitor complex (30). Comparison of the \( K_i \) values for AMP and ADP-ribose with the \( K_m \) value for NADH thus indicates that the nicotinamide ring plays a major role in the binding of the NADH molecule to ActVB. On the other hand, NMNH, the NADH analog lacking the AMP part of the molecule (Scheme 1), was neither a substrate nor an inhibitor, up to a concentration of 1 mM (Table IV). This now indicates that the nicotinamide ribose phosphate part of the NADH is not recognized by itself either. Taken together, these data suggest that the NADH molecule is recognized by ActVB as a whole and that individual contributions of the different parts of the molecule cannot provide enough interactions unless they are associated with the other parts of the NADH molecule.

**DISCUSSION**

The enzyme named Fre can be considered as a prototype of a whole class of flavin reductases, the enzymes catalyzing the reduction of riboflavin, FMN, and FAD by NADPH and NADH (14). This is the most extensively characterized flavin reductase so far (2, 3, 14, 16–18). Kinetic analysis and structural characterization in our laboratory have shown that this enzyme binds the flavin, with a preference for riboflavin, almost exclusively through interactions with the isoalloxazine ring (14) and reduced pyridine nucleotides, preferably NADPH, mainly through interactions with the nicotinamide moiety (17). These are unique binding modes. The enzyme proceeds by a sequential ordered mechanism, binding the NADPH first and providing a site that accommodates both substrates in a ternary complex from which the optimized hydride transfer occurs (2, 14).

ActVB belongs to a different and much less well characterized group of flavin reductases. These enzymes are generally associated with flavin-dependent monoxygenases and have no sequence homology with the Fre family (7–13). Here we report a detailed study of ActVB that allows comparison with Fre. As discussed below, we show that ActVB is different from Fre also with respect to substrate specificities and recognition.

The first question we have addressed is whether or not ActVB is a flavoprotein. Previous reports had not provided a clear answer to that question, and furthermore in our hands preparations of ActVB had highly variable amounts of protein-bound FMN. Furthermore, whereas some members of the ActVB family, such as HpaC (10), were isolated without bound cofactors, others, such as SnaC (8), were shown to contain significant amounts of flavin bound to the polypeptide chain after purification. The presence of FMN in ActVB could be explained either by a tight interaction between ActVB and FMN, allowing isolation of the flavin reductase with its substrate, or by a loss of a prosthetic FMN group from a flavoprotein during purification. It should be noted that some flavoproteins, such as sulfite reductase, may display some flavin reductase activity (15). However, in this case, the internal flavin cofactor mediates an unspecified electron transfer from NAD(P)/H to the exogenous flavin substrate as well as other acceptors (quinones, ferric iron, cytochrome c, etc.) and thus proceeds through a ping-pong mechanism (15).

The results presented here unambiguously demonstrate that

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### Table III

**Apparent kinetic constants for various flavin derivatives with NADH as electron donor**

| Flavin derivative | Properties | \( K_m \) \( \mu \text{M} \) | \( k_{cat} \) \( \text{s}^{-1} \) | \( K_i \) \( \mu \text{M} \) | \( K_d \) \( \mu \text{M} \) |
|-------------------|------------|--------------------------|-----------------|-----------------|-----------------|
| Riboflavin       | Substrate  | 8.1 ± 0.9                | 13 ± 0.6        |                 |                 |
| FMN              | Substrate  | 1.0 ± 0.1                | 9.2 ± 0.4       |                 |                 |
| FAD              | Substrate  | 8.7 ± 0.6                | 8.2 ± 0.7       |                 |                 |
| Lumiflavin       | Substrate  | 69 ± 8                   | 9.9 ± 0.3       |                 |                 |
| 1                | Substrate  | 3.7 ± 0.4                | 8.1 ± 0.2       |                 |                 |
| 2                | Substrate  | 15.7 ± 2.5               | 3.6 ± 0.1       |                 |                 |
| 3                | Substrate  | 11.4 ± 0.8               | 7.2 ± 1.3       |                 |                 |
| Lumichrome<sup>b</sup> | Inhibitor Competitive/FMN | 104 ± 17 | 87 ± 14 |                 |                 |
| Alloxazine<sup>c</sup> | Inhibitor Competitive/FMN | 93 ± 19 | 78 ± 18 |                 |                 |

<sup>a</sup> The concentration of NADH used for the determination of \( K_m \) and \( k_{cat} \) values was 200 μM.

<sup>b</sup> From the data of Fig. 6A.

<sup>c</sup> Experimental conditions as in Fig. 6A, except for alloxazine concentrations (0, 40, 90, 130, and 170 μM).

### Table IV

**Apparent kinetic and thermodynamic constants for NADH derivatives with FMN as electron acceptor**

| NADH derivative | Properties | \( K_m \) \( \mu \text{M} \) | \( K_d \) |
|-----------------|------------|--------------------------|---------|
| NADH            | Substrate  | 6.6 ± 0.5                | 7.8 ± 0.7 |
| AMP<sup>b</sup> | Inhibitor competitive/NADH | 3000 ± 500 |         |
| ADP-ribose<sup>c</sup> | Inhibitor competitive/NADH | 17,400 ± 4000 |         |
| NADPH           | Not a substrate up to 0.8 mM |                 |         |
|                 | Not a substrate up to 1 mM |                 |         |
| NMNH            | Not an inhibitor up to 1 mM |                 |         |

<sup>a</sup> The concentration of FMN used for the determination of the \( K_m \) and \( k_{cat} \) values was 50 μM. \( k_{cat} \) value was 9.2 s<sup>–1</sup>.

<sup>b</sup> From the data of Fig. 5A.

<sup>c</sup> Experimental conditions as in Fig. 5A, except for ADP-ribose concentrations (0, 0.8, 2, and 6 mM).
ActVB is not a flavoprotein but a flavin reductase displaying a low $K_d$ value for FMN. First, with all substrates, FMN, riboflavin, and FAD, the kinetic data fit nicely with a sequential and not with a ping-pong mechanism (Fig. 4). Second, FMN and riboflavin reductase activities do not depend on the amount of FMN bound to the protein used for the assay (Table II). Third, ActVB cannot bind more than one equivalent of FMN as shown by reconstitution experiments, in agreement with the presence of only one FMN binding site. Furthermore, this site is directly accessible to NADH, where electron transfer can occur as shown from the formation of a charge transfer complex (Fig. 2).

Kinetic analysis of ActVB with a variety of flavin and NADP(H) analogs allows the first detailed understanding of the protein-substrate interaction with this class of flavin reductases. Clearly, the isoalloxazine ring provides a major site of interaction with the polypeptide chain. Lumiflavin, lacking a lateral chain, is a substrate, and lumichrome and alloxazine are competitive inhibitors with respect to FMN, with $K_c$ values comparable with the $K_m$ value for lumiflavin (70–80 $\mu M$). In addition, the fact that $K_c$ values for lumichrome and alloxazine are comparable shows that the methyl groups of the isoalloxazine ring at positions 7 and 8 are not important for the protein-flavin interaction. On the other hand, the $K_m$ values for the natural substrates, riboflavin and FMN, are much lower (8 and 1 $\mu M$) (Table III). Thus, the ribityl chain also contributes to the binding. The catalytic efficiency of the enzyme with compound 1 and with FMN as substrates suggest that the 2'-OH of the sugar chain and the phosphate group play significant roles in the interaction of the substrate with the polypeptide chain. FMN is the better substrate in agreement with the finding that the purified preparations of ActVB contained FMN exclusively. This property represents a clear difference between the ActVB and the Fre flavin reductase families. Indeed, in the case of Fre, the recognition of the flavins occurs almost exclusively at the level of the isooalloxazine ring, with $K_m$ values for riboflavin and FMN comparable with that for lumiflavin and with the $K_d$ value for the inhibitor lumichrome, in the 0.5–2 $\mu M$ range (14).

The first and only available structural information on the ActVB family comes from the three-dimensional structure of the FeR protein, from A. fulgidus, in complex with the FMN substrate (22). Since ActVB displays significant sequence homology with FeR (22% identity and 34% similarity; data not shown), it should be possible to identify some key residues for flavin binding in ActVB. However, this approach proved to be rather limited, since the great majority of the residues involved in FMN binding in the FeR structure are not conserved in sequences of FeR homologs, and many of these residues interact with FMN through main chain nitrogen and oxygen atoms (22). Nevertheless, it is likely that ActVB should display structural similarities with FeR, with a FMN binding site that provides hydrogen bonds, ion pair interactions, and electrostatic dipole interactions to the various parts of the substrate, the isoalloxazine ring, the OH groups of the ribitol chain, and the phosphate group. In contrast, the structure of Fre, in complex with riboflavin, shows interactions of the polypeptide chain mainly with the isoalloxazine ring. All of these results are consistent with the kinetic analysis reported here.

As far as the binding of reduced pyridine nucleotides is concerned, again large differences between the ActVB and the Fre families are observed. Whereas Fre recognizes NADP(H) mainly through the nicotinamide ring, allowing NMNH to be a good substrate (17), ActVB interacts with the various parts of the molecule. This is shown from the drastic increase of the $K_d$ value for both inhibitors AMP and ADP-ribose and from the observation that NMNH is neither an inhibitor nor a substrate. It is also remarkable that ActVB is highly specific for NADH, whereas Fre can use both NADH and NADPH. Again we learn very little from the structure of the homolog, the FeR protein from A. fulgidus, since the residues interacting with NADP$^+$, in a crystal of the FeR-FMN complex soaked with NADP$^+$, are not conserved in ActVB (22). However, as illustrated by the characterization of a strong charge-transfer complex between reduced flavin and NAD$^+$ in the active site of ActVB (Figs. 2 and 3), it is likely that, as in FeR, the nicotinamide ring of the pyridine nucleotide in ActVB packs against the isoalloxazine ring of the flavin, with the C-4 atom of nicotinamide and the N-5 atom of the flavin at a few Å from each other in a stacking arrangement typical for direct hydride transfer in flavoproteins. A specific structural analysis of ActVB is required to better understand the various aspects of substrate recognition and mechanism discussed above.

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