Some Properties of Enacyloxin Oxidase and its Cofactor(s)

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A new extracellular quinoprotein oxidase named enacyloxin oxidase (ENX oxidase), which is involved in biosynthesis of ENX IIa, a congener of ENX, was found in the culture supernatant of *Frateuria* sp. W-315 and shown to be exoenzyme. ENX oxidase was shown to act oxidation of ENX IVa to ENX IIa. The enzyme was presumed to have a redox cofactor different from PQQ.

Abbreviations : DHase, dehydrogenase ; ENX, Enacyloxin ; FAD, Flavin adenine dinucleotide ; FMN, Flavin mononucleotide ; GDase, Glucose dehydrogenase ; HPLC, High performance liquid chromatography ; NAD(P), Nicotinamide adenine dinucleotide (phosphate) ; PQQ, pyrroloquinoline quinone ; TLC, Thin layer chromatography

Introduction

We have discovered a unique antibiotics named enacyloxin (ENX), which is a family of non-lactonic polyene antibiotics produced by *Frateuria* sp. W-315. ENX has more than 12 congeners and they are active against Gram-positive and Gram-negative bacteria.[1-5] ENX IIa has been shown to inhibit protein synthesis of *E. coli* by inhibiting binding of a.a.-tRNA to A site of ribosomes.[6,7] Very recently, our colleague including Parmeggiani and others (Ecole Polytechnique, France), Watanabe and others (Tohoku Univ., Japan) reported that ENX IIa is the first antibiotic found to have a dual specificity targeted to elongation factor Tu and the ribosome.[8]

In the early phase of production of the antibiotics, the main product secreted into the culture fluid was ENX IVa, while it gradually decreased in correspondence to the increase of ENX IIa with the lapse of the culture period.[9] The differences in chemical structure between ENX IIa and ENX IVa (Fig. 1) is oxidative status of

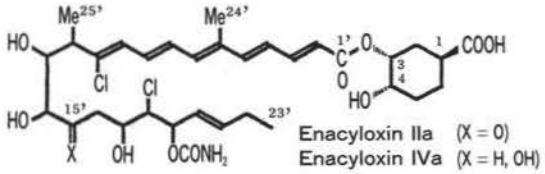

Fig. 1 Chemical Structures of Enacyloxins (ENXs)

C-15′ suggesting that bacterial extracellular oxidase or dehydrogenase catalyzes dehydrogenation of ENX IVa. Moreover, this change seemed to be a stoichiometric conversion of ENX IVa to ENX IIa. We named the enzyme enacyloxin oxidase (ENX oxidase) tentatively.

Duine *et al*[10] have reported that a cofactor of D-glucose DHase from *Acinetobacter calcoaceticus* was PQQ, and proposed the name of "quinoprotein" for PQQ-containing group of DHases. Several Gram-negative bacteria have quinoprotein DHases[11] in their periplasm fractions, but no extracellular quinoprotein has been reported.

In this paper, we describe some properties of ENX oxidase and its cofactor(s).

Materials and Methods

1. *Extraction of cofactor(s) and enzyme assay*
Cofactors were extracted from the partially

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purified enzyme preparation (precipitates after ultracentrifugation) with diethylether under acidic condition (pH5). Ether layer was evaporated to dryness *in vacuo* and the dried residue was dissolved in 1% solution of $NaHCO_3$ (named as ether fraction). Unextractable water layer was immediately neutralized to the original pH (pH7.2) (named as ether-treated enzyme).

ENX oxidase activity was determined by estimation of ENX IIa formed from ENX IVa using HPLC. A hundred μl of reaction mixture contained 20 μl of 0.1 mM ENX IVa, 10 μl of enzyme solution, 5 μl of ether fraction or 0.2 mM PQQ, which were added as cofactors, if necessary. Total volume was adjusted to 100 μl with Tris HCl buffer (50 mM, pH7.2).

2. *Others*

Bacterial growth was measured by absorbance at 660 nm using a Hitachi U-1000 spectrometer. A Beckman DU-65 spectrophotometer was used for spectrophotometric measurement of ENX oxidase. Changes of ENXs in the culture supernatant were measured using HPLC. One unit of ENX oxidase was defined as the amount that catalyzed the formation of 1 μg ENX IIa per min at 30°C. Protein concentrations were determined by Lowry's method.[12]

Results and Discussion

As shown in the preceeding paper,[13] we tried to identify the cofactor required for ENX oxidase reaction, cofactor such as NAD(P), FAD, and PQQ were added separately to the reaction mixture containing ENX IVa and ENX oxidase, and incubated. Any stimulative effect of each cofactor on the enzyme reaction, however, was not observed, suggesting that the cofactor of ENX oxidase would remain in the partially purified enzyme preparation (data not shown). Since carbonyl reagent is known as an inhibitor of quinoprotein by its reaction with carbonyl group of PQQ, effect of hydroxylamine on ENX oxidase was examined. The culture supernatant incubated for 1 h with hydroxylamine showed a big decrease of ENX oxidase activity as compared to that of without hydroxylamine, suggesting that the enzyme would have PQQ or PQQ-like substance as its cofactor.[13]

Cofactor of ENX oxidase was partially extracted with diethylether (ether fraction) indicating no covalent bond would be formed between the enzyme and cofactor. Reconstraction of the holoenzyme of ENX oxidase was carried out by mixing the apoenzyme (ether-treated enzyme) and the ether fraction. Using the treated enzyme, partial reconstruction of the enzyme activity was achieved by addition of the ether fraction or PQQ as cofactor (Table I). The ether fraction was analyzed by TLC on silica gel 60 (Merck, MeOH/AcOH = 70/1), together with PQQ (Fig. 2). Cofactor in the ether fraction was obtained from the area of Rf 0.67, while PQQ showed Rf of 0.13 under the same TLC conditions. This result suggests the presence of a new

Table I Effects of Ether Fraction and PQQ on Activities of the Partially Purified ENX Oxidase and Ether-treated ENX Oxidase

Components	ng ENX IIa formed/min/mg protein	ratio
Enzyme	2.25	1.00
Enzyme+ether fraction	2.27	1.00
Enzyme+PQQ	2.21	0.98
Ether-treated enzyme	0.85	1.00
Ether-treated enzyme+ether fraction	1.59	1.91
Ether-treated enzyre+PQQ	1.27	1.53

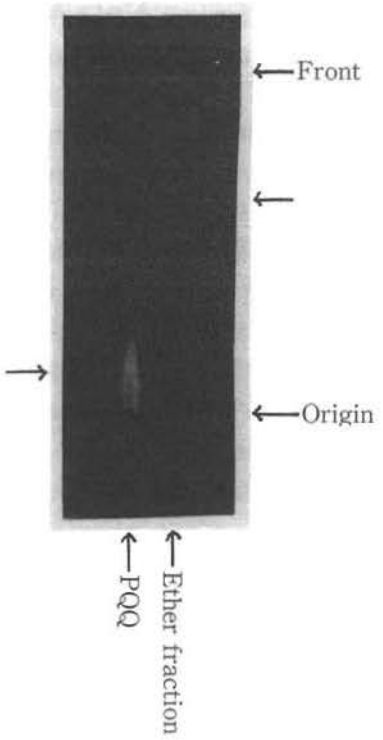

Fig. 2 Thin Layer Chromatogram of Ether Fraction and PQQ
The picture was taken under UV light.

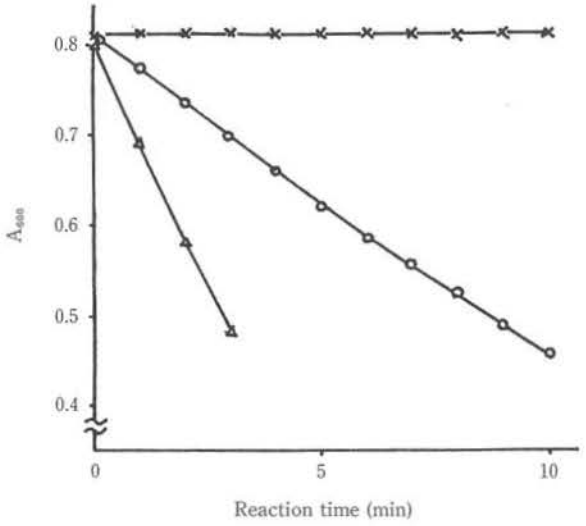

Fig. 3 Spectrophotometric Measurement of ENX Oxidase Activity
Two hundred μl of basal mixture (BM) contains 50 mM Tris-HCL buffer, 200 μM of tetramethyl-p-phenylenediamine (Wurster's Blue) and 5 μl of ENX oxidase. Reaction was carried out at 30°C after addition of the substrate (ENX IVa or D-glucose) and the absorbance at 600 nm was recorded every one minute automatically. Symbols ○, △ and × indicate BM plus ENX IVa (200 nM), BM enriched 2 times of the enzyme concentration plus ENX IVa (200 nM), and BM plus D-glucose (20 mM), respectively.

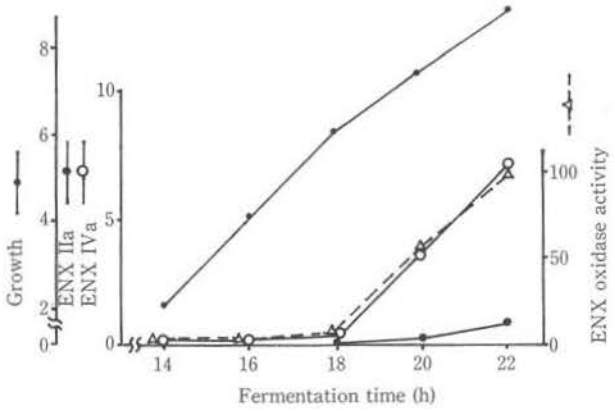

Fig. 4 ENX Production Correspondent to ENX Oxidase Activity in the Culture Supernatant
Symbols •, ○ and ●, and △ indicate cell growth at 660 nm, ng of both ENX IVa and ENX IIa per ml of the supernatant, and ENX oxidase activity shown as ng of ENX IIa formed per ml of the culture supernatant per h, respectively.

cofactor or activator different from PQQ. Klinman *et al*[14] reported that a new redox cofactor covalently bound to amine oxidase was 6-hydroxydopa. Chemical properties of new cofactor or activator in ether fraction are now under investigation.

During these studies, ENX oxidase activity was measured with TLC method,[9] then another method of the enzyme assay was carried out with tetramethyl-p-phenylenediamine (Wurster's Blue) as electron acceptor. The result indicates that ENX IVa could act as electron donor, but D-glucose could not react with the enzyme (Fig. 3). This means the absence of quinoprotein GDases in the enzyme preparation, because quinoprotein GDase are known to use Wurster's Blue as electron acceptor.[10]

Finally, we checked the time courses of ENX oxidase, ENX IVa and ENX IIa formations in the culture supernatant (Fig. 4). Even in a very early phase of the culture of *Frateuria* (18 h, early log phase), ENX oxidase activity began to appear in the culture supernatant together with ENX IVa, and after a while, ENX IIa began to appear in the same supernatant. Although quinoproteins are shown to be located in membrane or cytoplasm in the bacterial cells,[10] ENX oxidase activity, as above mentioned, began to be detected outside the cells in the early log phase, but it could not be detected in the bacterial cells throughout the

culture.

From the above results, ENX oxidase with its cofactor would function in the culture as one of the key enzyme in the biosynthesis of ENXs.

Acknowledgements

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