The flavin reductase ActVB from *Streptomyces coelicolor*: Characterization of the electron transferase activity of the flavoprotein form

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Abstract The flavin reductase ActVB is involved in the last step of actinorhodin biosynthesis in *Streptomyces coelicolor*. Although ActVB can be isolated with some FMN bound, this form was not involved in the flavin reductase activity. By studying the ferric reductase activity of ActVB, we show that its FMN-bound form exhibits a proper enzymatic activity of reduction of iron complexes by NADH. This shows that ActVB active site exhibits a dual property with regard to the FMN. It can use it as a substrate that goes in and off the active site or as a cofactor to provide an electron transferase activity to the polypeptide.

Keywords: ActVB from *Streptomyces coelicolor*; Flavin reductase; FMN substrate; FMN cofactor; Ferric reductase; Two-substrates enzyme mechanism

1. Introduction

Flavin reductases represent a broad class of enzymes defined by their ability to catalyze the reduction of free flavins, riboflavin, FMN or FAD, by NAD(P)H [1–4]. A clear distinction between a flavin reductase and a flavoprotein resides in the fact that the former uses flavin as a substrate whereas the latter uses it as a prosthetic group. In general, flavin reductase physiological functions have been directly associated with the chemical properties of their released product, the free reduced flavin [1]. Free reduced flavins are known to reduce natural iron complexes like ferrisiderophores very efficiently and thus flavin reductases have been proposed to play an important role in iron uptake in bacteria, as ferric reductase enzymes [1]. Flavin reductases have been also found to be essential for a recently emerging class of flavin dependent monooxygenases such as luciferase for light emission or enzymes involved in antibiotics synthesis, aromatic and sulfur oxidations and other reactions [2–4]. In those systems, the reduced free flavin generated by the flavin reductase binds to the active site of the monooxygenase and, in the presence of oxygen, is then converted into a flavinhydroperoxide species as the key intermediate for oxidation reactions [5]. Such a two-component system has been recently studied in the case of the last step of actinorhodin biosynthesis, a natural antibiotic produced by *Streptomyces coelicolor* [2,3,5]. It involves ActVB, as the flavin reductase [2,3], and ActVA-ORF5, as the monooxygenase component [5]. Although ActVB can be isolated with various amount of FMN bound, depending on the preparation, we unambiguously demonstrated that flavin reductase activity of ActVB did not involve the protein-bound FMN. The presence of protein-bound FMN was rather interpreted as a reflect of the strong affinity of ActVB for the oxidized FMN [3].

In this work, by studying the ferric reductase activity of ActVB, we show that its FMN-bound form exhibits a proper enzymatic activity namely the reduction of iron complexes by NADH, in the absence of additional free flavins. These data highlight a dual property of ActVB active site with regard to the FMN. In the presence of high concentrations of FMN, it can use it as a substrate that goes in and off the active site. In the absence of free flavin, the ActVB-bound FMN provides the polypeptide a flavoprotein character with an electron transferase activity.

2. Materials and methods

NADH, FMN, Fe^{3+}–EDTA, ferricyanide and bathophenanthroline were from Sigma. Pyoverdin, azotobactin and desferal were gifts from Dr. Isabelle Schalk (University of Strasbourg, France). The recombinant ActVB protein from *S. coelicolor* was purified as reported in [3]. ActVB–FMN content was determined spectrophotometrically using a $\varepsilon_{455\,nm}$=13.6 $mM^{-1}\,cm^{-1}$ [3]. Enzymatic activities were measured at 18 °C, under anaerobiosis conditions. Flavin and Fe^{3+}–EDTA reductase activities were determined from the decrease of the absorbance at 340 nm ($\varepsilon_{340\,nm}$=6.22 $mM^{-1}\,cm^{-1}$), as reported in [3]. For the low redox potential iron complexes, azotobactin, pyoverdin and desferal, the reduction reaction was carried out in the presence of the ferrous iron acceptor bathophenanthroline and followed from the increase of the absorbance at 535 nm ($\varepsilon_{535\,nm}$=25.0 $mM^{-1}\,cm^{-1}$), characteristic of the Fe^{2+}–bathophenanthroline complex [6]. Ferricyanide reduction was followed from the decrease of the absorbance at 420 nm ($\varepsilon_{420\,nm}$=1.01 $mM^{-1}\,cm^{-1}$). Anaerobic experiments were carried out in a Jacomex glove box equipped with an Uvikon XL spectrophotometer coupled to the measurement cell by optical fibers (Photonetics system).

3. Results

3.1. Fe–EDTA reductase activity of ActVB in the presence of free flavins

The ability of ActVB to reduce Fe^{3+}–EDTA complex in the presence of NADH and FMN was tested in anaerobiosis. The

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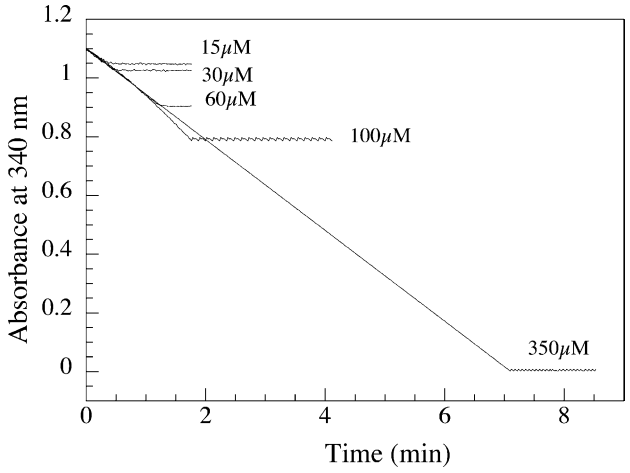

Fig. 1. Time course of NADH (200 μM) oxidation followed at 340 nm in anaerobiosis in the presence of 3 μM FMN, 0.054 μM ActVB and different concentrations of Fe^{3+}–EDTA: 15, 30, 60, 100 and 350 μM, in 50 mM Tris/HCl, pH 7.6.

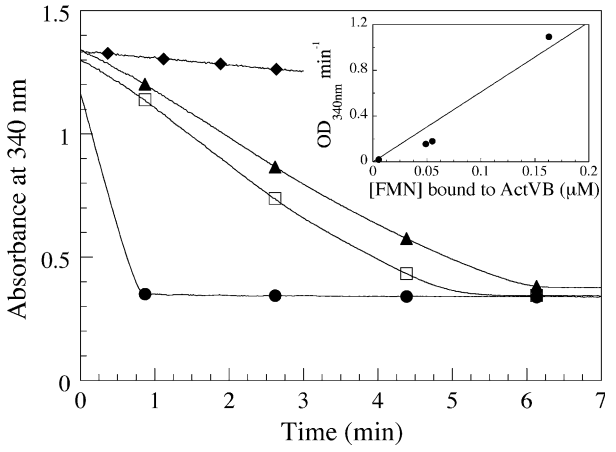

Fig. 2. Ferric reductase activity in the absence of free FMN, followed at 340 nm in anaerobiosis. The solution contains 200 μM NADH, 300 μM Fe^{3+}–EDTA in 50 mM Tris/HCl, pH 7.6. The reaction was started by addition of various preparations of ActVB-bound FMN protein. (◆) ActVB 0.054 μM, FMN-bound 0.005 μM, (▲) ActVB 0.490 μM, FMN-bound 0.05 μM, (□) ActVB 0.110 μM, FMN-bound 0.055 μM, (●) ActVB 0.327 μM, FMN-bound 0.16 μM. In the inset is shown the initial velocity of NADH oxidation as a function of the concentration of the ActVB-bound FMN.

reaction was followed spectrophotometrically from the decay of the absorbance at 340 nm, due to the oxidation of NADH. As shown in Fig. 1, the extent of NADH oxidation was found directly proportional to the amount of Fe^{3+}–EDTA present in the reaction mixture, giving a proportion of one NADH molecule reducing two molecules of Fe^{3+}–EDTA (Fig. 1). In the absence of Fe^{3+}–EDTA, only a slight amount of NADH was oxidized, corresponding to the amount of free FMN present in the assay mix (5 μM, data not shown). When the initial velocity of the reaction was studied in the presence of a fixed concentration of Fe^{3+}–EDTA (300 μM) as a function of NADH or FMN concentrations, typical Michaelis–Menten curves were obtained (data not shown). V_m and K_m values for NADH and FMN under those conditions were found to be identical to those reported using the standard flavin reductase activity assay in which the electron acceptor is oxygen (Table 1 and [3]). These data suggest that in the presence of free FMN, reduction of Fe^{3+}–EDTA occurs through a chemical reaction with the reduced FMN, provided by the flavin reductase activity of ActVB.

3.2. Fe–EDTA reductase activity of ActVB in the absence of free flavins

The ActVB polypeptide contains various amounts of bound FMN, depending on the enzyme preparation [3]. Reduction of Fe^{3+}–EDTA by NADH catalyzed by different ActVB preparations containing 0.1 or 0.5 mol of bound-FMN per mol of polypeptide chain was tested in the absence of free FMN. In anaerobiosis, in the presence of 300 μM Fe^{3+}–EDTA, the velocity of NADH oxidation was found to be directly proportional to the total concentration of FMN bound to ActVB (Fig. 2). It should be noted that under these conditions, the concentration of FMN bound to ActVB is several orders of magnitude smaller than the concentration of free flavins used in the flavin reductase activity assay described above (Fig. 1). These data show that the ActVB–FMN complex catalyzes the reduction of Fe^{3+}–EDTA by NADH in the absence of free flavin. From these data, a k_{cat} value of 17 s^{-1} for the ActVB protein containing one mol of bound-FMN per mol of polypeptide chain can be calculated.

The mechanism for the enzyme reaction was investigated by studying the initial velocity of NADH oxidation as a function of NADH and Fe^{3+}–EDTA concentrations, under anaerobiosis conditions. As shown in Fig. 3A, the double reciprocal plot of the initial velocity of NADH oxidation as a function of NADH concentration at several levels of Fe^{3+}–EDTA shows a series of parallel lines. In Fig. 3B, the initial velocity of the reaction as a function of Fe^{3+}–EDTA concentration at several levels of NADH also shows a series of parallel lines. These data are consistent with a ping-pong enzyme mechanism. The kinetic parameters, K_m and V_m values determined from

Table 1
Kinetic parameters for the ferric reductase activity of ActVB[a]

Varied substrate	In the presence of free FMN[b]		In the absence of free FMN					
	NADH	FMN	NADH[c]	Fe–EDTA[c]	Ferricyanide[d]	Pyoverdin[d,e]	Azotobactin[d,e]	Desferal[d,e]
k_{cat} (s^{-1})	9.2 ± 0.4	9.2 ± 0.4	8.9 ± 0.5	9.8 ± 0.4	42 ± 2	0.23 ± 0.03	3.7 ± 0.4	No activity
K_m (μM)	6.6 ± 0.5	1.0 ± 0.1	8.4 ± 0.5	105 ± 14	130 ± 20	128 ± 38	165 ± 40	–

[a]Measured in anaerobiosis in 50 mM Tris/HCl, pH 7.6. The ActVB preparation contains 0.5 mol of FMN bound per mol of polypeptide chain.
[b]With a fixed concentration of Fe^{3+}–EDTA (300 μM).
[c]Data from Fig. 3A and B.
[d]With a fixed concentration of NADH (200 μM).
[e]In the presence of 2 mM bathophenanthroline.