FORMATION OF DIHYDROXY DERIVATIVE OF ML-236B FROM ML-236B (COMPACTIN) BY LIPID PEROXIDATION

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As reported in our previous paper, pravastatin sodium (the generic name of CS-514, hereafter referred to as pravastain) was obtained by the hydroxylation of sodium ML-236B-carboxylate catalyzed by many kinds of microorganisms[1~4]. In contrast, the dihydroxy derivative of ML-236B (formerly referred to as M_3) was found together with pravastatin in all such microorganisms[5].

The present paper shows that the formation of dihydroxy derivative of ML-236B *in vitro* depends upon enzymatic-lipid peroxidation. Consequently, the mechanism of formation of this compound *in vivo* is suggested.

Recently, we described the cytochrome P-450$_{sca}$ monooxygenase system in *Streptomyces carbophilus* that hydroxylates sodium ML-236B-carboxylate to pravastatin[6]. Dihydroxy derivative of ML-236B is also obtained in case of *S. carbophilus*. At first, we determined whether the formation of dihydroxy derivative of ML-236B depends upon cytochrome P-450$_{sca}$ monooxygenase or not. According to that paper, *S. carbophilus* was cultured and cytochrome P-450$_{sca}$ was obtained, the amount of dihydroxy derivative of ML-236B formed in cytochrome P-450$_{sca}$ monooxygenase system was estimated. Cytochrome P-450$_{sca}$-dependent monooxygenase system contained 10 nmol/ml of the mixture of P-450$_{sca-1}$ and P-450$_{sca-2}$, spinach ferredoxin, spinach ferredoxin-NADP$^+$ reductase and NADPH generation system as previously described[6]. As shown in Table 1, the formation of this compound was independent of cytochrome P-450$_{sca}$ while the formation of pravastatin is dependent on cytochrome P-450$_{sca}$. Therefore, the formation of dihydroxy derivative of ML-236B is considered as being dependent on an other mechanism. The chemical reaction mechanism of the hydroxylation to dihydroxy derivative of ML-236B is still unknown. It is possible that it arise from formation of an epoxide of the tri-substituted olefin, followed by S_N2' addition of H_2O. And this is supported by the fact that oxidation by *meta*-chloroperbenzoic acid of sodium ML-236B-carboxylate and subsequent treatment by water affords dihydroxy derivative of ML-236B (data not shown). It is noteworthy that cytochrome P-450$_{sca}$ has complete site-selectivity for the 6-allylic position and not for the tri-substituted olefin.

Then, we examined the formation of dihydroxy derivative of ML-236B in lipid-peroxidation system. A typical incubation system was prepared as follows: To a solution of 2 mg lipid (phosphatidylcholine, or fatty acid), 800 μl enzyme preparation which contained 0.8 nmol of cytochrome P-450$_{sca}$, 0.26 mM NADP$^+$, 0.04 units of ferredoxin-NADP$^+$ reductase, 20 μg ferredoxin, 70 mM glucose-6-phosphate, 1.0 unit of glucose-6-phosphate dehydrogenase and 1 mM ADP, sodium ML-236B-carboxylate (1,000 μg/ml in final concentration) was added in a final volume of 1,000 μl. Phosphatidylcholine or other fatty acids were added to this system in the form of suspension. The reaction was heterogeneous. The mixture was preincubated for 5 minutes at 30°C, and the reaction was started by the addition of aqueous solution of ferrous sulfate. Incubation was carried out at 30°C for 60 minutes. The reaction was stopped by the addition of 10 μl 1.5 N NaOH. The co-factor requirement test for the formation of dihydroxy derivative of ML-236B in this system was made as follows: From the reconstitution system, each of ferrous ion, phosphatidylcholine, NADH generation system, ferredoxin-NADP$^+$ reductase, ADP and enzyme was eliminated, and the amount of dihydroxy derivative of ML-236B formed was determined. 10 nM of purified cytochrome P-450$_{sca}$ was added to this system, and the dihydroxy derivative of ML-236B was also assayed. The quantity of dihydroxy derivative of

Table 1. Formation of dihydroxy derivative of ML-236B without the addition of cytochrome P-450$_{sca}$.

	Dihydroxy derivative of ML-236B formed (μg/ml)
Reconstitution system[a]	5.439
– P-450$_{sca}$	5.716

[a] Reconstitution system contained purified cytochrome P-450$_{sca}$, ferredoxin, ferredoxin-NADP$^+$ reductase, NADPH generation system and sodium ML-236B-carboxylate as described in the previous paper[6]. This system did not contain any lipids.

Table 2. Co-factor requirement test.

	Relative amount of dihydroxy derivative of ML-236B (%)
Reconstitution system[a]	100.0
− $FeSO_4$	13.1
− Phosphatidylcholine	5.6
− $FeSO_4$, − phosphatidylcholine	4.3
− NADPH generating system	11.4
− Ferredoxin-$NADP^+$ reductase	29.7
− Cell-free extract	46.2
− Cell-free extract, + $P\text{-}450_{sca}$[b]	45.4

[a] Reconstitution system contained cell-free extract of *Streptomyces carbophilus*, NADPH generation system, ferredoxin, ferredoxin-$NADP^+$ reductase, phosphatidylcholine, ADP, $FeSO_4$ and sodium ML-236B-carboxylate.

[b] $P\text{-}450_{sca}$ was used at 10 nmol/ml.

ML-236B was determined by HPLC using a radial-pack cartridge (C18) (Millipore Waters Division). The column was eluted with 21% acetonitrile - 79% aqueous triethylammonium phosphate (0.1%, pH 3.2) at a flow rate of 1.0 ml/minute and dihydroxy derivative of ML-236B was detected by absorbance at 210 nm. The amount of pravastatin was also determined by HPLC analysis as previously reported[6].

As shown in Table 2, dihydroxy derivative of ML-236B was formed without the addition of the *S. carbophilus* cell-free extract or a cytochrome $P\text{-}450_{sca}$. The production of dihydroxy derivative of ML-236B was strictly dependent on phosphatidylcholine or ferrous ion. Removal of electron donor or electron transport protein also significantly reduced the hydroxylation activity.

Lipid hydroperoxidation in hepatic microsomes is known to be remarkably accelerated either by ferrous ion in the presence of NADPH and ADP. As shown in Table 3, when ferrous ion or ADP was omitted from the complete system, hydroxylation to dihydroxy derivative of ML-236B was significantly reduced. Potassium cyanide, at a seven times higher concentration than that of ferrous ion, inhibited this hydroxylation. The data in Table 3 also shows that this hydroxylation occurs in the presence of linoleoate (18 : 2), but that only a little hydroxylation occurs in the presence of stearoeate (18 : 0). This result strongly suggested the participation of unsaturated fatty acid and its ester in the hydroxylation. It has already been suggested that

Table 3. Difference between the amount of dihydroxy derivative of ML-236B formed by unsaturated fatty acid and that formed by saturated fatty acid.

	Relative amount of dihydroxy derivative of ML-236B (%)
Reconstitution system[a]	100.0
− $FeSO_4$	4.0
− ADP	22.4
+ KCN	12.9
Reconstitution system[b]	30.3
− $FeSO_4$	22.6
− ADP	33.6
+ KCN	17.8

[a] The reaction mixture contained linoleic acid (18 : 2), NADPH generation system, ferredoxin, ferredoxin-$NADP^+$ reductase, ADP, $FeSO_4$ and sodium ML-236B-carboxylate.

[b] The reaction mixture contained stearic acid (18 : 0), NADPH generation system, ferredoxin, ferredoxin-$NADP^+$ reductase, ADP, $FeSO_4$ and sodium ML-236B-carboxylate.

Both of the reaction mixtures did not contained the cell-free extract of *Streptomyces carbophilus*.

Table 4. Formation of dihydroxy derivative of ML-236B by cell-free extract of *Streptomyces carbophilus*.

	Relative amount of dihydroxy derivative of ML-236B (%)
Reconstitution system[a]	100.0
−Cell-free extract	40.4
* Cell-free (100°C, 10 minutes)	39.2
−$FeSO_4$	35.2
+KCN	18.7
−NADPH generating system	16.2

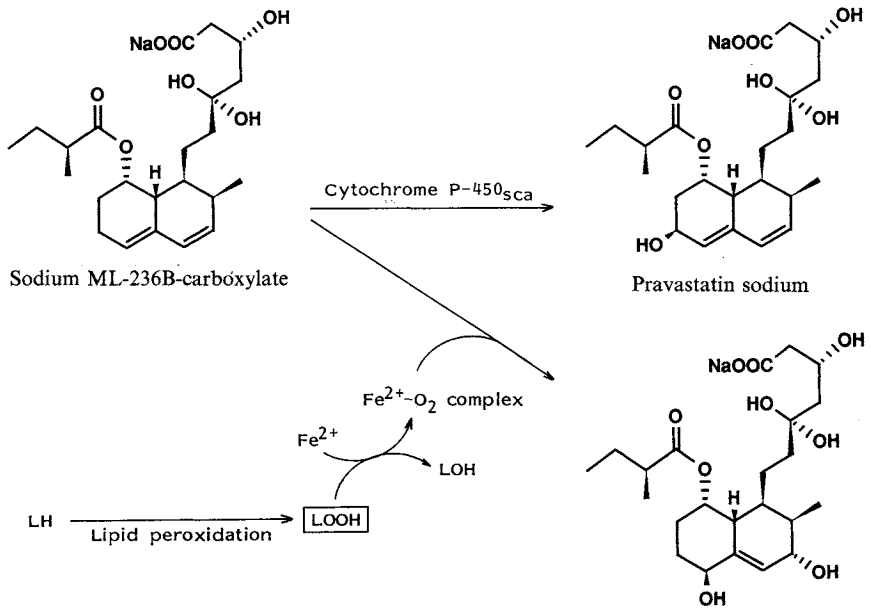
[a] Reconstitution system contained cell-free extract of *S. carbophilus*, NADPH generation system, phosphatidylcholine, ADP, $FeSO_4$ and sodium ML-236B-carboxylate.

* With heated cell-free extract.

ADP might serve as a chelating agent for the formation of lipid hydroperoxide from polyunsaturated fatty acyl lipids[7]. Potassium cyanide is likely to block the formation of active oxygen iron complex required to catalyze the epoxidation of sodium ML-236B-carboxylate[7]. Therefore, the dihydroxy derivative of ML-236B would be formed by lipid-peroxidation in these *in vitro* systems.

Table 4 also shows the result of using a reconstitution system which does not contain electron transport protein, ferredoxin and ferredox-

Fig. 1. A proposed formation mechanism of dihydroxy derivative of ML-236B.
LH is an abbreviation of unsaturated fatty acid or its ester.



in-NADP$^+$ reductase. In this system, significant formation of dihydroxy derivative of ML-236B was observed and it was dependent on the cell-free crude extract *S. carbophilus* or NADPH. This result strongly suggested that there would be a microbial counterparts of spinach ferredoxin and spinach ferredoxin-NADP$^+$ reductase in *S. carbophilus*. Thus, from the facts shown in our experiments, a formation mechanism for lipid hydroperoxidation from ML-236B to dihydroxy derivative of ML-236B in *S. carbophilus* may be proposed as illustrated in Fig. 1.

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