Journal of Industrial Microbiology & Biotechnology (1998) 20, 373–375

http://www.stockton-press.co.uk/jim

A new hydroxylase system in *Actinomadura* sp cells converting compactin to pravastatin

Y Peng[1,2] and AL Demain[1]

[1]*Fermentation Microbiology Laboratory, Biology Department, Massachusetts Institute of Technology, Cambridge, MA 02139, USA*

The hydroxylase in cell-free extracts of *Actinomadura* sp strain 2966 converts compactin to pravastatin. It requires NADPH as coenzyme and Mg^{2+} as cofactor; Mn^{2+} can partially replace Mg^{2+}. In contrast with the inducible cytochrome P-450 system of *Streptomyces carbophilus* which catalyzes the same overall reaction, this constitutive hydroxylase is stimulated by ATP and ascorbic acid and is not inactivated by CO.

Keywords: *Actinomadura*; compactin; hydroxylase; microbial transformation; pravastatin

Introduction

Pravastatin significantly reduces plasma cholesterol levels in humans and is of great importance in the therapy of hypercholesterolemia [1,8,9,10]. Pravastatin is obtained via hydroxylation of compactin by *Streptomyces carbophilus* which contains a cytochrome P450 monooxygenase system [2,7]. We found that a newly isolated microorganism, *Actinomadura* sp strain 2966, is able to carry out the same overall reaction in very high yield [4,11]. In this paper, we present our studies on the hydroxylase of *Actinomadura* sp using cell-free extracts which indicate a different type of hydroxylase than is present in *S. carbophilus.*

Materials and methods

Chemicals

Compactin was obtained from Fluka, Buchs, Switzerland, and pravastatin from Bristol-Myers Squibb, Princeton, USA.

Organisms, growth and bioconversion conditions

Actinomadura sp strain 2966 was isolated from soil in South Korea as a rare actinomycete [11]. It has been deposited as ATCC 55678. NZ medium agar, used for preparing slants, contained the following ingredients (per liter): glucose 10 g, soluble starch 20 g, yeast extract 5 g, N-Z amine 5 g, and agar 18 g (pH 7.3). YM medium, used as liquid seed medium and growth medium, contained the following ingredients (per liter): glucose 10 g, yeast extract 3 g, malt extract 3 g, peptone 5 g (pH 6.5).

The organism was inoculated from a slant (incubated for 7 days at 30°C) into 250-ml Erlenmeyer flasks containing 20 ml of seed medium. After growth at 30°C with shaking (220 rpm) for 2 days, 2 ml of the seed culture was inoculated into 500-ml Erlenmeyer flasks containing 50 ml of growth medium. After 2 days growth under the same conditions as the seed culture, cells were harvested from 10 flasks by centrifuging the medium at $23000 \times g$ for 30 min. Cells were washed twice with water, then once with buffer A (80 mM Tris-HCl buffer, pH 7.4, supplemented with 20% glycerol and 2 mM dithiothreitol (DTT)), and stored at –80°C.

Cell-free extract preparation

Cells were taken from the –80°C freezer, thawed and 40 g (wet weight) was resuspended in 50 ml of buffer A. Cells were disrupted in a French Pressure cell. The homogenate was centrifuged at $105000 \times g$ in a Beckman Model L5-50 ultracentrifuge. The supernatant fluid was stored at –80°C as the cell-free extract.

Preparation of compactin

Compactin (100 mg) was dissolved in 1.5 ml dimethylformamide and 8.5 ml of 0.1 N NaOH was added. The solution was stirred at 70°C until the compactin dissolved. This solution was stored at 4°C for up to 2 months. Dimethylformamide had no effect on the enzymatic conversion described in this paper.

Determination of compactin and pravastatin

Compactin and pravastatin concentrations were determined by HPLC [4].

Results

Enzymatic conversion depends on the presence of a nicotinamide coenzyme

The conversion activity of cell-free extracts was tested using NADH or NADPH. The reaction mixture included 0.23 mM (=93 μg ml^{-1}) compactin, 160 μl of cell-free extract and 0.26 mM NADH or NADPH in a total volume of 220 μl. The reaction mixture was incubated in a 15-ml glass test tube, at 30°C, in a water bath shaker operated at 250 rpm. The results (Table 1) show that cell-free hydroxylation activity was observed but only in the presence of

Correspondence: Dr AL Demain, Fermentation Microbiology Laboratory, Biology Department, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[2]Present address: Institute of Biology, Hebei Academy of Sciences, Shijiazhuang, PR China
Received 22 December 1997; accepted 15 June 1998

Table 1 Activity of NADH and NADPH as H^+ donor in the enzymatic conversion of compactin to pravastatin[a]

Time (h)	Pravastatin (μg ml^{-1}) with:		
	No H^+ donor	0.26 mM NADH	0.26 mM NADPH
5.5	0	2.8	12.8
21.5	0	2.8	11.9

[a]Compactin at 0.23 mM, 160 μl cell-free extract, 220 μl total volume, 30°C, agitated at 250 rpm.

a proton donor; NADPH was more active than NADH in this respect.

Hydroxylase activity is constitutive

In our previous work [4], we observed that the bioconversion by intact cells was linear for 16 h. Pravastatin could be detected 5 min after compactin was added to the culture. We concluded that either induction of the enzyme is very fast or is not required at all. To determine whether the enzyme in strain 2966 is indeed constitutive, extracts were made from cells previously exposed to compactin as well as from cells that had not been exposed.

In this experiment, cells grown for 2 days were separated into two flasks. Compactin was added to one of these flasks at a concentration of 300 μg ml^{-1} (0.74 mM). Both flasks were incubated at 29°C, 250 rpm for 3 h. Cells were harvested and extracts were made and the enzymatic conversions were carried out. The data (not shown) clearly demonstrated that prior exposure to compactin is not required and the *Actinomadura* hydroxylase is constitutive.

Stimulation of enzymatic conversion by Mg, Fe, ATP and ascorbate

A number of factors were tested in the enzymatic conversion reaction because magnesium is a common cofactor for many enzymes, ATP may provide energy for the conversion, Fe^{3+} and Fe^{2+} are in the active site of many oxygenases, and ascorbic acid is a reducing agent often required for dioxygenase reactions. ATP plus ascorbic acid markedly improved conversion whereas $MgCl_2$ showed a slight stimulation; Fe^{3+} and Fe^{2+} had no stimulatory effect (Table 2).

An experiment on the optimization of ATP concentration in the absence of ascorbate showed that activity increased up to a concentration of ATP of 1.8 mM. In the next experiment, the effect of ascorbic acid concentration was tested in the absence of ATP; 23 mM ascorbic acid was optimal for the conversion. Table 3 shows that ATP, ascorbate and $MgCl_2$ are all needed for highest activity. The optimum $MgCl_2$ concentration was 9.1 mM.

Table 2 Stimulation of hydroxylation by $MgCl_2$, ATP plus ascorbic acid[a], and Fe salts

Additive	Concentration (mM)	Pravastatin (μg ml^{-1})
None	–	3.2
$MgCl_2$	2.4	4.4
ATP +	1.9	11.3
ascorbate	12.0	
$FeSO_4$	0.09	2.3
$FeCl_3$	0.10	3.3

[a]Compactin at 0.23 mM, NADPH at 0.72 mM, 160 μl cell-free extract, 220 μl total volume, 30°C, agitated at 250 rpm, 30 min.

Table 3 Effect of ATP, ascorbic acid, $MgCl_2$ and pravastatin on conversion[a]

ATP (mM)	Ascorbate (mM)	$MgCl_2$ (mM)	Pravastatin (μg ml^{-1})
0	0	0	9.5
2.3	0	0	16.2
3.6	0	0	19.6
0	23	0	18.4
2.3	23	0	30.4
3.6	36	2.3	37.6

[a]Compactin at 0.23 mM, NADPH at 0.78 mM, 160 μl cell-free extract, 220 μl total volume, 30°C, agitated at 250 rpm, 60 min.

Effect of other possible cofactors

α-Ketoglutarate (1 mM, 5 mM), flavin adenine dinucleotide (FAD; 0.5 mM), flavin mononucleotide (FMN; 0.5 mM), $CoCl_2$ (1 mM, 20 mM), and $NiCl_2$ (1 mM, 20 mM) were tested for their effects on the conversion but none was noted. On the other hand, $CuSO_4$ almost totally inhibited the reaction at 2.3 mM.

Effect of chelators

To confirm the importance of a metal ion for the activity of the enzyme, the effect of ethylenediaminetetraacetic acid (EDTA) on enzyme activity was tested. The metal chelator was added to the cell-free extract in different concentrations and incubated for 10 min at room temperature before the reaction was started. Magnesium was excluded from the reaction mixture. The reaction was inhibited by increasing concentrations of EDTA from 0.1 to 2 mM presumably by removal of Mg^{2+} carried over from the cells and present in the cell-free extract. The inhibitory effect of EDTA preincubation was reversed by adding $MgCl_2$ to the reaction mixture (data not shown). Similar reversal was observed with $MnSO_4$ (Table 4). From this result, it was presumed that Mn^{2+} could replace Mg^{2+} in the conversion reaction. To

Table 4 Reversal of EDTA inhibition by Mn^{2+} in the absence of $MgCl_2$[a]

Preincubation with EDTA	$MgCl_2$ concentration in reaction mixture (mM)	$MnSO_4$ concentration in reaction mixture (mM)	Pravastatin (μg ml^{-1})
No	0	0	7.0
No	9.1	0	15.9
Yes (1 mM)	0	0	1.1
Yes (1 mM)	0	5	11.8
Yes (2 mM)	0	0	0.4
Yes (2 mM)	0	5	11.2

[a]Compactin at 0.23 mM, NADPH at 0.78 mM, ATP at 3.6 mM, ascorbate at 25 mM, 160 μl cell-free extract, 220 μl total volume, 30°C, agitated at 250 rpm, 60 min.

Table 5 Effect of CDTA preincubation, dialysis and Mg^{2+} in conversion[a]

Pretreatment of cell-free extract	Mg^{2+} added to reaction mixture	Pravastatin (μg ml^{-1})
Untreated	9.1 mM	18.9
Dialysis	0	4.5
CDTA	0	1.7
CDTA, dialysis	0	1.0
CDTA, dialysis	9.1 mM	12.7

[a]Reaction conditions as in Table 4 footnote.

confirm this, Mn^{2+} and Mg^{2+} were compared for cofactor activity. In the absence of EDTA pretreatment, Mn^{2+} showed about 85% of the activity observed with Mg^{2+}. Ni^{2+}, on the other hand, showed little to no activity as an Mg^{2+} replacement. In a further experiment, it was found that a combination of a sub-optimal level of $MgCl_2$, (2 mM) and 15 mM $MnSO_4$ gave full activity. Such was not the case with a combination of 2 mM $MgCl_2$ and 1 or 5 mM $NiCl_2$.

Experiments were also done with cyclohexanediaminetetraacetic acid (CDTA) which has a higher binding affinity for metal ions than EDTA. The cell-free extract was dialyzed, or pretreated with 5 mM CDTA, or treated with CTDA and then dialyzed. Dialysis was against 10% polyethylene glycol (PEG 4000) in order to eliminate CDTA from the cell-free extract. Mg^{2+} was added to the reaction mixture at its usual concentration of 9.1 mM. The results in Table 5 show that dialysis, or CDTA treatment, or both, markedly decreased activity presumably due to removal of Mg^{2+} from the cell-free extract. Addition of Mg^{2+} to the CDTA-treated and dialyzed cell-free extract markedly increased enzyme activity.

Effect of temperature

The conversion was carried out at 28°C, 30°C and 37°C for 60 min to determine the effect of temperature. Pravastatin produced amounted to 10.8, 13.2 and 11.1 mg ml^{-1} respectively.

Effect of pH

A pH value of 7.5 is the optimum temperature for the conversion of compactin to pravastatin. The range tested was 5.0 to 8.5.

Lack of effect of CO on enzyme activity

In order to determine whether or not the hydroxylase is a heme protein, we tested the effect of CO on hydroxylase activity. Heme-containing enzymes would be expected to be inhibited by CO [3]. A cell-free extract was gently flushed with CO for 30 s before testing its activity. The subsequent conversion reaction was not influenced by the CO treatment.

Discussion

Cell-free extracts made from *Actinomadura* sp are able to hydroxylate compactin to pravastatin. The studies presented above show that the hydroxylase requires NADPH and is stimulated by ATP, ascorbate and Mg^{2+} as cofactors; Mn^{2+} can replace Mg^{2+}. The enzyme thus differs from the cytochrome P450 hydroxylating system which catalyzes the conversion of compactin to pravastatin in *S. carbophilus* in the following ways: (i) it does not need induction; (ii) it is stimulated by ATP and ascorbic acid. Unlike most heme enzymes including P450 monooxygenases [3], it is not inactivated by CO. These properties suggest that the *Actinomadura* enzyme is not only different from that in *S. carbophilus* but is also a unique hydroxylase [5,6].

Acknowledgements

The authors thank Dr Aiqi Fang for her advice and assistance on the preparation of this manuscript.

References

1 Koga T, Y Shimada, M Kuroda, Y Tsujita, K Hasegawa and M Yamazaki. 1990. Tissue-selective inhibition of cholesterol synthesis *in vivo* by pravastatin sodium, a specific inhibitor of HMG-CoA reductase. Biochim Biophys Acta 1045: 115–120.
2 Matsuoka T, S Miyakoshi, K Tanzawa, M Hosobuchi, K Nakahara and N Serizawa. 1989. Purification and characterization of cytochrome P-450$_{sca}$ from *Streptomyces carbophilus*. Eur J Biochem 184: 707–713.
3 Ortiz de Montellano P. 1995. Inhibition of cytochrome P450 enzymes. In: Cytochrome P450, Structure, Metabolism and Biochemistry, 2nd edn (P Ortiz de Montellano, ed), pp 305–364, Plenum Press, NY.
4 Peng Y, J Yashphe and AL Demain. 1997. Biotransformation of compactin to pravastatin by *Actinomadura* sp 2966. J Antibiot 50: 1032–1035.
5 Sariaslani FS. 1989. Microbial enzymes for oxidation of organic molecules. Crit Rev Biotechnol 9: 171–257.
6 Sariaslani FS and CA Omer. 1992. Actinomycete cytochromes P-450 involved in oxidative metabolism: biochemistry and molecular biology. Crit Rev Plant Sci 11: 1–16.
7 Serizawa N and T Matsuoka. 1991. A two component-type cytochrome P-450 monooxygenase system in a prokaryote that catalyzes hydroxylation of ML-236B to pravastatin, a tissue-selective inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Biochim Biophys Acta 1084: 35–40.
8 Serizawa N, K Nakagawa, K Hamano, Y Tsujita, A Terahara and H Kuwano. 1983. Microbial hydroxylation of ML-236B (compactin) and monacolin K. J Antibiot 36: 604–607.
9 Serizawa N, S Serizawa, K Nakagawa, K Furuya, T Okazaki and A Terahara. 1983. Microbial hydroxylation of ML-236B (compactin): studies on microorganisms capable of 3β-hydroxylation of ML-236B. J Antibiot 36: 887–891.
10 Tsujita Y, M Kuroda, Y Shimada, K Tanzawa, M Arai, I Kaneko, M Tanaka, H Masuda, C Tarumi, Y Watanabe and S Fujii. 1986. CS-14, a competitive inhibitor of 3-hydroxy-methylglutaryl coenzyme A reductase: tissue selective inhibition of sterol synthesis and hypolipidemic effect on various animal species. Biochim Biophys Acta 877: 50–60.
11 Yashphe J, J Davis, Y Peng and AL Demain. 1997. New micro-organisms which convert compactin to pravastatin. Actinomycetologica 11: 20–25.