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Biomimetic experiment of enoyl-reduction process by F420-dependent enzyme obtained from *Saccharopolyspora erythraea* and the biosynthetic implication*

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Abstract

To approach the involvement of 5-deazaflavin coenzyme (F420) on the enoyl-reduction process in the step 4 of the erythronolide B in Saccharopolyspora erythraea, *an investigation was carried out using biomimetic experiment. Three a,b-unsaturated-bearing substances, i.e. ethyl crotonate, citral, and dimethyl glutaconate, were chosen as the hydride-acceptor substances in the experiment to mimic the step 4 intermediate: 7,9-dihydroxy-2,4,6,8-tetramethyl-5-oxo-undec-2-enoyl-S-ACP. In the experiment crude extract isolated from* Sac. erythraea *was used to catalyze the hydride transfer from the NADPH, as the hydride donor, to the acceptors through F420. The hydride transfer was indicated by A_{401} of the coenzyme, before and after the transfer process which was under dim light and oxygen free condition. The results showed that coenzyme F420 has a role in the enoyl-reduction process of an a,b-unsaturated carbonyl structure.*

Keywords: Saccharopolyspora erythraea – *F420 – enoyl-reduction – biomimetic experiment.*

Introduction

5-Deazaflavin coenzyme has been found in some *Methanogenbacterium* sp. (Daniels *et al.*,1985; Eirich *et al.*,1978;1979; Kern *et al.*, 1983), and Actinomycetes, mostly *Streptomyces* sp. (Eker *et al.*, 1980; Kuo *et al.*, 1989, Lambert 1987; Lin and White, 1985; McCormick and Morton, 1982; Miller *et al.*, 1960; Novotna *et al.*, 1989; Sudibyo and Jenie,1996a; 1996b; 1997). In *Saccharopolyspora erythraea* the coenzyme has been structurally elucidated as F420 (Sudibyo and Jenie, 1996a; 1996b; 1997) which is also found in methanogen bacteria. F420 mostly involves in the redox process of the methanogen bacteria (Eirich and Dugger, 1984; Jacobson *et al.*, 1882; Jin *et al.*, 1983; Schauer and Ferry, 1982; 1986; Jones and Stadtman, 1980; Yamazaki and Tsai, 1980a; 1980b). The coenzyme is dependent to NADPH in the several redox systems of the *Methanobacterium* sp. (Eirich and Dugger, 1984; Yamazaki and Tsai, 1980a; 1980b) and *Streptomyces* sp. (Lambert, 1987).

The 5-deazaflavin coenzyme found in some Streptomyces sp. has a role in the secondary metabolite biosynthesis of the microbes. In *S. aurofasciens* and *S. rimosus* (chlortetracycline and oxytetracycline producer respectively) the coenzyme involves in the last step of the tetracycline biosynthesis. The mechanism in that step is an

* This topic is a part of Sudibyo's doctoral thesis

enoyl-reduction process involving an hydride transfer into an α,β-unsaturated carbonyl chlortetracycline (Miller *et al.*, 1960; McCormick and Morton, 1982) (Figure 1) carbonyl structure of 5a,11a-dehydro-chlortetracycline (Miller et al.,1960; McCormick and Morton, 1982) (Figure 1) and 5a,11a - dehydrooxytetracycline (Rhodes *et al.*, 1981) (Figure 2).

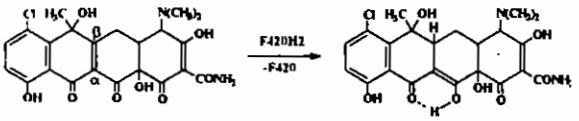

Figure 1. The conversion of 7-chloro-5a,11a-dehydrochlortetracycline into chlortetracycline, the last step of the chlorteracycline biosynthesis in *S. aurofasciens* (Miller *et al.*, 1960; McCormick and Morton, 1982)

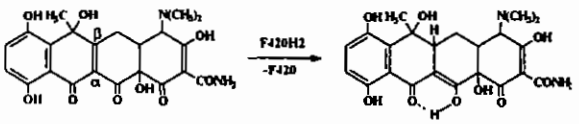

Figure 2. The conversion of 5a,11a-dehydro-oxytetracycline into oxytetracycline, the last step of the oxyteracycline biosynthesis in *S. rimosus* (Rhodes *et al.*, 1981)

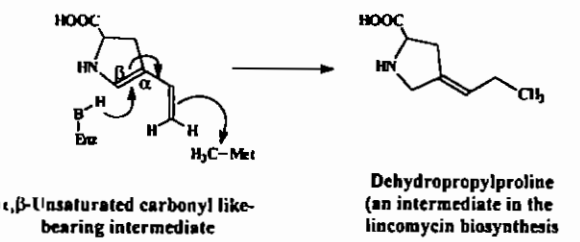

Figure 3. Conversion of an α,β-unsaturated carbonyl like-bearing intermediate into dehydropropylproline, an intermediate of lincomycin biosynthesis in *S. lincolnensis* (Brahme *et al.*, 1984)

S. lincolnensis a lincomycin producer, also contains 5-deazaflavin coenzyme. This coenzyme was reported to involve in the lincomycin biosynthesis (Coats *et al.*, 1989; Kuo *et al.*, 1989). Based on the lincomycin biosynthetic pathway (Brahme *et al.*, 1984), the coenzyme might work on the reduction of a diene system, an α,β–unsaturated carbonyl like structure (Figure3).

Donadio*et al.* (1991), by a genetic study of erythronolide B biosynthesis, reported that an enoyl-reductase is involved in the step 4 of the biosynthesis. It means that in the step 4 there should be an enoyl-reduction process. Based on that, the mechanism of the enoyl-reduction process in the step 4 can be assumed as in figure 4. In this biosynthetic pathway, the α,β-unsaturat-ed carbonyl-bearing intermediate is 7,9-dihydroxy-2,4,6,8-tetramethyl-5-oxo-undec-2-enoyl-S-ACP (a) which undergoes enoyl-reduction process to produce a fully saturated counterpart of 7,9-dihydroxy-2,4,6,8-tetramethyl-5-oxo-undecanoyl-S-ACP (b) (Figure 4). It was also reported that NADPH is involved in the enoyl-reduction process (Donadio and Katz, 1992; Donadio *et al.*, 1993). Since erythronolide B, the erythromycin aglycon, is produced in *Sac. erythraea* (which also produces 5-deazaflavin), a question rises whether 5-deazaflavin coenzyme in *Sac. erythraea* also involves in the step 4, that is the enoyl-reduction process. To find out the involvement of the coenzyme to the enoyl-reduction process in the *Sac. erythraea*, a biomimetic experiment was designed. To mimic the α,β-unsaturated-bearing intermediate of erythronolide B biosynthesis (a) (Figure 4), several α,β-unsaturated carbonyl-bearing substances were chosen, i.e. ethyl crotonate, citral, and dimethyl glutaconate (Figure 5).

In this experiment a crude enzyme isolated from *Sac. erythraea* was used to catalyze a process of hydride transfer from NADPH (as the hydride donor) to the mimical substances as the hydride receptor through F420. The hydride transfers were monitored using the absorbancy of F420 in wavelength

of 401 nm. Because F420 is sensitive to the light, and the process is oxygen sensitive, therefore the experiments were carried out under dim light and oxygen free condition.

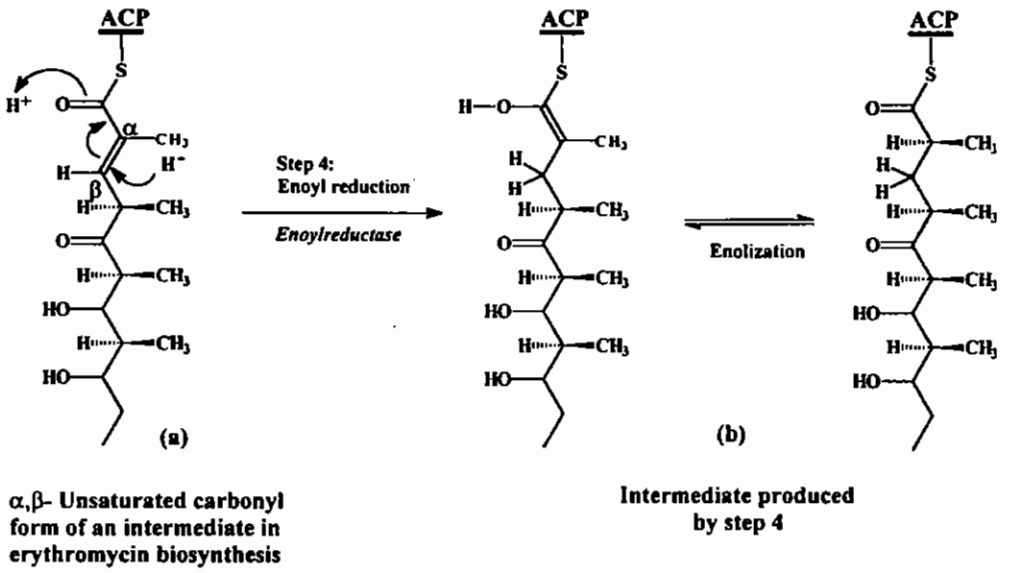

Figure 4. The enoyl-reduction process in the step 4 of the erythromycin biosynthesis

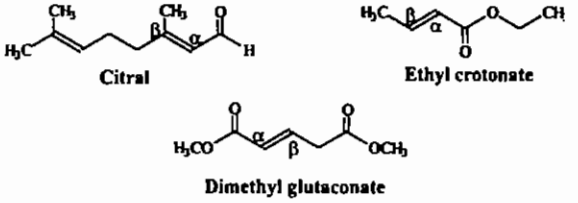

Figure 5. The chemical structures of the a,b-unsaturated carbonyl-bearing substances used as the hydride acceptor

Materials and Method

Microbe. Saccharopolyspora erythraea ATCC 11635. Chemicals: Ethyl crotonate (Merck), Citral (Merck), Dimethyl glutaconate (Merck) NADPH (Sigma), coenzyme F420 was a kindly give from Dr. Lacy Daniels, Department of Microbiology, Iowa University, USA. **Buffer solutions.** Lysis, reaction, and storage buffers were made by modification of those of Malhotra et al. (1992)

Media: Starter medium: Nutrient Broth 0.8%; inoculum medium: Yeast Extract 0.25%, Malt Extract (Sigma) 0.25%, Peptone 0.5%, Glucose (Merck) 0.5%, $MgCl_2$ (Merck) 0.5 mM/l; fermentation medium: Tryptic Soy Broth 3%, Sucrose (Merck) 1.5%. Instead of stated, all materials were Difco Lab. products. Cell production. Lyophilized *Sac. erythraea* was inoculated to make a starter. This culture was incubated at 28o-30°C on a rotary-shaking incubator until the growth was indicated. The starter was transferred into the *inoçulum* medium, and incubated at the same temperature for 2 days. The *inoculum* finally was transferred into the fermentation medium and fermented in a 5 l fermenter for 2 days under 400 rpm agitation, temperature 28°-30°C and pH 7.0. The foaming was controlled using antifoam B Solution (Sigma). The cells was separated by centrifugation on 10.000 rpm and 4°C, for 30 minutes. Then it was washed two times with cold demineralized water, and centrifuged

for the separation. **Crude enzyme.** The washed cells was suspended in lysis buffer and sonicated three times at for 15 minutes. The sonicated suspension was centrifuged at 20,000 rpm and 4°C, for 30 minutes. The supernatant (crude enzyme) was separated from the debris, and stored at temperature -70°C until it was used for the experiment. **Hydride transfer experiment.** Except the crude extract, all the reactants were dissolved in the cold (0°-4°C) activity buffer. All steps in the experiment were under dim light and oxygen free conditions, that is by flushing all the solutions used (except the crude enzyme) in tightly closed tubes with oxygen free-nitrogen gas for 15-30 minutes. The F420 absorbance of each experimental step was measured at 401 nm (the isobestic point of F420) at temperature of 25°C for 10 minutes and 1 minute interval time. Three steps were carried out in every experiment, which measured the F420 absorbance in the solutions containing respectively: (1). F420 + NADPH, (2). F420 + NADPH + crude enzyme, and (3). F420 + NADPH + crude enzyme + one of the α,β-unsaturated carbonyl substances (the hydride acceptors).

The measuring absorbance was corrected using the same but no F420 content solution in every determination.

Results and Discussion

The biomimetic experiment was carried out in order to investigate the involve-ment of F420 in the enoyl reduction of α,β-unsaturated carbonyl structures. In principle, an enoyl reduction is a **Michael addition reaction** in which hydride ion acts as a nucleophile. The NADPH in this study was used as the hydride donor which transfers the hydride to the oxidized form of the F420, and it will transform the coenzyme into the reduced form of $F420H_2$ (Figure 6). The new hydride donor resulted, $F420H_2$, then transferred the hydride ion into an α,β-unsaturated carbonyl molecule through Michael addition mechanism to bring about an enoyl reduction process. This reduction process is catalyzed by the enoyl-reductase which is in the crude enzyme isolated from *Sac. erythraea*.

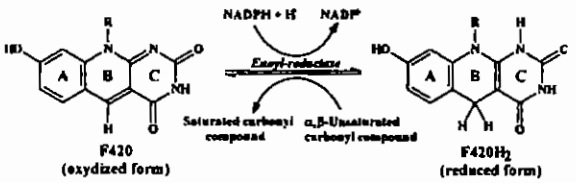

Figure 6. The hydride transfer from NADPH to 5-deazaflavin

To monitor the hydride transfer processes in this experiment, an ultra-violet spectroscopic approach was used. This approach is based on the changing of F420's absorbance at 401 nm (A_{401}) when the F420 is accepting or donating the hydride ion. Hypochromic effect will occur when F420 accepts an hydride, since conjungated diene system in ring B and C is broken, and therefore decreasing the aromaticity of the pyridine moeity of ring B. As the result, it decreases the A_{401}. Conversely, hyperchromic effect will restore the absorbance of the F420, when the $F420H_2$ donates the hydride ion into the α,β-unsaturated carbonyl molecule (Figure 8). The measuring of A_{401} in each step of the experiment was 10 minutes, with 1 minute intervals. The average of the absorbances resulted in the each step was recorded as in the Table 1.

The experiment I showed that a mixture of NADPH and the F420 exhibited A_{401} of 0.806. Addition of crude enzyme isolated from *Sac. erythraea* into the mixture decreased the absorbance into 0.713. The hypochromic occurrence leads to a conclusion that an hydride transfer from NADPH into the F420 to give $F420H_2$ has occured. However, when the hydride acceptor: methyl

crotonate was then added into the enzymatic mixture, an hyperchromic effect occur to restore the absorbance into 0.860 (Table 1). In this case, an hydride transfer has occurred from the $F420H_2$ into the α,β-unsaturated carbonyl molecule: methyl crotonate.

Table 1. The biomimetic study results (experiment I, II, and III) which show the hydride transfer from NADPH to the hydride acceptors (ethyl crotonate, citral, and dimethyl glutaconate) by F420 and enoyl-reductase contained inthe crude enzyme that is isolated from *Sac. erythraea*

Experiment I : **Using ethyl crotonate as the hydride acceptor**	**A_{401} average**
NADPH + F420	0.806
NADPH + F420 + Crude enzyme	0.713
NADPH + F420 + Crude enzyme + Ethyl crotonate	0.860
Experiment II : **Using citral as the hydride acceptor**	**A_{401} average**
NADPH + F420	0.960
NADPH + F420 + Crude enzyme	0.680
NADPH + F420 + Crude enzyme + Citral	1.340
Experiment III : **Using dimethyl glutaconate as the hydride acceptor**	**A_{401} average**
NADPH + F420	0.470
NADPH + F420 + Crude enzyme	0.400
NADPH + F420 + Crude enzyme + Dimethyl glutaconate	0.420

Similar result was shown in the experiment II, when citral was used as the hydride acceptor. An hypochromic effect has changed the absorbance of the mixture, from 0.960 into 0.680, when the crude enzyme was added into a mixture of NADPH and the F420. Again, the absorbance of the mixture was increased into 1.340, when citral was added (Table 1). This means that the hydride transfer into citral has occurred.

In experiment III, dimethyl glutaconate was used as hydride acceptor. Again hypochromic effect occurred when the crude enzyme was added into the mixture of NADPH and the F420. However, addition of dimethyl glutaconate into the enzymatic mixture did not give significant absorbance change, *i.e.*, only from 0.400 into 0.420 (table 1). The difficulty of dimethyl glutaconate to accept hydride ion might be due to the presence of an enol form of dimethyl glutaconate. This enol form would increase resonance stabilization so that the α,β-unsaturated carbonyl moiety within the ester become inert toward the nucleophile (see Figure7).

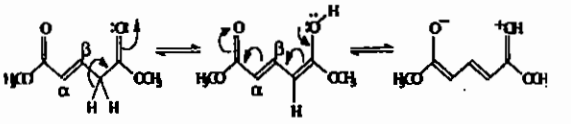

Figure 7. The tautomeric process in dimethyl glutaconate to give the enol form

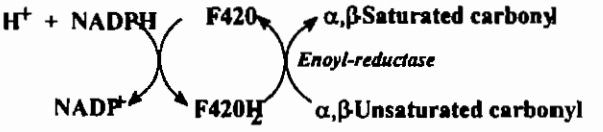

Figure 8. The hydride transfer from NADPH into an α,β-unsaturated carbonyl structure (an enoyl-reduction process) by F420

From this biomimetic experiment, it was concluded that the F420 acts as intermediary agent during the hydride transfer process from NADPH into the α,β-unsaturated carbonyl compound, under enzymatic control (see Figure 8).

Conclusion

This experiment shows that coenzyme F420 does involve in the enoyl-reduction process of *Sac. erythraea*. Based on the previous research, that there is a co-occurrence of the erythromycin and 5-deaza-

flavin productions (Sudibyo, 1997) by *Sac. erythraea*, it can be concluded that F420 might have a role in the enoyl-reduction process of the step 4 of erythronolide B biosynthesis.

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