A soluble multicomponent monooxygenase of *Actinomadura madurae* capable of converting compactin to pravastatin *in vitro*.

By

Edward D. Yaworski

A thesis

submitted to the Faculty of Graduate Studies

in partial fulfillment of the requirements

for the Degree of

Masters of Science

Department of Chemistry

University of Manitoba

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A soluble multicomponent monooxygenase of *Actinomadura madurae* capable of converting compactin to pravastatin *in vitro*.

BY

Edward D. Yaworski

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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ABSTRACT

Pravastatin is a potent, tissue-selective inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key enzyme in the biosynthesis of cholesterol. This active pharmaceutical ingredient is prepared from compactin by a hydroxylation reaction that is carried out by many micro-organisms including the filamentous bacterium, *Actinomadura madurae*. The work described in this thesis was aimed at characterizing the enzyme system that performs this hydroxylation in *A. madurae*. Using ion-exchange chromatography, a multicomponent compactin hydroxylase was partially purified from cell extracts of *A. madurae*. Independent assays were developed to reconstitute the bioconverting activity and used to test for the separated components. The hydroxylase system, which appears to be constitutive in *A. madurae* culture, contains at least three components. One of the protein components has been purified to homogeneity and it has similarities in sequence, composition, and molecular weight to the ferredoxins from several species of *Streptomyces*. This protein has a molecular weight of 11,876 Da by mass spectrometry and 23 kDa by SDS-PAGE, indicative of a dimeric form. From the dramatic effect of an exogenous reductase from spinach on the bioconversion rate, it is likely that a flavoprotein reductase constitutes the second electron transfer component. It was not possible to determine whether the terminal monooxygenase was a heme or a non-heme enzyme. The multi-component system characterized here is typical of cytochrome P450 (CYP)

systems in some respects, but the activity was unaffected by carbon monoxide under reducing conditions, whereas CYP activity is classically inhibited by this treatment.

ACKNOWLEDGMENTS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Organisms of terrestrial and aquatic ecosystems are exposed to a myriad of potentially toxic chemicals in the environment. Thousands, if not millions, of tons of inorganic and organic pollutants are generated each year by the many industries and modes of transportation. Some organisms are also capable of producing toxins, such as the mycotoxins of fungi, endotoxins of bacteria, and the neurotoxins of snake venom, that are hazardous to other life forms. Other chemicals produced by cellular processes are often harmful to the organism itself. Ammonia is a classical metabolic poison produced by the breakdown of proteins that can be lethal in cases of urea cycle disorder. Even endogenous biomolecules, such as hormones and prostaglandins, are treated as toxins when inactivated and excreted. Organisms of all levels of complexity have evolved detoxification systems to negate the deleterious effects of chemicals introduced by the environment (xenobiotics) or generated *in vivo* (endobiotics). The general strategy, which appears to be somewhat conserved between organisms, involves the conversion of toxins into more polar metabolic intermediates or products that are readily excreted. Discussed herein is a group of diverse and versatile oxidative enzymes called the oxygenases that partake in the process of detoxification by catalyzing an array of structural modifications to a broad range of organic substrates. They also play important roles in bioremediation, biosynthesis, and the design of novel therapeutics.

Bioremediation is the process by which microbes are exploited to degrade environmental toxins. Many microorganisms, including soil bacteria of the *Pseudomonas, Nocardia, Acinetobacter,* and *Mycobacterium* genera, contain oxidative systems that are capable of transforming organic chemicals into metabolic intermediates (Alexander, 1999a). The oxygenases are major players in this process. For example, a culture of *P. putida* is capable of using camphor as the sole source of carbon for growth and respiration. The first step of the catabolic sequence, a hydroxylation of camphor at the 5-exo position, is catalyzed by an oxygenase (Hedegaard and Gunsalus, 1965). Another soil bacterium, *Rhodococcus rhodochrous*, utilizes phenolic ether compounds by first catalyzing by way of oxygenase an *O*-dealkylation to produce catechol (Eltis *et al.*, 1993). Owing to relaxed substrate specificity, these same enzymes can detoxify a host of organic chemicals by transforming them into biologically compatible products for transport to the extracellular environment (Alexander, 1999b). Often all that is required is a subtle change to the structure. Hydroxylation by a culture of *Aspergillus niger* converts 2,4-dichlorophenoxy-acetic acid to 2,5-dichloro-4-hydroxyphenoxyacetic acid (Faulkner and Woodcock, 1964). The insecticide, lindane (hexachlorocyclohexane) is desaturated by *Escherichia coli* to 2,3,4,5,6-pentachloro-1-cyclohexene (Francis *et al.*, 1975). Tapping into the vast repertoire of microbial reactions, a culture growing in the presence of a carbon source can be used to structurally modify other non-primary, chemical substrates, a process called cometabolism (*vide infra*).

The detoxification system in humans is complex, versatile, and adaptable to a broad spectrum of chemicals from novel xenobiotics (drugs) to endogenous biomolecules. The toxins that tend to accumulate in the body are typically lipophilic or fat-soluble in nature. Many have been linked to tissue damage and the pathogenesis of cancer, immune dysfunction syndrome, and Parkinson's disease (Liska, 1998). As a means of defense, the body converts these potentially hazardous chemicals into water-soluble compounds or conjugates that are more readily excreted. This is accomplished primarily in the liver by two phases of metabolism (Liska, 1998). In phase-I, oxygenases and oxidases introduce or expose polar functional groups as in the hydroxylation of steroids, benzene, phenobarbital, and ibuprofen, and the *N, O*-dealkylation of morphine, caffeine, and codeine (Katzung, 1995). These reactions also have the potential to generate oxidants and carcinogens that are even more toxic than the parent compounds, a process called metabolic activation (Pelkonen and Raunio, 1997). In the metabolism of acetaminophen, a small amount of a reactive electrophile forms that in the absence of intracellular nucleophiles attacks cellular macromolecules, such as proteins (Katzung, 1995). The phase-II reactions are catalyzed by transferases which, act to further enhance the substrate's hydrophilicity by adding small polar molecules such as glutathione, glucuronic acid, glycine, and sulfate (Liska, 1998). These conjugation reactions also function as protective antioxidants by neutralizing some of the destructive species formed in phase-I. The two phases of metabolism work together or independently to deactivate xeno- and endobiotics.

Oxygenases

Metalloproteins possess one or more metal atoms that are essential for their structure and function. They are ubiquitous in nature and are involved in many biological processes including detoxification, biosynthesis, oxygen transport, electron transfer, and metal storage. In the past sixty years, approximately 80 or so zinc enzymes have been identified including the alcohol dehydrogenase, aldolases, peptidases, and DNA- and RNA-polymerases (Cotton and Wilkinson, 1988). The radical scavenger, superoxide dismutase and the terminal oxidase, cytochrome *c* oxidase of animals are examples of copper proteins. Iron proteins are by far the most abundant metallo-biomolecules in nature. They include hemoglobin and myoglobin, the ferredoxins, ferritins, cytochromes, peroxidases and catalases, as well as the diverse group of oxygenases. Oxygenases, also called hydroxylases, bind and activate molecular oxygen for insertion into substrate, an ability that is essential for many biological processes since most organic substrates have very little reactivity with molecular oxygen in its stable, triplet ground state (Sono *et al.*, 1996). Monooxygenases, commonly referred to as mixed-function oxidases (MFO) because they have oxygenase and oxidase activity, incorporate one atom of dioxygen into substrate while the other is reduced to water. The dioxygenases, on the other hand, incorporate both oxygen atoms into one or in some instances two substrates (Que and Ho, 1996). Hydroxylases are categorized into heme and non-heme enzymes. The heme-containing enzymes are monooxygenases that comprise the cytochrome P450 super-family. Both mono- and dioxygenases constitute the

group of non-heme enzymes that are further categorized into mono- and dinuclear iron-centers.

Cytochrome P450 Oxygenases

Cytochrome P450 (CYP) enzymes were first discovered in animal liver microsomes in 1958 (Klingenberg). Since then, they have been identified in most animals, plants, insects, fungi, and certain bacteria (Omura, 1999). Although termed hydroxylases, CYP monooxygenases actually catalyze an impressive array of reactions (Fig. 1). In animals, they catalyze many of the phase-I functionalization reactions to detoxify chemical contaminants. Others perform mainly hydroxylations in the biosynthesis (adrenal cortex, gonads) and catabolism (liver) of endogenous biomolecules, including steroid hormones, bile acids, and fatty acids (Omura, 1999). Matched to their functions, the detoxifying CYP's are promiscuous in nature whereas those involved in biosynthesis have narrow substrate specificity (Black and Coon, 1987). In plants, they participate in the detoxification of herbicides and insecticides and in the synthesis of hormones and other secondary metabolites. Bacterial CYP's are involved in the biodegradation and detoxification of environmental pollutants and have provided much insight into the physical and chemical properties of their mammalian counterparts. A distinct spectral feature, from which these pigments (P) were named, is an absorption maximum at 450 nm when bound by carbon monoxide under reduced (ferrous) conditions (Omura and Sato, 1964). The active site of these metalloenzymes contains a generic iron-protoheme IX common to many

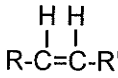
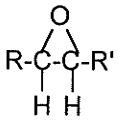
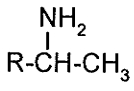
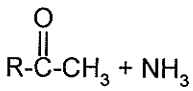
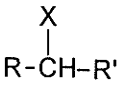
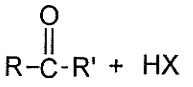
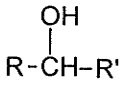
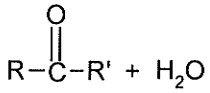
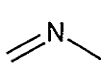
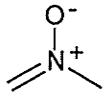
reaction	substrate	product(s)
Hydroxylation	alkyl, aryl: R-H	R-OH
	alkyl, aryl: R-NH_2	R-NHOH
Epoxidation		
O-Dealkylation	R-O-CH_3	ROH + CH_2O
N-Dealkylation	R-NH-CH_3	R-NH_2 + CH_2O
Deamination		
Dehalogenation		
Oxidation		
N-Oxidation		

Figure 1. Some of the reactions catalyzed by cytochrome P45O enzymes (Sono *et al.*, 1996).

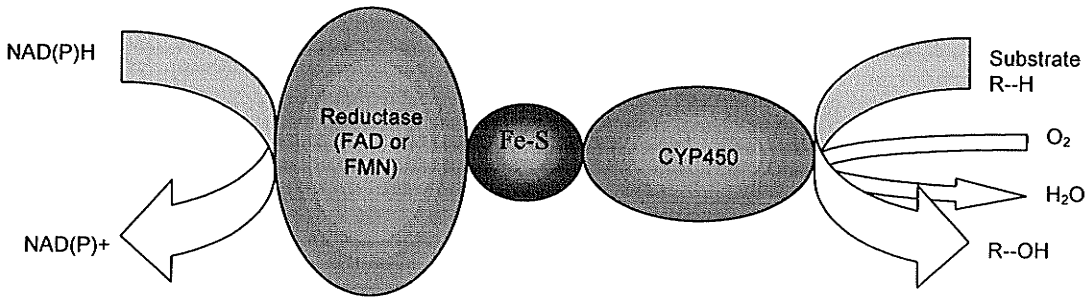
holoproteins, including the non-enzymatic hemoglobin, myoglobin, and certain electron-transfer cytochromes. The unique spectral property and inherent reactivity are attributed to the electron-donating nature of a highly conserved, axial, thiolate anion ligand provided by a cysteine of the apoprotein (Poulos *et al.*, 1985). Water serves as the sixth ligand, trans to cysteine, in the resting state.

The CYP holoenzymes of eukaryotes are usually bound to the membrane of the endoplasmic reticulum (microsomal) or inner-mitochondrion, whereas those of prokaryotes are primarily cytosolic (Fulco, 1991). They are the terminal component of a hydroxylase system that includes one or more ancillary proteins for the gated transfer of two electrons from reduced pyridine nucleotides (Fulco, 1991) (Fig. 2). Most bacterial and mitochondrial systems utilize two generally soluble proteins for transfer. The electrons are first received by an FAD or FMN containing oxidoreductase and then shuttled to the CYP-terminal oxidase via a small plant-type ferredoxin protein with a $(cys)_2FeS_2Fe(cys)_2$ cluster.

One of the first mammalian, three-component systems isolated was the steroid side-chain cleaving (SCC) monooxygenase from adrenal cortex mitochondria (Suzuki and Kimura, 1965). The components were $P450_{scc}$ (M_r = 60,000), adrenodoxin reductase (M_r = 54,000), and adrenodoxin* (M_r = 12,500) (Kimura *et al.* 1978). In bacteria, the isolated camphor hydroxylase system from *P. putida* (Cushman *et al.*, 1967; Katagiri, *et al.*, 1968) consisted of $P450_{cam}$,

*The initial CYP nomenclature system was based on the enzyme's origin, reaction catalyzed, or substrate.

A.



B.

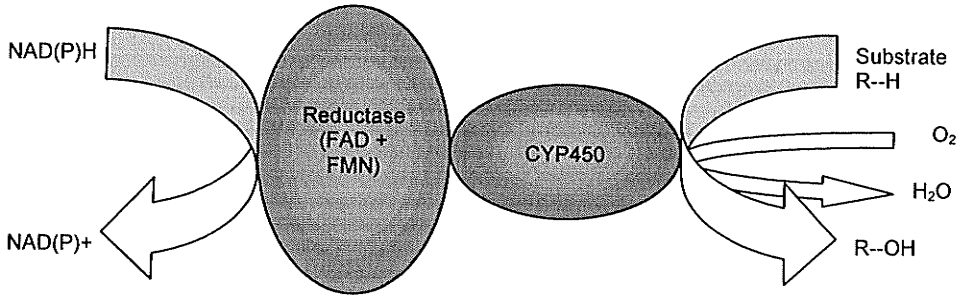


Figure 2. Components of cytochrome P450 monooxygenase systems. (A) mitochondrial and bacterial and (B) microsomal.

(M_r = 47,000), putidaredoxin reductase (M_r = 43,000), and putidaredoxin (M_r = 12,500). Microsomal systems, on the other hand, contain only two components with the CYP receiving electrons directly from a membrane-associated reductase containing both FAD and FMN. A solubilized, fatty acid ω-hydroxylase from rabbit liver microsomes (Lu and Coon, 1968) contained a 48 kDa-CYP (Black and Coon, 1987) and a reductase. This system also required a co-purified membrane lipid component, phosphatidylcholine for *in vitro* activity (Strobel *et al.* 1970). Similar two-component bacterial systems have also been isolated as soluble components from *Streptomyces carbophilus* (Serizawa and Matsuoka, 1991) and *Bacillus megaterium* (Narhi and Fulco, 1986). The fatty-acid hydroxylase system of *B. megaterium* ($P450_{BM-3}$) is unique in that both the CYP and reductase are comprised in a single fusion protein with a molecular weight of approximately 119 kDa.

Suited to their roles in metabolism and detoxification, the expression of most CYP systems can be chemically induced, usually by potential substrates. The hepatic, drug-metabolizing monooxygenases are induced by a number of chemicals including barbiturates, caffeine, ethanol, DDT, and various organic solvents (Black and Coon, 1987). As a source of carbon, the bicyclic mono-terpene camphor and its analogues induce the expression of the plasmid-encoded, 5-exo-monooxygenase system in *P. putida* (Koga *et al.*, 1985). Many chemicals can induce enzymes in different organisms as the result of broad and overlapping substrate specificities. Phenobarbital, a classical hepatic-CYP

inducing agent, also induces the $P450_{sca}$ of *S. carbophilus* (Watanabe and Serizawa, 1998) and $P450_{BM-3}$ of *B. megaterium* (Narhi and Fulco, 1981); however, phenobarbital is not a substrate for the latter. In some microorganisms the expression of oxygenase appears to be constitutive. Often, however, a foreign chemical is introduced at some point during the cultivation to cause the induction. An example is the versatile $P450_{soy}$ system of *S. griseus*, which was induced by genistein, a trace component of the soybean flour used in the growth medium (Sariaslani and Kunz, 1986). CYP activity can also be inhibited through the competitive interference with the binding of substrate and dioxygen. Inhibition by carbon monoxide under reduced conditions is a classical method used to help identify members of this super-family, however CYP enzymes may vary in sensitivity (Hansson and Wikvall, 1982). Other common chemical inhibitors include cimetidine, methadon, and SKF-525A (2-diethylaminoethyl-2,2-diphenyl valerate hydrochloride).

The heme-center of most resting CYP enzymes exists in the low-spin ferric state. Exactly how CYP activates dioxygen and hydroxylates substrate remains under investigation, since many of the key intermediary species have yet to be characterized. It is proposed, however, that a reactive heme-electrophile is responsible for the insertion of an oxygen atom between the C—H bond of a substrate (Sono *et al.*, 1996). Briefly, the reaction cycle (Fig. 3) starts with the binding of substrate by the resting iron center causing the displacement of the axial water and a shift to the high-spin state. The center then nets an electron

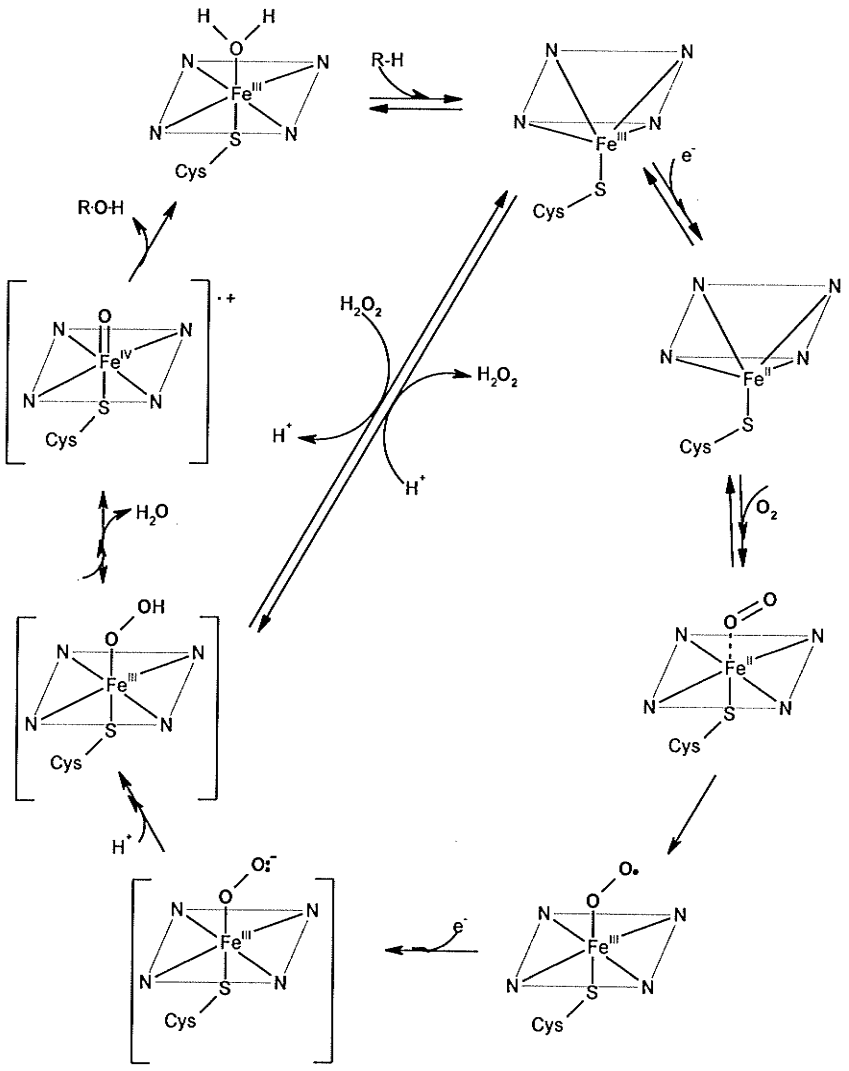


Figure 3. Proposed catalytic cycle of cytochrome P450 enzymes. The bracketed species represent structures of putative intermediates (Sono *et al.*, 1996).

(Fe^{II}) and binds dioxygen to yield a low-spin oxy-ferrous [Fe^{II}-O_2] or ferric-superoxide, [Fe^{III}-O-O•] complex. Following further reduction by a second electron and the protonation to ferric-hydroperoxide, the O—O bond is heterolytically cleaved causing the formation water and a putative, highly reactive ferryl π radical, $[Fe^{IV}=O]^{\bullet+}$, equivalent to Fe^{V}. The radical resides on the porphyrin ring or an amino acid side chain. The required protons are provided either by the direct contact with acidic distal residues, usually aspartic acid, or through a proton relay involving water (Gerber and Sligar, 1994). Oxygen is then inserted into the substrate by the oxygen-rebound process (Fig. 4), also referred to as the radical recombination process. The closely related heme-peroxidases contain a proximal histidine ligand as well as distal, non-ligated histidine and arginine or glutamine residues (Ortiz de Montellano, 1995a). These enzymes in the Fe^{III} oxidation state use an organic reductant to catalyze the reduction of hydroperoxides (ROOH) to the corresponding alcohol (ROH) and water, or two water molecules in the case of hydrogen peroxide (H_2O_2). Several CYP monooxygenases are also turned-over by hydroperoxides (Coon *et al.*, 1996) by a process called "peroxide-shunt" (Ortiz de Montellano, 1995b).

Non-heme Oxygenases

It has been only over the last ten years or so that the interest in these enzymes has rivaled the more popular CYP monooxygenases. And for the most part, the limelight seems to have been centered on the microbial systems for potential application in bioremediation and the pharmaceutical industry. Like

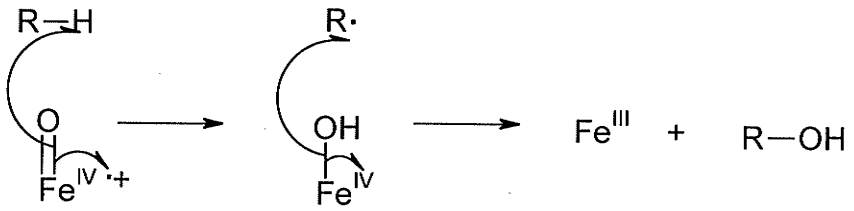


Figure 4. The oxygen rebound mechanism for the insertion of the oxygen atom into substrate (Ortiz de Montellano, 1995).

their CYP counterparts, they catalyze in addition to hydroxylation many different reactions in simple and complex organisms. The stearoyl-acyl carrier protein Δ^9 desaturase of higher plants inserts a double bond into the fatty acid of stearoyl-acyl carrier protein (ACP) to convert it to oleoyl-ACP (Fox *et al.*, 1993). Isopenicillin N synthase of fungi catalyzes the oxidative ring closure to form lactam in the biosynthesis of isopenicillin N, the precursor to penicillins and cephalosporins (Aharonowitz and Cohen, 1992). Glycine betaine is a plant osmoprotectant that is formed by a two-step oxidation process that includes the conversion of choline to betaine aldehyde by the choline monooxygenase (Meng *et al.*, 2001). Unlike the CYP monooxygenases, many are capable of inserting both atoms of dioxygen into a substrate. The lipoxygenases of plants and animals catalyze the hydroperoxidation of certain polyunsaturated fatty acids (Nelson and Seitz, 1994). During the metabolic processing of aromatic chemicals, the bacterial catechol dioxygenases catalyze the ring fission of *cis*-dihydroxylated arene substrate to dicarboxylic acid (Hirose *et al.*, 1994). Non-heme oxygenases do not exhibit the intense $\pi \rightarrow \pi^*$ absorption spectral characteristic of heme centers (Solomon *et al.*, 2000), a shortcoming that has hindered the study of function and the catalytic mechanism. They contain at their catalytic core one or two prosthetic iron centers.

Mononuclear Enzymes

The oxygenases of this center-type are primarily dioxygenases. Well studied are the substrate-inducible, aromatic ring-hydroxylating and cleaving

dioxygenases of many bacteria (Cerniglia, 1992). They function together along with other oxidative enzymes to metabolize a wide range of aromatic hydrocarbons including toluene (Fig. 5). Other prokaryotic and eukaryotic dioxygenases play integral roles in biosynthesis. Many of these catalyze the monohydroxylation of a substrate with the second oxygen of dioxygen incorporated into a co-substrate. So does the prolyl 4-hydroxylase from vertebrates that couples the hydroxylation of proline in collagens to the oxidative decarboxylation of α-ketoglutarate (Myllyharju and Kivirikko, 1997). The aromatic ring hydroxylating dioxygenases (ARHD) are part of a group of hydroxylase systems that are collectively referred to as Rieske-type oxygenases (Que and Ho, 1996). In addition to a mononuclear iron, these enzymes contain a Rieske-type iron-sulfur domain that is characterized by absorption maxima at 325, 458, and 560 nm when oxidized; the visible-absorption spectra of plant ferredoxins have peaks at 330, 422, and 460 nm (Fee *et al.*, 1986). Rieske protein with the $(cys)_2FeS_2Fe(his)_2$ cluster was first purified from beef heart mitochondria and described by Rieske *et al.* (1964). Together the mononuclear iron center and Rieske domain are termed the iron-sulfur protein (ISP) hydroxylase. Several Rieske-type dioxygenases also behave as monooxygenases by catalyzing the monohydroxylation of aliphatic and aromatic hydrocarbons (Wackett *et al.*, 1988; Spain *et al.*, 1989; Lee and Gibson, 1996). The environment of the catalytic mononuclear iron center is difficult to generalize for the members have diverse ligands and structures. Often, however, they do have more than one coordination site available to bind substrate and dioxygen.

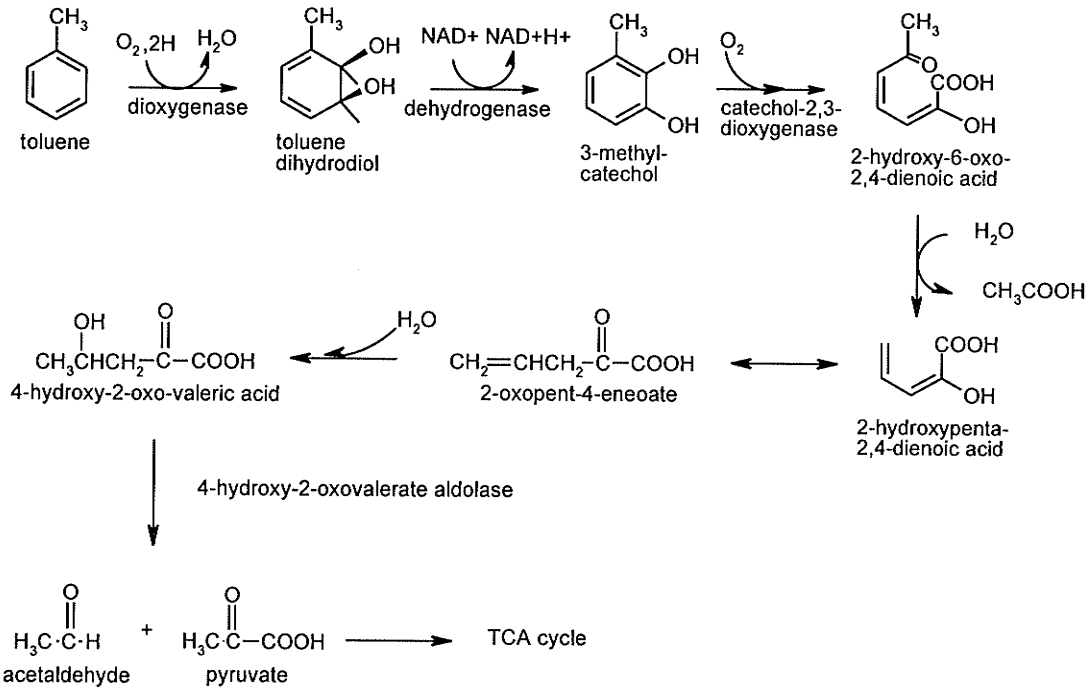


Figure 5. Catabolic degradation of toluene by *P. putida* (Zylstra and Gibson, 1991).

In contrast to the CYP monooxygenases, many of the mononuclear-type enzymes are the sole hydroxylase component. The aromatic ring-cleaving catechol 2,3-dioxygenase from *Pseudomonas* sp., an enzyme consisting of four identical 32 kDa-subunits (Harayama and Kok, 1992), does not require an electron source for activity (Que and Ho, 1996). Some dioxygenases accept electrons from non-enzymatic reductants, as is the case with a *Streptomyces* proline 3-hydroxylase (M_r = 35,000) that requires ferrous iron and ascorbic acid in addition to α-ketoglutarate for *in vitro* activity (Mori *et al.* 1997). Rieske-type oxygenases, on the other hand, do require reducing equivalents from NAD(P)H and have the necessary ancillary proteins to facilitate the transfer. Most common are the soluble, three-component bacterial systems. The toluene metabolizing *P. putida* contains a toluene 2,3-dioxygenase system consisting of a large oligomeric, ISP-hydroxylase (M_r = 147,196), an FAD-reductase (M_r = 42,942), and a ferredoxin (M_r = 11,900), all with assigned roles as in the CYP systems (Butler and Manson, 1997). A similar system also from a species of *Pseudomonas*, the naphthalene dioxygenase, contains a reductase (M_r = 35,552) with an incorporated [Fe_2S_2] cofactor (Butler and Manson, 1997). The iron-sulfur clusters outside the ISP are generally of the plant or Rieske-type. Other Rieske oxygenases use a two-in-one, reductase and ferredoxin protein as such to deliver electrons directly to the terminal component. The two-component, phthalate dioxygenase from *P. cepacia* consists of a tetrameric ISP-hydroxylase (M_r = 192,000) and a FMN, Fe_2S_2-reductase (M_r = 34,000) (Butler and Manson, 1997).

Although less well understood, the proposed activation mechanisms of the mononuclear iron center are much more diverse than the heme-CYP mono-oxygenase (Que and Ho, 1996). For one thing, the substrate often binds along with dioxygen to the iron and in specific cases, they are active in the ferric state and do not require a reductant. A catechol substrate for the aromatic ring-cleaving dioxygenases coordinates to the iron in a bidentate manner displacing ligands. Some fission enzymes in the ferrous state then bind and activate dioxygen for the nucleophilic attack of the substrate. Others in the ferric state are unable to activate dioxygen, since ferrous is a prerequisite for binding. So instead, they withdraw an electron from the substrate to activate it for the direct electrophilic attack by dioxygen. The α-keto acid dependent enzymes add an extra dimension to the mechanistic studies. These enzymes bind both a cosubstrate and dioxygen and then activate the latter for nucleophilic attack of the former to generate a reactive electrophile (Fe^{IV}=O), which then inserts an oxygen atom into a substrate. The Rieske-systems are similar to CYP mono-oxygenases and therefore, the proposed mechanism of monohydroxylation is much the same (Fig. 6). However, in this case, both of the reducing equivalents are acquired after dioxygen is bound and the second electron serves to reduce the enzyme back to the resting (Fe^{II}) state. Whether or not a non-porphyrin center can support the formation of a high-valent, iron-oxo species remains a topic of debate.

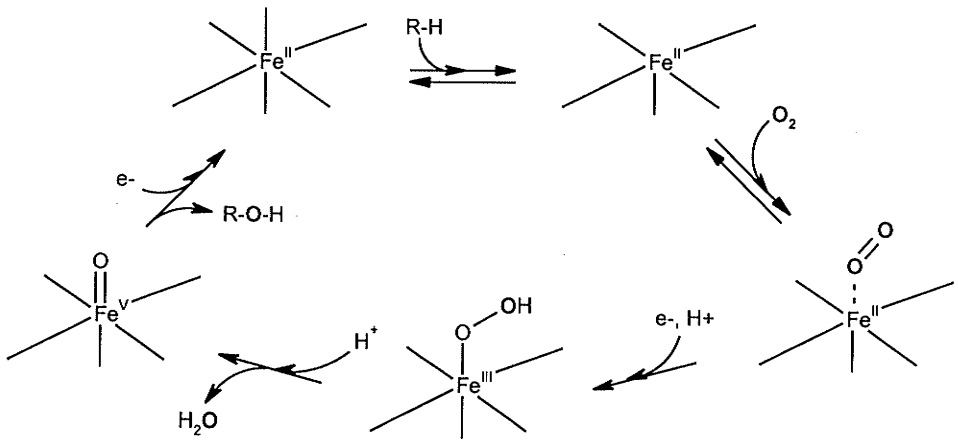


Figure 6. Postulated monohydroxylation mechanism for Rieske oxygenases (Que and Ho, 1996).

Dinuclear Enzymes

Substrate-inducible, bacterial monooxygenases constitute the majority of oxygenases of this center-type. One of the first hydroxylases isolated and by far the best characterized is the methane monooxygenase (MMO) of many methanotrophs, including *Methylococcus capsulatus* and *Methylosinus trichosporium* (Dalton, 1980). MMO catalyzes the hydroxylation of methane to methanol, the carbon source for growth and respiration. Another well-studied alkane-monooxygenase, the ω-hydroxylase of *P. oleovorans*, is also capable of hydroxylating a terminal carbon of aliphatic hydrocarbons to produce primary alcohols (Ruettinger *et al.*, 1977). These are then further oxidized to fatty acid and processed by the β-oxidation pathway yielding acetyl-CoA for intermediary metabolism. In keeping with the general metabolic strategy for aromatic substrates, many bacteria possess aromatic ring hydroxylating monooxygenases (ARHM). These enzymes catalyze a single or double-monohydroxylation leading to a *cis*-dihydrodiol intermediate, which is then converted by dehydrogenase to catechol, a substrate for the ring cleaving dioxygenases. Examples are the toluene 2-monooxygenase of *Burkholderia* (formerly Pseudomonas) *cepacia* (Newman and Wackett, 1995) and the plasmid-encoded phenol hydroxylase of *Pseudomonas* sp. (Shingler, 1989). The dinuclear center-type oxygenases are part of a group of diiron-carboxylate proteins that includes the non-enzymatic hemerythrin, an oxygen transport protein in marine invertebrates containing two irons tethered by carboxylate and oxide/hydroxide bridges (Nordlund and Eklund, 1995). Typical of the dinuclear oxygenases, each iron is five or six-coordinate

and ligated by a single histidine and several carboxylate oxygens and oxo-or hydroxo-bridges (Wallar and Lipscomb, 1996). The overall ligand environment of the dinuclear site is relatively symmetrical.

Essentially all of the diiron-carboxylate oxygenases are multicomponent and receive reducing equivalents from NAD(P)H. As expected, the usual players are involved in the electron transfer to the terminal hydroxylase component. The alkane ω-hydroxylase from *P. oleovorans* is a three-component system that resembles the mitochondrial CYP monooxygenases. It contains a soluble electron-transfer chain consisting of rubredoxin (M_r = 19,000) and rubredoxin reductase (M_r = 55,000) and an integral membrane hydroxylase component (M_r = 40,800) (Ruettinger *et al.*, 1977). Rubredoxin is a mononuclear iron-sulfur protein without inorganic sulfur, ie. $Fe(cys)_4$. A system similar to this is also proposed to partake in the catabolism of long-chain alkanes by species of *Acinetobacter* (Ratajczak *et al.*, 1998). Unique to the binuclear oxygenases, many systems include a small, cofactorless protein that is believed to be regulatory in function. The soluble, three-component system of MMO includes such an effector protein (M_r = 15,800) as well as a dimeric hydroxylase (M_r = 245,000) and a reductase (M_r = 38,400) containing FAD and an $[Fe_2S_2]$ cofactors (Lipscomb, 1994). More recently, soluble, four-component systems have been identified. The toluene-4-monooxygenase from *P. mendocina* (Pikus *et al.*, 1996) contains a dimeric hydroxylase (M_r = 220,000), a reductase (M_r = 36,000) containing FAD and an $[Fe_2S_2]$ cluster, a cofactorless effector protein (M_r =

11,600), and a Rieske-type ferredoxin (M_r = 12,500) (Wallar and Lipscomb, 1996). Other similar four-component systems include an alkene monooxygenase from a strain of *Xanthobacter* (Small and Ensign, 1997) and the phenol hydroxylase from *Acinetobacter radioresistens* (Pessione *et al.* 1999).

The catalytic core of the MMO has been used to provide much of the mechanistic information pertaining to oxygen activation and insertion. Although several mechanisms have been proposed, it is the radical recombination process (Fig. 7) similar to the CYP systems that is most widely accepted (Wallar and Lipscomb, 1996). In the resting state, the diiron-carboxylate enzyme is fully oxidized and is diferric. Upon reduction to diferrous, the active species capable of binding dioxygen, the ligand environment undergoes rearrangement with the loss of an oxygen bridge and the shift of a carboxylate ligand. Therefore, it is speculated that this step also provides the necessary protein flexibility to enable substrate entry. The binding of dioxygen then leads to heterolysis of the O—O bond, release of water, and the formation of the reactive species, a diiron-electrophile analogous to $[Fe^{IV}—Fe^{IV}]=O$, which then abstracts a hydrogen atom from the substrate and inserts the oxygen atom by the radical recombination process. Like CYP monooxygenases, MMO can be continuously turned over by hydrogen peroxide via the peroxide-shunt pathway (Froland *et al.*, 1992). However, unlike CYP's a single turnover can be obtained using non-enzymatic reductants such as sodium dithionite (Fox *et al.*, 1989). The roles of the

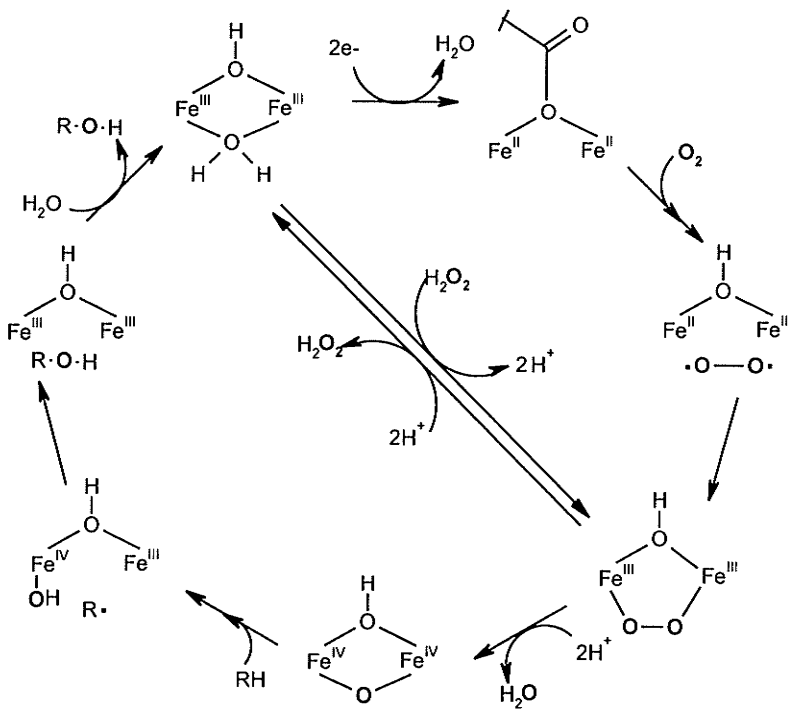


Figure 7. Proposed reaction cycle of methane monooxygenase (Wallar and Lipscomb, 1996).

reductase and small effector protein of the MMO system appear to go beyond electron transfer (Froland *et al.*, 1992). Each can affect both the activity and specificity of the reaction when the system is turned over by a non-enzymatic reductant or hydrogen peroxide. It is proposed that these components alter the structure of the hydroxylase and stabilize the intermediates of the reaction cycle.

Cometabolism

Cometabolism is the incidental transformation of xenobiotic compounds by enzymes involved in normal metabolism that provides no nutritional benefit to the microorganism (Alexander, 1999c). The MMO of methanotrophic bacteria is often used to exemplify this "fortuitous" type of metabolism. A culture of *M. capsulatus* utilizing methane can oxidize a broad spectrum of chemicals including alkanes up to pentane in size, short-chain alkenes, simple ethers, as well as heterocyclic and aromatic hydrocarbons (Dalton, 1980). The cometabolites formed are excreted into the culture medium where they accumulate. Why cometabolism occurs in microbes is an open question. Is it a means of detoxification or perhaps an attempt at catabolism in the absence of a complete set of enzymes required for mineralization? In nature, mixed microbe populations are the most successful in degrading complex chemicals. Microorganisms incapable of utilizing contaminants excrete them after structural modification to undergo further functionalization and the eventual mineralization by other microbes. The process of cometabolism provides researchers with a

plethora of regio- and enantioselective, enzymatic reactions for use as alternatives to chemical synthesis.

Biocatalysis is the basis of many industrial bioconversion processes for the preparation of personal care products, food additives, surfactants, and polymers (Thayer, 2001). Another increasingly common application is in the development of therapeutics. As an example, the solubility and bioavailability of an anti-leukaemic agent, 506U78, was enhanced by acylation using lipase from the yeast *Candida antarctica* (Mahmoudian, 1999). Other drugs prepared at least in part by bioconversion include amoxicillin, carbovir, *s*-atenolol, *s*-naproxen (Wackett and Hershberger, 2001).

This thesis describes the partial characterization of a hydroxylase system that produces the cholesterol-lowering drug, pravastatin. Pravastatin is a potent and specific inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key enzyme in the biosynthesis of cholesterol (Tsujita *et al.*, 1997). It is prepared commercially by a two-step fermentation process. The first fermentation is of *Penicillium citrinum* and is used to prepare compactin, a secondary metabolite of the mevinic acid family. In the second step, compactin is hydroxylated to pravastatin by a culture of the soil bacterium *Streptomyces carbophilus* (Fig. 8). The enzyme responsible for this conversion was identified as a compactin-inducible, soluble, two-component, CYP ($P450_{sca}$) system (Matsuoka *et al.*, 1989; Serizawa and Matsuoka, 1991). More recently, another

soil actinomycete, this one a species of *Actinomadura*, was found to carry out the conversion of compactin to pravastatin (Peng *et al.*, 1997). Apotex Fermentation Inc. (AFI) later identified the organism as *A. madurae*. Presented here is a preliminary purification of a monohydroxylase from *A. madurae* capable of converting compactin to pravastatin *in vitro*. The hydroxylase was collected in cell extract and fractionated by way of DEAE-ion exchange chromatography using developed *in vitro* assays to measure activity.

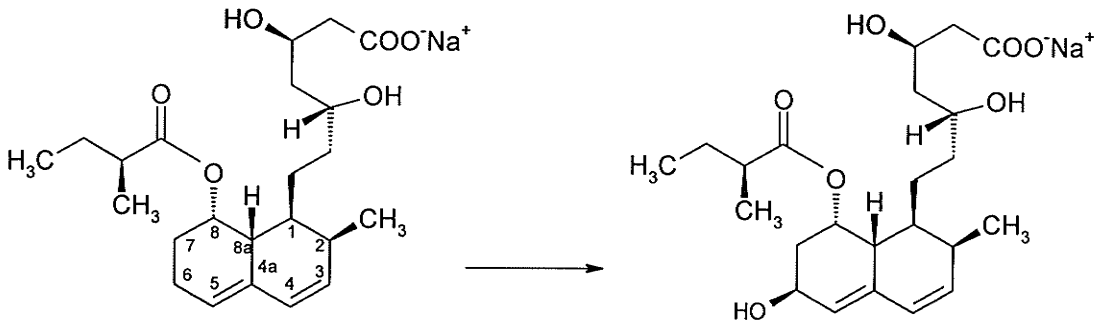


Figure 8. Microbial hydroxylation of compactin at the 6β-position to form pravastatin.

MATERIALS AND METHODS

Materials

AFI provided the strain of *A. madurae* and the lactones of compactin and pravastatin for the high-pressure liquid chromatography (HPLC) standard curves and the bioconversion reaction.

The following were purchased from sources indicated: agar, malt extract, and yeast extract from Difco Laboratories; Hy-Soy from Quest International; adenosine 5'-triphosphate (ATP), ammonium bicarbonate (NH_4HCO_3), ascorbic acid, 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), dextrose, diethylaminoethyl Sephacel (DEAE-Sephacel), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), N-(2-hydroxyethyl)- piperazine-N'-2-ethanesulfonic acid and sodium salt (HEPES), magnesium chloride ($MgCl_2$), β-mercaptoethanol (βME), reduced - nicotinamide adenine dinucleotide (NADH) and phosphate (NADPH), sodium dithionite, sodium thioglycolate, spinach ferredoxin, spinach ferredoxin-NADP+ reductase, streptomycin sulfate, Triton X-100, and bovine trypsin – TPCK treated from Sigma Chemical Co.; glycerol, organic solvents, phosphoric acid, sodium chloride, sodium phosphate-monobasic, and water-HPLC grade, from Caledon Laboratories Ltd; bovine serum albumin (BSA), Coomassie brilliant blue-R250 (CBB-R250), polyvinylidene difluoride (PVDF) membrane, protein dye concentrate, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffers and materials for gels, and Tris from Bio-Rad Laboratories Inc.;

SDS-PAGE protein ladder standards from Gibco-BRL Ltd.; carbon monoxide from Aldrich Chemical Co.; iodoacetamide from Becton Dickinson and Co.; 95% ethanol from Brenntag Canada Inc.; trifluoroacetic acid (TFA) from Pierce; centrifugal ultrafilters (3.5 and 0.5-mL) with molecular weight cut off (MWCO) 10 and 30 kDa from Pall Corp. Rolled oats (Robin Hood) and sucrose (Rogers) were purchased from nonspecific vendors.

HPLC-grade organic solvents were used in the HPLC method and the electroblotting and trypsin in-gel digestion procedures. HPLC-grade water was used in the HPLC method, Milli-Q in the SDS-PAGE and electroblotting procedures, Nano-pure for mass spectrometry solutions, and reverse osmosis water for all other applications.

Buffers and Stock Solutions

Buffer-1 (0.02 M Tris, 0.1% Triton X-100, pH 6.0) was used in the seed bank and inoculum preparations. *Buffers-2* (0.1 M HEPES, 2 mM DTT, 20% glycerol, pH 7.4) and -*3* (0.1 M HEPES, 2 mM DTT, pH 7.4) were used in the cell extract preparations and purification study. *Buffer-4* (25 mM phosphate, pH 4.0) was used in the HPLC test method. *Buffers-5* (0.5 M Tris-HCl, pH 6.8), -*6* (1.5 M Tris-HCl, pH 8.8), -*7* (62.5 mM Tris-HCl, 2% SDS, 25% glycerol, 0.01% bromophenol blue, pH 6.8), and -*8* (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) were used in the SDS-PAGE procedure. *Buffer-9* (10 mM CAPS, 20% methanol, pH 11) was used in the electrotransfer procedure. *Buffers-10* (25 mM

NH_4HCO_3) and *–11* (100 mM NH_4HCO_3) were used in the trypsin in-gel digestion.

The stock solutions of compactin, 230 and 9.10 mM, used in the bioconversion trials were prepared from compactin lactone. Briefly, lactone (MW 390.52) was combined with water and sodium hydroxide pellets added to make 1.1 molar equivalence. With stirring, the mixture was heated to and maintained at 55 – 60°C until dissolved. The solution was then cooled to room temperature and the pH adjusted to 7.4 with 5% phosphoric acid. HPLC analysis was performed to determine the concentration of the 9.10 mM solution. Other bioconversion solutions included NADPH (31 mM), ascorbic acid (960 mM), ATP (140 mM), $MgCl_2$ (270 mM), and ferredoxin-NADP+ reductase (8 u/mL). ATP and NADPH solutions were prepared fresh. Ferredoxin-NADP+ reductase solution was stored at –20°C and renewed as needed.

Organism Growth, Maintenance, and Cell Harvest

A seed bank of *A. madurae* spores was prepared in *buffer-1* supplemented with glycerol (10%) and DMSO (5%) and stored in freeze vials at -80°C. Working culture was maintained at 4°C on oatmeal-agar slants prepared as follows: rolled oats (30 g) were extracted with water (1.5 L) by stirring for 40 minutes at room temperature and the mixture filtered through cheesecloth to yield extract. Agar (20 g) was added to extract (1 L) and dissolved by heating. The hot medium was dispensed into large test tubes, autoclaved, and then

allowed to solidify at room temperature. Slants were inoculated with seed suspension (0.2 mL) and incubated at 28°C for 14 days. Inoculum was prepared from slants by scraping the spores into *buffer-1* (10 mL/3 slants) using a sterile, wooden applicator stick. A sample of the pooled spore suspension was then diluted (1/10) with 20% sucrose/*buffer-1* and the optical density at 600 nm (OD600) determined using a spectrophotometer. The bulk spore suspension was diluted with *buffer-1* to achieve a theoretical OD600 of 0.71 and then further diluted (1/3) with sterile water.

Cultivation was performed at 37°C in 250 mL shake flasks containing YM medium (50 mL). The YM medium consisted of yeast extract (3.0 g/L), malt extract (3.0 g/L), Hy-Soy (5.0 g/L), and dextrose (11.0 g/L); adjusted to pH 7.0 prior to autoclaving. Flasks were inoculated with spore suspension (3.0 mL) and incubated on a platform shaker at 200 rpm for 72 hours. After this, the flasks were pooled, dispensed (5.0 mL) into fresh YM medium, and incubated for another 25 hours. The combined culture was then cooled to 5 – 10°C and the cells collected by centrifugation at 2,000 x *g* for 10 minutes. Following washes with cold water (2 x 4 volumes) and *buffer-2* (1 x 4 volumes), the harvested cells were frozen by liquid nitrogen and stored at –80°C until required.

Preparation of Cell Extract

Cells to be extracted in the absence of glycerol were first washed with cold *buffer-3* (1 x 4 volumes). Whole cells were suspended in cold *buffer-2* or -*3* (1 volume) and disrupted by two passes at 20,000 psi through a French pressure cell. Cellular remnants were removed by centrifugation at 20,000 x g for 20 minutes at 4°C and the extract frozen by liquid nitrogen and placed at -80°C.

Concentration and Desalting of Protein Solutions

Solutions were concentrated at 4°C to a minimum volume using 3.5 and/or 0.5-mL centrifugal ultrafilters at 7,500 and 13,000 x g, respectively, and the retentates then reconstituted in *buffers-2*, *-3*, or *-11*. Units used to desalt solutions of protein destined for mass spectrometry were pre-washed with buffer to remove any membrane contaminants.

Bioconversion Reaction

Compactin was converted to pravastatin *in vivo* and *in vitro*. The pravastatin formed was measured by the HPLC test method and used to assess the hydroxylase activity. Bioconversion by culture in the second stage of growth was carried at 37°C on a shaker at 200 rpm for 6 hours with an initial compactin concentration of 1.3 mM. To allow for the possibility that the activity must be induced, the bioconversion was performed after 25 hours of growth. The reaction was stopped by dilution (1/5) of an aliquot with 50% aqueous acetonitrile. For cell extract and solutions from chromatography, the

bioconversion was performed in an aliquot (100 µL) supplemented with NADPH (0.7 mM), $MgCl_2$ (3.3 mM), and spinach ferredoxin-NADP+ reductase (0.2 u/mL, occasionally omitted). Compactin was added to make 0.21 mM and a total reaction volume of 108.8 µL. In the case of fractions, the hydroxylase system was first reconstituted by combining fractions or fraction concentrates (see Results section). Bioconversion was carried out at 30°C on a water bath shaker at 150 rpm for 60 minutes. An equal volume of 95% ethanol was added to stop the reaction. To determine the contribution of the HPLC system to the pravastatin concentration, a "deactivated" bioconversion solution was prepared (ethanol added before compactin) and analyzed with the sample set.

General Purification Procedure

All chromatography was performed at 4°C and the collected fractions were frozen by liquid nitrogen and stored at –80°C. For the preliminary purification studies, cell extract (8 – 10 mL) was loaded onto a DEAE-Sephacel column (1.9 x 5.0 cm) equilibrated in *buffer-2* or *-3*. The column was washed with buffer (30 mL) and the protein eluted with a linear gradient of NaCl (0 – 0.6 M, 70 mL), collected in 10 or 1-mL fractions. In a later study, cell extract (40 mL) was loaded onto a DEAE-Sephacel column (2.5 x 10.0 cm) equilibrated in *buffer-3*. The column was washed with buffer (100 mL) and the protein eluted with a linear gradient of sodium chloride (0 – 0.6 M, 245 mL), collected in 35-mL fractions. To further purify a hydroxylase component to homogeneity, the respective fractions were pooled and desalted. The material was then re-chromatographed on a

DEAE-Sephacel column (0.5 x 6.2 cm) using first a gradient of sodium chloride consisting of several linear segments: 0 – 0.2 M (2.5 mL), 0.2 – 0.3 M (2.5 mL), 0.3 – 0.4 M (5 mL), and ending isocratic: 0.4 M (3 mL); collected in 0.5-mL fractions.

Analytical Procedures

The dried biomass content of culture was determined in triplicate. Culture samples (10 mL) were filtered on preweighed, glass microfibre filters under moderate vacuum. After rinsing with water, the laden filters were dried at 105°C for 12 to 16 hours and the net weights determined. YM medium was also filtered and the residues dried to determine the contribution of the medium.

All spectrophotometric measurements were carried out using a Shimadzu UV-160 recording spectrophotometer with 1 cm cuvettes. The procedure used to estimate protein concentration is based on the method of Bradford (Bradford, 1976). A prepared solution of BSA protein standard (1.36 mg/mL) was aliquoted and stored at –20°C until needed. Protein dye concentrate was diluted (1/5) with water immediately before use. The standard curve solutions, up to 1.09 mg/mL BSA protein, and samples were mixed with dye solution (50 µL in 2.5 mL) and the absorbance at 595 nm measured after a 5 – 10 minute incubation period. Preparations of the standard solutions and samples were performed in triplicate, volume permitting.

A Waters LC Module-1 HPLC system equipped with a Waters 474 scanning fluorescence detector, set at 238 nm, and a Merck LichroCART RP-C18 column (4 µm, 4.0 x 75 mm) was used to determine the concentrations of compactin and pravastatin. Prior to analysis, the samples were centrifuged at 13,000 x *g* for 5 minutes to pellet the insoluble material. An aliquot (5 µL) was injected into the instrument and the components were resolved at 30°C using a programmed gradient of mobile phase-A (35% methanol/*buffer-4*) and –B (10% *buffer-4*/methanol) consisting of several linear segments (Table 1). Concentrations were derived from high or low range standard curves, 12.5 – 1250 and 2 – 200 µM, prepared by hydrolyzing the lactones of compactin and pravastatin.

The cytochrome P450 concentration was estimated by the method of Omura and Sato (1964). Briefly, a sample of test solution (2.5 mL) was purged with carbon monoxide for 1 minute and then reduced with sodium dithionite (2 mg). The reduced-CO versus reduced difference spectrum was acquired (350 – 600 nm) and the cytochrome P450 content estimated using 91 $cm^{-1}mM^{-1}$ as the molar absorptivity:

$$\text{CYP450 (nmol/L)} = 1000/91 \text{ (nmol/L)} \times [\text{OD450} - \text{OD490}]$$

Table 1. HPLC gradient method used to resolve compactin and pravastatin.

Time (min.)	Flow (mL/min.)	MP-A[a] (%)	MP-B[b] (%)
0	1.0	100	0
12	1.0	60	40
13	1.5	0	100
15	1.5	0	100
16	1.5	100	0
20	1.0	100	0

[a]35% methanol/*buffer-4*
[b]10% *buffer-4*/methanol

SDS PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (1970). The hand-cast gels (7.3 x 8.0 x 0.075 cm) comprised a stacking (4.0%T, 0.1%C) and resolving (12.0%T, 0.3%C) region prepared in *buffers-5* and *–6*, respectively. Samples were concentrated by ultrafiltration, diluted (1:2) with reducing sample buffer (50 µL βME/950 µL *buffer-7*), and denatured for 4 minutes at 95°C prior to loading (5 – 25 µL). Electrophoresis was carried out in *buffer-8* using a Bio-Rad Mini-PROTEAN-II Cell under constant voltage (130 V) for approximately 70 minutes or until the tracking dye reached the end of the gel. To view the bands, the gels were placed in an aqueous staining solution containing CBB-R250 (0.05%), methanol (40%), and acetic acid (10%) and gently mixed on a platform shaker for 30 minutes. The gels were then destained with an aqueous solution of methanol (30%) and acetic acid (10%) until an almost colorless background was obtained. The standard protein ladder used to estimate protein size consisted of 12 bands (10 – 120 kDa) in 10 kDa increments and a 200 kDa band; 5 µL of standard solution was loaded per lane.

Electroblotting

Protein was electro-transferred from polyacrylamide gel to PVDF membrane according to the procedure of Matsudaira (1987). SDS-PAGE was performed as described above with the following steps to minimize the modification of protein. The monomer solutions were degassed under vacuum

for 15 minutes prior to polymerization and the gels were allowed to set for approximately 24 hours prior to use. Sodium thioglycolate was added to the running buffer to a final concentration of 0.5 mM. Following electrophoresis, the gel was equilibrated in *buffer-9* for 30 minutes; an exchange was made with fresh buffer after 15 minutes. The electro-transfer to PVDF was carried in *buffer-9* under constant voltage (25 V) for 60 minutes using a Bio-Rad Mini Trans-Blot Cell with a cooling block. The blot was stained for 50 seconds in an aqueous solution of CBB-R250 (0.1%), methanol (40%), and acetic acid (1%) and then destained with 50% methanol until the background was pale blue. After drying, the bands were excised using a new, grease-less scalpel blade and placed in pre-rinsed microfuge tubes.

Amino Acid Analysis and N-terminal Sequencing

Amino acid analysis and N-terminal sequencing were carried out at the W.M. Keck Facility at Yale University. Protein hydrolysis was performed *in vacuo* at 115°C for 16 hours by 6N HCl (100 µL) containing phenol (0.2%) and norleucine (2 nmol); the latter an internal standard to correct for the losses from sample transfers and drying. Following the digestion, the hydrolysate was dried by Speedvac, reconstituted in Beckman sample buffer (100 µL) containing homoserine (2 nmol) as the internal standard, and run on a Beckman 7300 amino acid analyzer. Data analysis was performed with Perkin Elmer/Nelson data acquisition software. Sequencing of the amino-terminus was carried out on

an Applied Biosystems Procise 494 instrument equipped with an on-line HPLC for the identification of phenylthiohydantoin (PTH) -amino acid derivatives.

Trypsin In-gel Digestion

Protein was digested and the peptide fragments extracted from a CBB-R250 stained, polyacrylamide gel slice based on the procedure described by Shevchenko *et al.* (1996). After washing thoroughly with water (5 x 1 mL), the band was incubated in *buffer-10* (100 µL) for 15 minutes and then crushed into small pieces. Acetonitrile (100 µL) was added, incubated for 15 minutes, and the solution removed following centrifugation at 10,000 x g for 2 minutes. The gel pieces were washed with 50% acetonitrile/*buffer-10* (3 x 200 µL) and then dried in a vacuum centrifuge. Reduction and alkylation with DTT and iodoacetamide, respectively, were performed in *buffer-11*. The digestion was performed with 0.05 µg of trypsin in *buffer-10* at 40°C for 45 minutes. After this, the preparation was overlaid with *buffer-10* and placed at 37°C for overnight digestion. TFA (10 µL) was added, mixed and the supernatant collected after a brief incubation period. The gel pieces were extracted with 0.1% TFA by sonication for 30 minutes and the supernatant collected. Extraction was then repeated with 30 and 60% acetonitrile/0.1% TFA and finally acetonitrile. The extracts were combined and dried in a vacuum centrifuge.

Mass Spectrometry (MS)

Nano-electrospray ionization (nano-ESI) – MS was carried out using an electrospray time-of-flight mass spectrometer modified to allow nanospray. Data analysis was performed by TOFMA. Matrix assisted laser desorption ionization (MALDI) – MS was carried out on a MALDI QqToF built at the University of Manitoba (prototype for SCIEX orthogonal MALDI) according to the procedure of Shevchenko *et al.* (2000). Peptides were matched by a database search with ProFound (http://prowl.rockefeller.edu).

RESULTS

PROCEDURE DEVELOPMENT

The procedures provided by AFI for the growth and maintenance of the organism helped to expedite the study. However, additional development work was necessary before the purification could start. Firstly, the success at purification was greatly dependent on the amount of hydroxylase provided by the cultivation process. With this in mind, the age of second stage culture was determined for good bioconversion activity. From the assumption that the hydroxylase is soluble, a procedure was then developed to extract the cellular makeup. The enzyme assay used to measure the bioconversion activity of culture was also provided by AFI. An assay like this was also required for the solutions collected from chromatography. Using cell extract, an *in vitro* assay for the conversion of compactin to pravastatin was developed. Purification by chromatography often leads to dilute solutions of protein with high salt content. Again working with extract, a method for concentrating and desalting protein solutions was developed. The stability of the hydroxylase was of utmost concern for the conservative expression of a sensitive enzyme could disable the study. Experiments were performed to determine if special treatment was necessary to preserve the bioconverting content of whole cells and cell extract.

Culture Growth

Growth of culture in the first stage was performed unchanged from the AFI procedure. A typical culture of *A. madurae* contained a mixture of filamentous and pellet-like mycelia. By a single measurement, the dried biomass content was 8.41 g/L after 72 hours of growth at 37°C. The growth duration of second stage culture for the optimum expression of the hydroxylase was determined using the *in vivo* bioconversion assay. Culture was grown at 37°C for a total of 50 hours during which, at timed intervals, compactin was added to a concentration of 1.3 mM in triplicate flasks and allowed to convert for 6 hours. The bioconversion reaction was carried out starting at 5 hours of growth and then every 5 hours for up to 30 hours and finally at 40 and 50 hours of growth. Samples of the bioconversion mixtures were removed after 3 and 6 hours for HPLC analysis to assess the reaction rate. Only the 3-hour conversion results are averaged and reported (Table 2) as most flasks contained no compactin after 6 hours. To assess the amount of growth during the conversion, flasks were harvested at the start and end of the incubation period and the biomass content determined (Table 3). The initial biomass content was used to represent the growth of the culture and calculate the specific activities (Fig. 9). From this study, it was decided to attempt to purify the hydroxylase from extract of second stage culture grown for 25 hours, when the bioconversion rate in culture begins to plateau.

Table 2. Bioconversion by second stage cultures after 3 hours of incubation with compactin at 1.3 mM.

Culture age (hours)	Average content [std.dev.] (mM)	
	pravastatin[a]	compactin
5	0.02 [<0.01]	1.32 [0.03]
10	0.08 [<0.01]	1.27 [0.04]
15	0.21 [0.01]	1.05 [0.06]
20	0.36 [0.01]	0.79 [0.02]
25	0.47 [0.02]	0.61 [0.04]
30	0.56 [0.02]	0.48 [0.03]
40	0.53 [<0.01]	0.50 [0.03]
50	0.43 [<0.01]	0.66 [<0.01]

[a]These data were used to calculate the specific activities plotted in Figure 9.

Table 3. Dried biomass content of the bioconversion cultures before and after the 6 hour incubation period.

Culture age (hours)	Average, dried biomass content [std.dev.] (g/L)	
	start[a]	end
5	0.94 [0.37]	0.93 [0.16]
10	1.69 [0.21]	3.09 [0.31]
15	3.64 [0.48]	3.92 [2.22]
20	4.86 [0.31]	4.92 [0.27]
25	5.95 [0.19]	5.95 [0.20]
30	6.60 [0.31]	6.87 [2.42]
40	7.83 [0.24]	7.47 [0.19]
50	8.23 [0.15]	7.66 [0.38]

[a]These data are plotted in Figure 9.

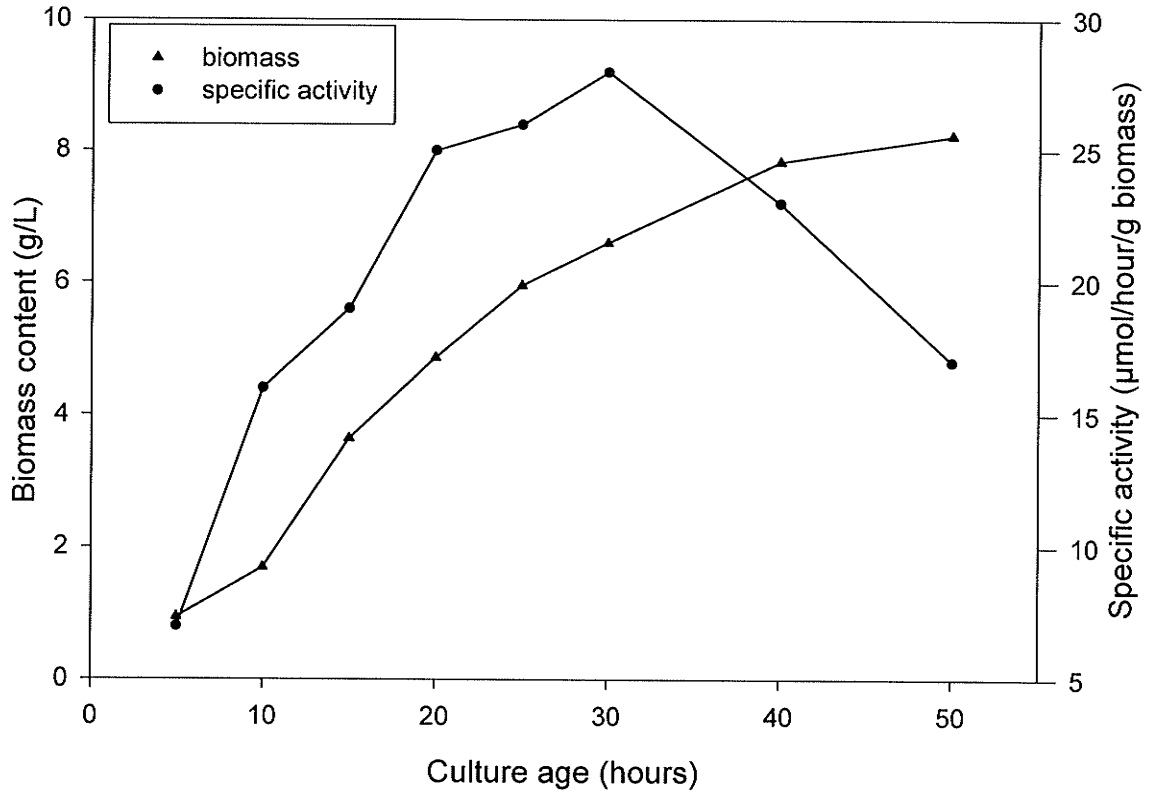


Figure 9. Growth and specific activities of second stage culture. The dried biomass data are from Table 3 (start) and the specific activities were calculated using the 3-hour bioconversion data from Table 2.

Two large batches of culture, approximately 4 L each, were prepared for the bioconversion and purification studies. The first preparation, *batch-A*, contained 61 and 5.17 g/L wet and dried biomass, respectively. The dried biomass content of *batch-B* was not determined; however, it contained 59 g/L wet biomass. At harvest, the culture was portioned and the cells collected (9 – 10 g ea.) were stored at –80°C (refer to stability study) after washing with water and buffer.

Cell Extraction

A French press, sonicator, and mortar and pestle were evaluated as disruption methods for the extraction of hydroxylase into buffer. The optical density at 260 nm (OD260) and microscopic examination were used to assess the effectiveness of each method. Washed cells used in the French press and sonicator preparations were first suspended in chilled *buffer-2* (4°C, 4 volumes). Two passes were performed at 20,000 psi through a pressure cell. On ice, the sonication was performed in 1-minute pulses for 10 minutes and then continuous for 5 minutes. The temperature of the mixture reached at maximum 7°C. Cells ground by mortar and pestle were first mixed with an equal weight of washed sea sand and then frozen by liquid nitrogen. Following pulverization, the powder was suspended in *buffer-2* to extract. The cell debris was removed from the extracts by centrifugation. To clarify the extracts, streptomycin sulfate (2% wt/vol) was dissolved and the centrifugation repeated after a brief incubation period. Buffer dilution (1/10) of the French press, sonicator, and mortar and pestle extracts

gave OD260 readings of 1.3, 0.6, and 0.5, respectively. Spent cells from the press procedure were colorless and shard-like in appearance, whereas much of the mycelia remained intact by the other procedures. Clearly, the French press was the superior method for preparing cell extract.

The extracts of this study were prepared with an equal volume of buffer on 9 to 10 g of wet cells, with the exception being the final preparation where 40 g was used. Extracts of *batch-A* and *–B* cells contained 8.4 – 13.9 and 5.2 – 8.7 mg/mL protein, respectively. Freshly prepared extract was promptly frozen by liquid nitrogen and placed an –80°C (refer to stability study) and typically, processed within 15 – 20 hours.

In vitro Bioconversion Reaction

The bioconverting activity was most likely a typical mixed-function oxidase, requiring oxygen and a co-reductant like NADH or NADPH. Using cell extract as the source of enzyme, a host of chemicals were tested to facilitate the conversion of compactin to pravastatin. Lysate (175 µL) supplemented with NADPH or NADH (0.8 mM), ascorbic acid (24 mM), ATP (3.6 mM), and $MgCl_2$ (6.8 mM) was unable to convert compactin at 0.23 mM; reaction volume 200 µL. An experiment was performed to determine if the hydroxylase of a culture must be induced with compactin. A second stage culture grown for 25 hours was fed compactin to a concentration of 1.3 mM and incubated for 6 hours. All of the compactin was consumed within the duration, but only 0.70 mM pravastatin was

formed. The cells were harvested and extracted and the bioconversion reaction tested. Again, no activity was observed using the described reaction solution. Keeping with the "induction" step for preparing cells, the testing continued with other components.

Hydrogen peroxide (10, 25, and 50 mM) and spinach ferredoxin (1.6 mg/mL) were ineffective. Pravastatin formation was first observed with the addition of spinach ferredoxin-NADP+ reductase (0.2 u/mL); reaction volume 205 µL. However, later experiments revealed that the exogenous reductase was not absolutely necessary for activity, although it did increase the amount of pravastatin formed. Including spinach ferredoxin did not improve the activity at this point. NADPH provided almost twice the activity as NADH and doubling the concentration of NADPH had no effect. An NADPH-regenerating system consisting of glucose 6-phospate (14 and 28 mM) and glucose 6-phosphate dehydrogenase (0.2 and 0.4 u) was also tested without effect.

Next, the need to pre-incubate the cells with compactin was tested. Extracts were prepared from "induced" and "non-induced" cells and their activities determined. Compactin incubated culture contained 6.45 g/L dried biomass and the respective extract, 8.2 mg/mL protein. Similarly, a culture grown for 31 hours without compactin contained 6.74 g/L biomass and 9.0 mg/mL protein in the extract. A set of bioconversion trials was performed in each extract for 30, 60, 90, and 120 minutes (Fig. 10). The specific activities of the

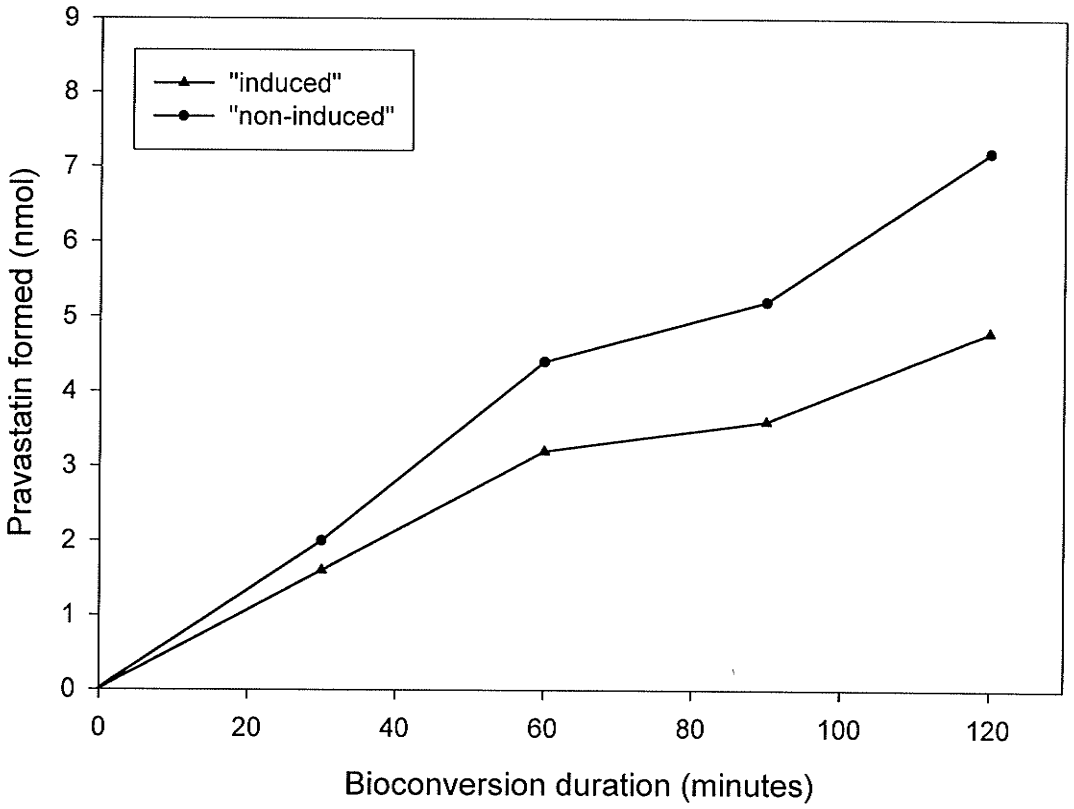


Figure 10. Bioconversion by cell extracts prepared from "induced" and "non-induced" cells. The reaction solutions contained NADPH (0.8 mM), ascorbic acid (24 mM), ATP (3.6 mM), $MgCl_2$ (6.8 mM), ferredoxin-NADP+ reductase (0.2 u/mL), and compactin (0.23 mM).

"induced" and "non-induced" preparations were 37 and 47 nmol/min/g protein, respectively, for up to 60 minutes. Past this, the enzyme activity began to drop off. Thus, the pre-exposure of cells to compactin does not improve the content of bioconverting activity.

The bioconversion assay at hand consistently converted compactin to pravastatin. However, it contained too many components and provided only marginal activity and therefore the development continued. The reductase cofactors, FAD and FMN, were each tested at 10 µM and had a slight negative effect. At the same time, it was discovered that halving the concentrations of ATP, $MgCl_2$, and ascorbic acid increased the activity; reaction volume 192.5 – 197.5 µL. Ascorbic acid was later found to depress the activity, whereas ATP had no effect. As the final modification, the lysate volume was reduced because of the high cost of spinach ferredoxin-NADP+ reductase. The bioconversion adopted from these findings contained lysate (100 µL), NADPH (0.7 mM), $MgCl_2$ (3.3 mM), ferredoxin-NADP+ reductase (0.2 u/mL, occasionally omitted), and compactin (0.21 mM); reaction volume 106.3 – 108.8 µL.

The extract preparations from *batch-A* and *–B* cells varied in converting ability. Presented in Table 4 are the bioconversion results from some of the extracts prepared. Adding glycerol to the extraction buffer (*buffer-2*) did not appear to provide an advantage at this point. *Buffer-2* was used for most of the preparations simply because it was the first buffer of choice and there was plenty

Table 4. Bioconversion activity of some of the extract preparations.

Cell batch	Extract	Per mL of extract		Specific activity (nmol/min/g)
		protein (mg)	activity[a] (nmol/min)	
A	1	8.4	0.89	110
	2	10	1.4	140
	3	9.5	0.76	80
	4	14	1.3	94
	5	7.9	1.3	160
	6	8.5	0.89	100
B	1	8.7	0.62	71
	2	7.1	0.61	86
	3[b]	5.2	0.38	73

[a]Bioconversion solutions contained NADPH (0.7 mM), $MgCl_2$ (3.3 mM), ferredoxin-NADP+ reductase (0.2 u/mL), and compactin (0.21 mM).
[b]Prepared in *buffer-3*; the other preparations were in *buffer-2*.

of it. When added, ferredoxin-NADP+ reductase increased the activity by 2 – 3 fold. A solution of the reductase (8 u/mL) was prepared fresh each time until the bioconversion by a *batch-B* extract showed similar activities with fresh and aged (-20°C, 5 months) solution. To demonstrate the reproducibility of the assay for cell extract, the bioconversion was performed in triplicate at three concentrations of compactin: 0.11, 0.21, and 0.41 mM. Using the data, K_m and V_{max} values of 0.33 mM and 270 nmol/min/g protein were estimated by non-linear regression (Fig. 11).

Concentration and Desalting of Protein Solutions

Ultrafiltration (UF) as a method for concentrating and desalting solutions of the hydroxylase was tested using cell extract. *Buffer*-2 cell extract (500 µL) was filtered to a minimum volume using centrifugal ultrafilters with 10 and 30 kDa MWCO at 13,000 x *g*, 4°C. The process was very slow and required 2 – 3 hours to complete. Retentates from the two membrane cut off's were then dissolved in 500 µL of each their respective filtrate and fresh buffer and the bioconversion reaction performed (Table 5). The filtration reduced the activity by 50% in comparison to the cell extract. Interestingly though, a part of the lost activity was restored by recombining the retentate and filtrate. This indicated that a small component was removed by the procedure. The filtration of an extract in *buffer-3* was also very slow and again a large part of the activity was lost.

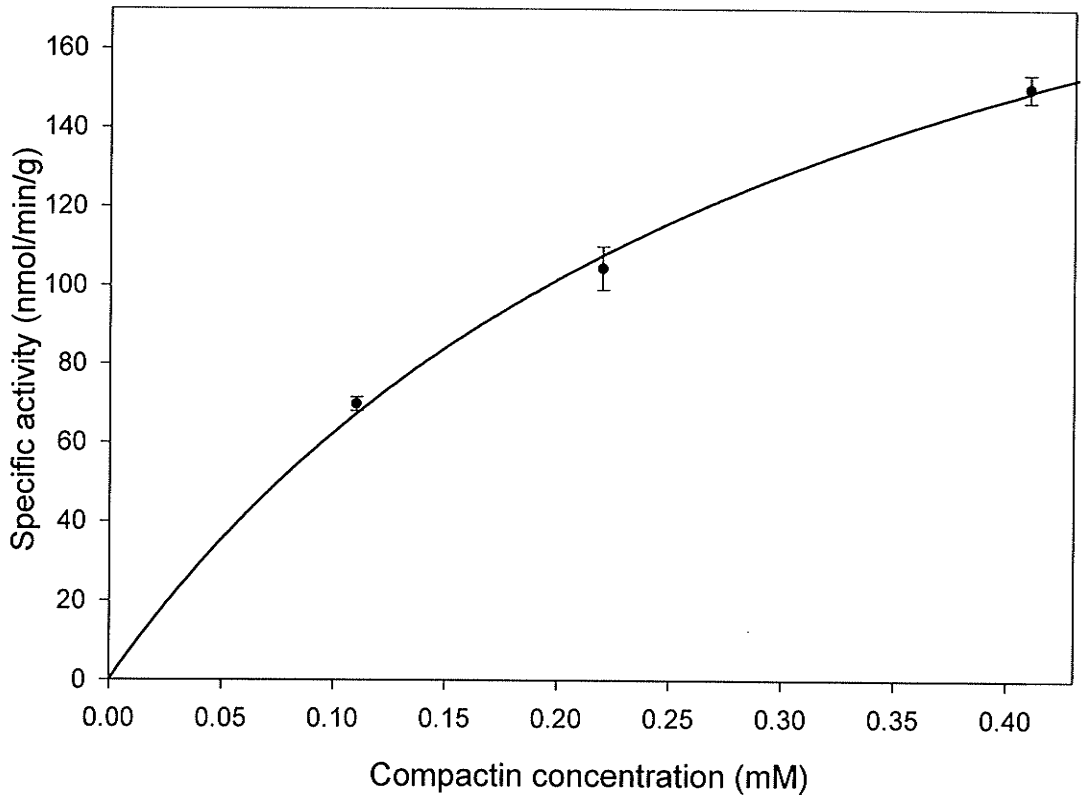


Figure 11. Effect of compactin concentration on the bioconversion rate by cell extract. The reaction solutions contained NADPH (0.7 mM), $MgCl_2$ (3.3 mM), ferredoxin-NADP+ reductase (0.2 u/mL), and compactin at 0.11, 0.21, and 0.41 mM.

Table 5. Bioconversion by *buffer*-2 cell extract after UF.

Filter MWCO (kDa)	Solution[a]	Activity[b] (nmol/min)
	extract	0.28
10	buffer	0.14
	filtrate	0.22
30	buffer	0.14
	filtrate	0.20

[a]After filtration, the retentates were dissolved in buffer and ultrafiltrate.
[b] Bioconversion solutions contained NADPH (0.8 mM), ascorbic acid (12 mM), ATP (1.8 mM), $MgCl_2$ (3.4 mM), and compactin (0.23 mM).

A diafiltration experiment was performed to see if the displaced component could be washed from the hydroxylase. *Buffer-2* extract was filtered using 10 kDa centrifugal units and the concentrates then dissolved in fresh buffer to make the starting volume. The filtration and dissolution was repeated two additional times. Enough filter units were used to allow a retentate from each filtration to be reconstituted in buffer and the accompanying filtrate for use in bioconversion trials (Table 6). The greatest loss of activity was from the first filtration where after only 33% of the cell extract activity remained. This could be increased 2-fold by recombining the concentrate and filtrate. The reduction in activity from the last filtration (wash) was caused by something other than the displaced component.

Potential cofactors, including spinach ferredoxin (0.1 mg/mL), FAD and FMN (10 μM), nicotinamide (1.0 mM), cysteine (1.0 mM), $FeSO_4$ (0.1 mM), $FeCl_3$ (0.1 mM), $ZnSO_4$ (0.1 mM), $CuSO_4$ (O.1 mM), ascorbic acid (1.0 mM), combinations of ascorbic acid with $FeSO_4$ and $FeCl_3$, NADPH (2 x 0.7 mM), and $MgCl_2$ (2 x 3.3 mM), were tested and unable to replace the lost component(s). Most, with the exception of nicotinamide, cysteine, $FeSO_4$, $FeCl_3$, and the additional $MgCl_2$, had a negative effect on activity. To assess the stability of the small component, the bioconversion was performed in solutions of the hydroxylase prepared by dissolving UF-concentrate in fresh and aged (-80°C, 16 days) ultrafiltrate. Similar activities were observed for each indicating that the component is quite stable.

Table 6. Bioconversion by diafiltered *buffer*-2 cell extract.

Filtration	Solution[a]	Per mL of solution		Specific activity (nmol/min/g)
		protein (mg)	activity[b] (nmol/min)	
	extract	8.4	0.43	51
1	buffer	6.7	0.14	21
	filtrate	7.8	0.28	36
2	buffer	7.1	0.12	17
	filtrate	8.1	0.14	17
3	buffer	6.2	0.089	14
	filtrate	6.4	0.089	14

[a]After the filtration, the retentates were dissolved in buffer and filtrate.
[b]Bioconversion solutions contained NADPH (0.7 mM), $MgCl_2$ (3.3 mM), and compactin (0.21 mM).

The concentration of cell extract by UF was a slow process that greatly reduced the converting content. A large part of this lost activity was accounted for by the removal of a small component from the hydroxylase system. It was anticipated, however, that the filtration rate of hydroxylase solution would improve after the first purification step and that the small component would be lost anyway during the purification. Therefore, UF was to be tried with column fractions.

Hydroxylase Stability

A small batch of culture was prepared, divided into three equal portions and the cells harvested. One cell portion was extracted immediately and the activity determined while the others were stored at –80°C. After 14 and 27 days of storage, another portion was extracted and the bioconversion performed (Table 7). The hydroxylase was very stable in whole cells.

In a similar study, a *buffer-2* extract was prepared and divided into four portions after the bioconversion was performed in an aliquot. Two portions were frozen by liquid nitrogen and placed at –80°C and the others stored at 4°C. After 2 and 4 days, the activities of the extracts were determined at each temperature (Table 8). The preservation of active hydroxylase was good in extract at –80°C and did not require protease inhibitors.

Table 7. Stability of the hydroxylase in whole cells[a] at –80°C.

Age of cells (days)	Per mL of extract		Specific activity (nmol/min/g) x 10^{-1}
	protein (mg)	Activity[b] (nmol/min)	
fresh	11	1.2	11
14	9.9	1.1	11
27	9.7	1.2	12

[a]Cells were stored in sealed polypropylene centrifuge tubes.
[b]Bioconversion solutions contained NADPH (0.8 mM), ascorbic acid (12 mM), ATP (1.8 mM), $MgCl_2$ (3.4 mM), ferredoxin-NADP+ reductase (0.2 u/mL), and compactin (0.23 mM).

Table 8. Stability of the hydroxylase in *buffer-2* cell extract[a].

Storage temp. (°C)	Activity[b] per mL extract (nmol/min)	
	2 days	4 days
4	0.19	0.023
-80	0.43	0.38

[a]Extract was stored in sealed polypropylene centrifuge tubes.
[b]Bioconversion solutions contained NADPH (0.8 mM), ascorbic acid (24 mM), ATP (3.6 mM), $MgCl_2$ (6.8 mM), ferredoxin-NADP+ reductase (0.2 u/mL) and compactin (0.23 mM). Pravastatin formed by fresh extract – 0.53 nmol/min/mL.

INHIBITION BY CARBON MONOXIDE

The requirement for NAD(P)H in the bioconversion assay and the positive effect of exogenous reductase on the activity were good evidence of a multicomponent system. Inhibition by carbon monoxide was tested to determine if a cytochrome P450 enzyme was responsible for the conversion. *Buffer-2* extract containing 11.1 mg/mL protein was diluted (1/5) with buffer to attain an OD400 reading of 0.56. After saturating with carbon monoxide, the solution was reduced with sodium dithionite and the reduced-carbon monoxide versus reduced spectrum acquired; the extracted data are re-plotted in Figure 12. The cell extract contained 0.7 nmol/L or 60 pmol/g protein of cytochrome P450 content. Prior to testing the inhibition of activity with cell extract, the formed carbon monoxide complex was found to be stable for at least 20 minutes in a dilute extract at room temperature. Cell extract that was reduced with and without carbon monoxide present tested with similar activities. Therefore, a non-heme enzyme may be responsible for the hydroxylation. Interestingly, the addition of sodium dithionite reduced the activity of the extract by nearly 70%.

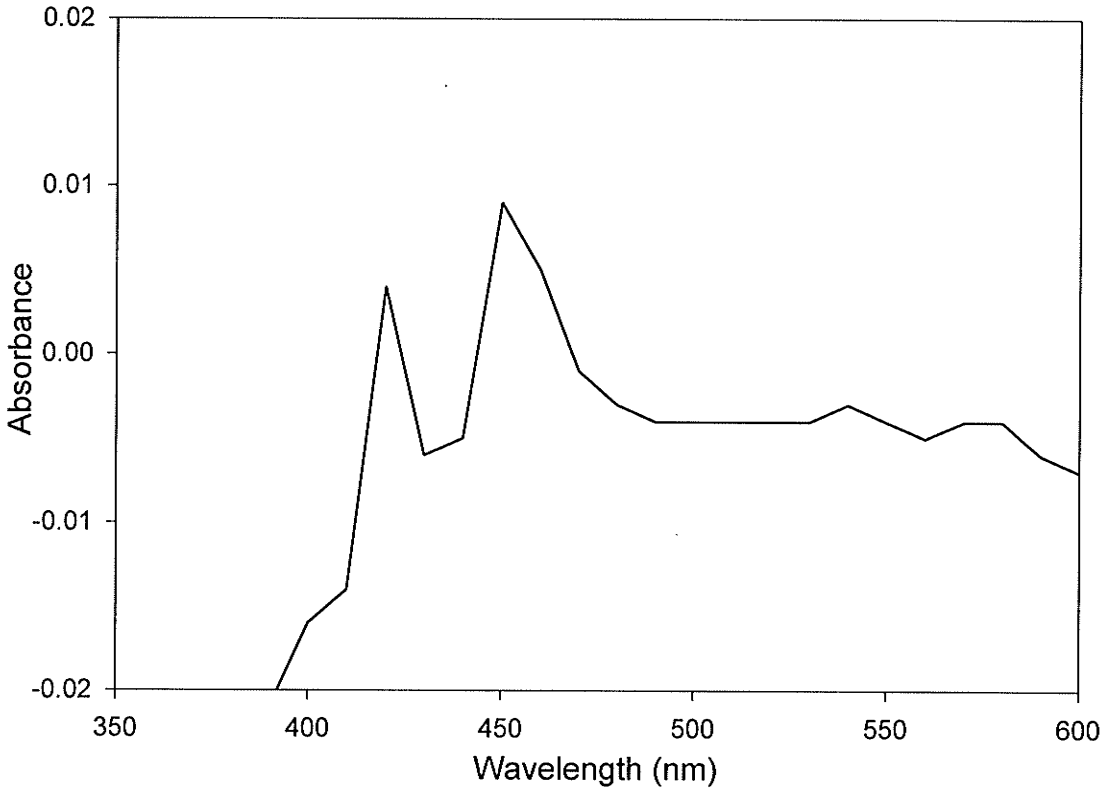


Figure 12. Reduced-CO versus reduced difference spectrum of cell extract. A 2.5 mL aliquot of extract was purged with carbon monoxide for 1 minute and then reduced with 2 mg of sodium dithionite. The spectrum of the solution was acquired with a reduced solution in the reference cell.

PURIFICATION OF THE HYDROXYLASE

Method Development

In this section, ion-exchange chromatography (IEC) was evaluated to resolve the components of crude cell extract. An experiment was performed first to show that the hydroxylase could be recovered with bioconversion activity from the ion-exchange resin. A column of DEAE-Sephacel (1.9 x 5.0 cm) was charged with cell extract protein (89 mg) in *buffer-2* (8.0 mL) and then washed with buffer (30 mL) and 0.4 M NaCl/buffer solution (40 mL). Approximately 72% of the protein was recovered from the column (Table 9); the flow-through and buffer wash each contained 3%. The NaCl wash was concentrated 5-fold by UF and the bioconversion performed. Little activity (~20%) was recovered in this preliminary experiment and the specific activity decreased 3.4-fold. Combining the NaCl concentrate and flow-through, also concentrated 5-fold, did not increase the activity. Much of the lost activity probably resulted from the removal of the small hydroxylase component that was previously identified in ultrafiltrate.

The next task was to fractionate the components of crude extract and then reconstitute the bioconverting activity. Cell extract (8 – 10 mL) was loaded onto a DEAE-Sephacel column (1.9 x 5.0 cm) equilibrated in buffer. After washing with buffer (30 mL), the protein was eluted with a linear gradient of NaCl (0 – 0.6 M, 70 mL), collected in 10-mL fractions. Several columns were run and each provided fractions that failed to convert compactin to pravastatin using the developed assay. Even the small endogenous cofactor present in 10 kDa

Table 9. Recovery of the hydroxylase from DEAE-Sephacel resin.

Solution	Volume (mL)	Protein (mg)	Total activity[a] (nmol/min)	Specific activity (nmol/ min/g) x 10^{-1}
cell extract	8.0	89	8.2	9.2
0.4 M NaCl eluate, 5x concentrated	9.0	59	1.6	2.7

[a]Bioconversion solutions contained NADPH (0.8 mM), ascorbic acid (12 mM), ATP (1.8 mM), $MgCl_2$ (3.4 mM), ferredoxin-NADP+ reductase (0.2 u/mL), and compactin (0.23 mM).

ultrafiltrate had no effect when added as a freeze-dried concentrate. Hydroxylase activity, however, was observed when several of the fractions were pooled. Cell extract protein (100 mg) in *buffer-2* was chromatographed (Fig. 13) and equal aliquots of the proteinaceous fractions 3, 4, and 5 and tailing fractions 6 and 7 were combined into eluate composite (EC) solutions –1 and –2, respectively. Five-fold concentrates were prepared and the bioconversion tested in each and in combination, with and without ferredoxin-NADP+ reductase (Table 10). The monooxygenase system contained at least two components that were distributed between EC-1 and –2 (Fig. 14).

It was now possible to identify the column fractions that contained the hydroxylase components. Fractions, in combination and/or single, were tested with their complementary EC solution to hydroxylate compactin. Concentrates were prepared of EC-1 and –2 solutions, 10-fold, and column fractions 3 – 7, 5-fold. The bioconversion solutions contained 90 µL of concentrated test fraction and 10 µL of EC-concentrate (Table 11). These preliminary results indicated that the monooxygenase system consisted of three components: two in EC-1, one in each of f-3 and f-5 and common to f-4, and one in f-6 and f-7 of EC-2. The components of f-3, f-5, and EC-2 were designated *A, B,* and *C*, respectively (Fig. 15).

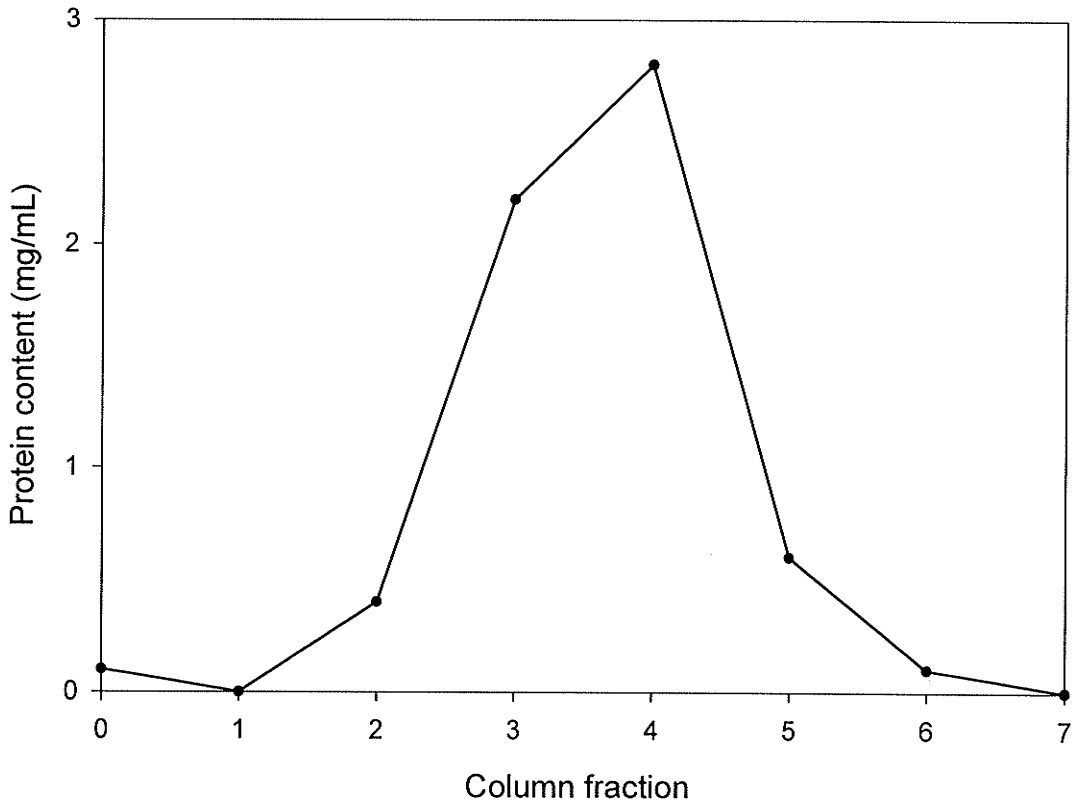


Figure 13. Cell extract protein distribution amongst the DEAE-IE column fractions. Fraction f-0 represents the column wash prior to elution.

Table 10. Bioconversion by combinations of the eluate composite solutions.

Reaction solution		Activity[a] (nmol/min) x 10^2	
5x-solution volume (µL)		reductase (0.2 u/mL)	
EC-1	EC-2	+	-
100	0	0	0
75	25	3.2	2.2
50	50	4.1	2.1
25	75	3.3	1.6
0	100	0	0

[a]Bioconversion solutions contained NADPH (0.7 mM), $MgCl_2$ (3.3 mM), and compactin (0.21 mM).

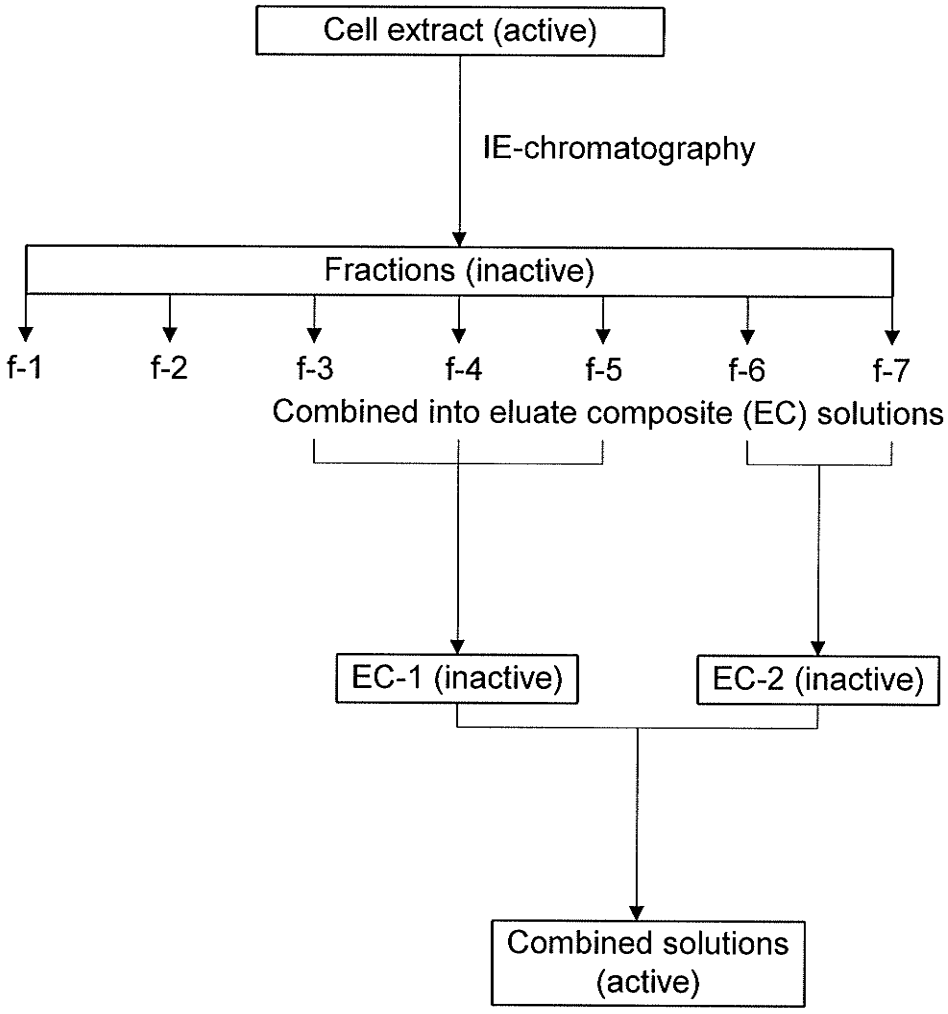


Figure 14. Fractionation of cell extract protein by ion-exchange chromatography and reconstitution of bioconversion activity.

Table 11. Bioconversion by the DEAE-IEC fractions.

Trial	Fraction(s) tested	Activity[a] (nmol/min) x 10^2
a	3	0
b	4	1.7
c	5	0
d	3 + 4	1.5
e	3 + 5	3.2
f	4 + 5	1.7
g	3 + 4 + 5	2.3
h	6	0.76
i	7	1.2

[a]Bioconversion solutions contained NADPH (0.7 mM), $MgCl_2$ (3.3 mM), ferredoxin-NADP+ reductase (0.2 u/mL), and compactin (0.21 mM). EC-2 concentrate was added to trials a – g and EC-1 to trials h and i.

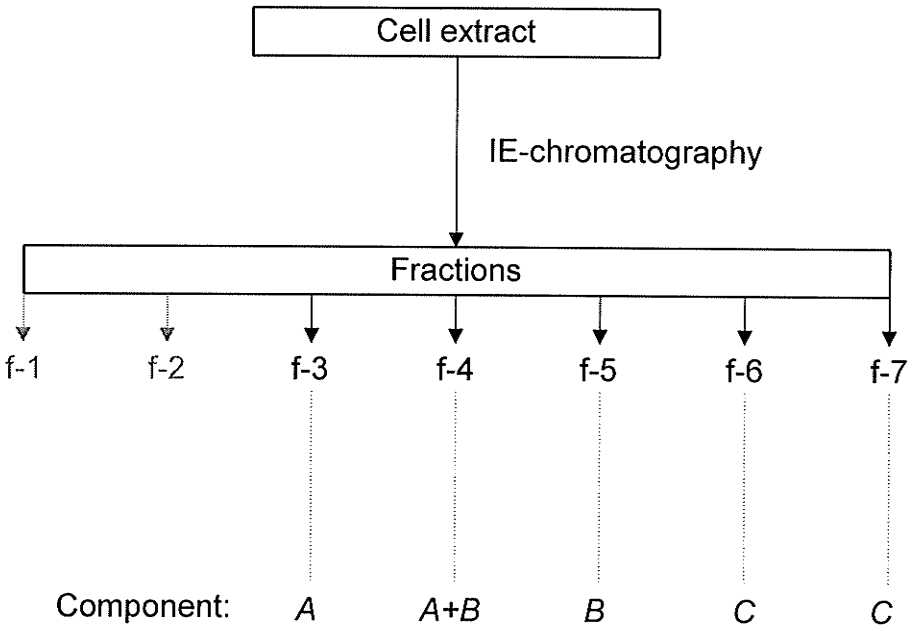


Figure 15. Column fractions containing the hydroxylase components.

Assay Development

With the confirmation of multiple hydroxylase components came the need for independent assays to permit testing during their purification. Informal kinetic experiments using the protein of f-3, f-5, EC-1, and EC-2 to represent the components *A*, *B*, *A+B*, and C, respectively, were performed to determine the dependencies of the bioconversion activity. One component was tested in each experiment with the others in excess, at least to start. From this, the amounts of the complementary components were determined for a range of linearity. First, the effect of EC-1 and –2 protein on the reaction rate was studied. A non-limiting concentration of EC-2 protein was then used to study the effect of f-3 and f-5 protein.

EC-1 (component A + B)

A solution of EC-1 containing 2.5 mg/mL protein was prepared by combining equal aliquots of fractions 3, 4, and 5 and then concentrated 50-fold by UF. Likewise, a solution of EC-2 containing 0.1 mg/mL protein was prepared from fractions 6 and 7 and concentrated 2.5-fold. The dependence of activity on EC-1 protein was studied by adding up to 10 µL of the 50x-solution to 90 µL of the EC-2 concentrate and when necessary, buffer was added to make 100 µL. The bioconversion reaction was performed with the exogenous reductase to maximize the amount of pravastatin formed (Table 12). In the presence of 20 µg of EC-2 protein, the EC-1 assay was linear up to at least 1300 µg of protein (Fig. 16).

Table 12. The effect of EC-1 protein on the bioconversion rate.

50x-solution volume (µL) concentration factor	1 0.5x	2.5 1.25x	5 2.5x	10 5x
protein (µg)	130	325	650	1300
activity[a] (nmol/min) x 10^2	0.31	1.8	3.3	6.7

[a]Bioconversion solutions contained NADPH (0.7 mM), $MgCl_2$ (3.3 mM), ferredoxin-NADP+ reductase (0.2 u/mL), compactin (0.21 mM), and EC-2 protein (20 µg). These data are plotted in Figure 16.

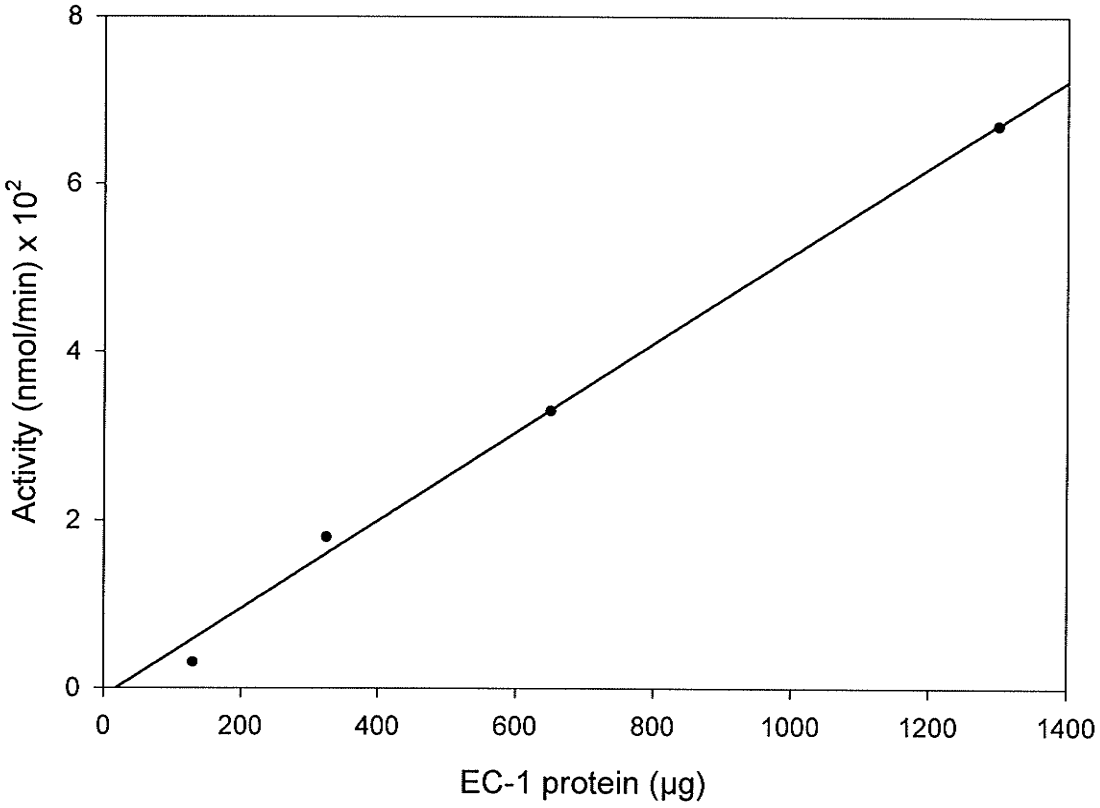


Figure 16. Effect of EC-1 protein in bioconversion solution containing EC-2 protein (20 µg). The data are from Table 12.

EC-2 (component C)

In this experiment, a 50x-solution of EC-2 was used to prepare a reaction curve in EC-1 solution that was concentrated 2.5-fold. Again, buffer was added to make 100 µL and the bioconversion was performed with ferredoxin-NADP+ reductase (Table 13). The EC-2 assay with 560 µg of EC-1 protein was linear up to approximately 25 µg (Fig. 17). These results are in rough agreement with the previous experiment, although the range of linearity here was slightly greater than expected seeing that the EC-1 protein content was less than half of the highest concentration in Figure 16.

f3 and f5 (components A and B)

Fresh column fractions, f-3 and f-5 and EC-2 solution were prepared that contained 2.2, 0.6, and 0.1 mg/mL protein, respectively. Reaction curves were established with the protein of f-3 and f-5 (ie. f-3/f-5 and f-5/f-3). Twenty-fold concentrated solutions of f-3 and f-5 were prepared and added, up to 7.5 µL, to 87.5 µL of the complementary fraction. Fifty-fold concentrated solution of EC-2, 5 µL (25 µg protein), was added to each reaction and buffer to make 100 µL. The bioconversion reaction was performed with and without ferredoxin-NADP+ reductase to determine if the enhanced activity was necessary. The pravastatin formed was detectable, although the amounts were small and in some cases out of the HPLC-curve range. However, the accuracy of the results was compromised by an HPLC system impurity that interfered with the detection of pravastatin. To process the data, the averaged amount of this impurity, as

Table 13. The effect of EC-2 protein on the bioconversion rate.

50x-solution volume (µL) concentration factor	1 0.5x	2.5 1.25x	5 2.5x	10 5x
protein (µg)	5	12.5	25	50
activity[a] (nmol/min) x 10^2	1.0	2.1	4.5	6.3

[a]Bioconversion solutions contained NADPH (0.7 mM), $MgCl_2$ (3.3 mM), ferredoxin-NADP+ reductase (0.2 u/mL), compactin (0.21 mM), and EC-1 protein (560 µg). These data are plotted in Figure 17.

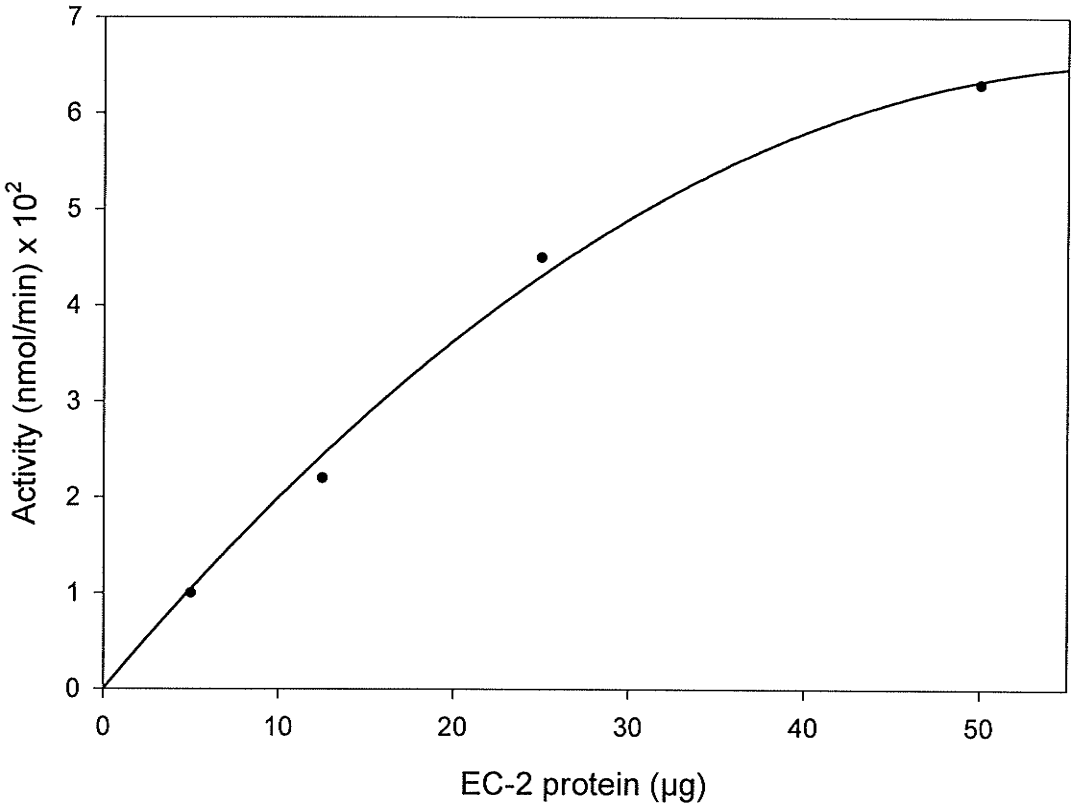


Figure 17. Effect of EC-2 protein in bioconversion solution containing EC-1 protein (560 µg). The data are from Table 13.

determined from the blanks run before and after the sample set, was subtracted from the pravastatin concentrations (Table 14 and 15). The f-3 assay with 50 µg of f-5 protein was linear up to approximately 110 µg (Fig. 18), while the f-5 assay was linear up to at least 90 µg with 190 µg of f-3 protein (Fig. 19).

The experiment was repeated with fresh f-3, f-5, and EC-2 solutions containing 2.3, 0.8, and 0.1 mg/mL protein, respectively. Several changes were incorporated to increase the amount of pravastatin formed and a different HPLC system was used to measure the concentration. This time the f-3 reaction curve was prepared in a 2.5x concentrated solution of f-5. Up to 10 µL of the f-3, 20x-solution was added to 90 µL of an f-5, 2.5x-solution. For f-5, a 50x-solution was added, up to 10 µL, to 90 µL of f-3. The EC-2, 50x-solution was added at 5 and 10 µL (25 and 50 µg protein) to ensure that it was non-rate limiting. Exogenous reductase was supplied to maximize the pravastatin formation (Tables 16 and 17); reaction volumes 113.8 – 118.8µL. At first sight, the results of this experiment (Fig's 20 and 21) are the opposite of those from the earlier one, but in fact the two runs are consistent. Increasing the amount of f-5 to 180 µg from 50 µg improved the f-3 linear range to at least 480 µg. The experiment in Figure 21 has almost the same amount of f-3 (210 µg) as that in Figure 19 (190 µg), and the linear range for f-5 is below 100 µg in both cases.

Table 14. The effect of f-3 protein on the bioconversion rate.

20x-solution volume (µL)	1.25	2.5	5	7.5
concentration factor	0.25x	0.5x	1x	1.5x
protein (µg)	55	110	220	330
activity[a] (nmol/min) x 10^2				
+ reductase	0.03	0.22	0.40	0.43
- reductase	0.1	0.15	0.22	0.25

[a]Bioconversion solutions contained NADPH (0.7 mM), $MgCl_2$ (3.3 mM), compactin (0.21 mM), f-5 protein (50 µg), and EC-2 protein (25 µg). These data are plotted in Figure 18.

Table 15. The effect of f-5 protein on the bioconversion rate.

20x-solution volume (μL)	1.25	2.5	5	7.5
concentration factor	0.25x	0.5x	1x	1.5x
protein (μg)	15	30	60	90
activity[a] (nmol/min) x 10^2				
+ reductase	0	0.53	0.68	1.1
- reductase	0.1	0.28	0.53	0.72

[a]Bioconversion solutions contained NADPH (0.7 mM), $MgCl_2$ (3.3 mM), compactin (0.21 mM), f-3 protein (190 μg), and EC-2 protein (25 μg). These data are plotted in Figure 19.

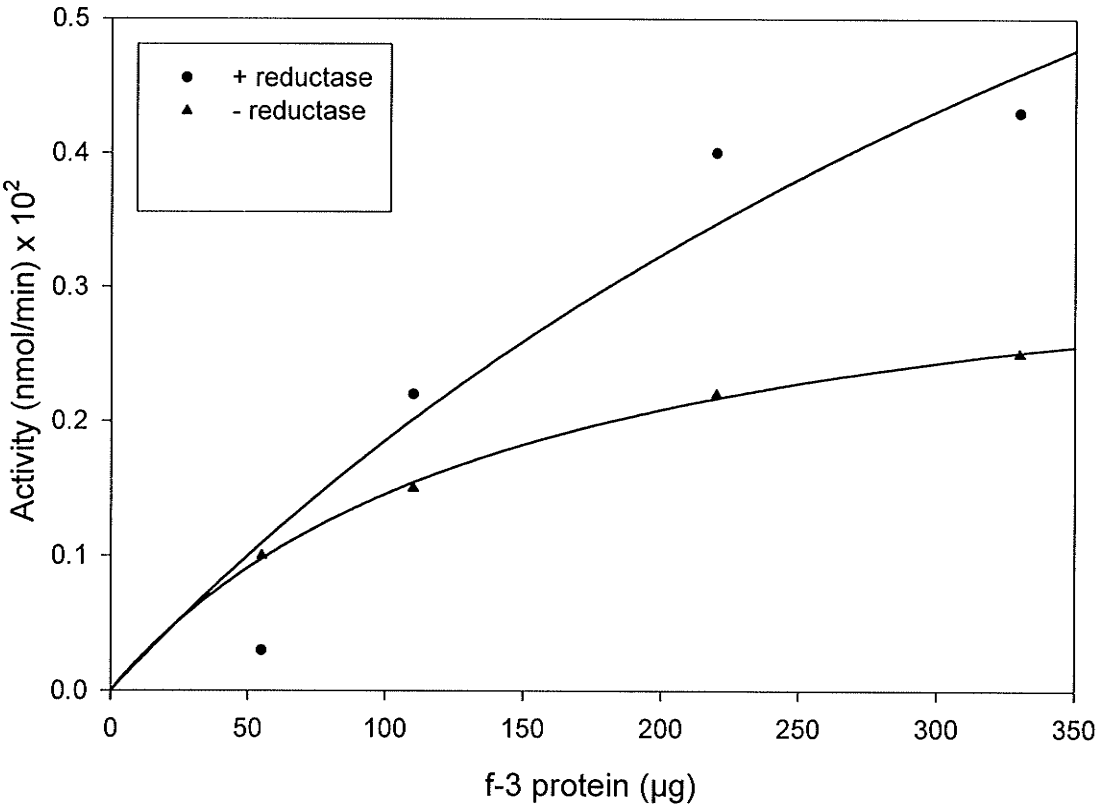


Figure 18. Effect of f-3 protein in bioconversion solution containing f-5 (50 µg) and EC-2 (25 µg) protein. The data are from Table 14.

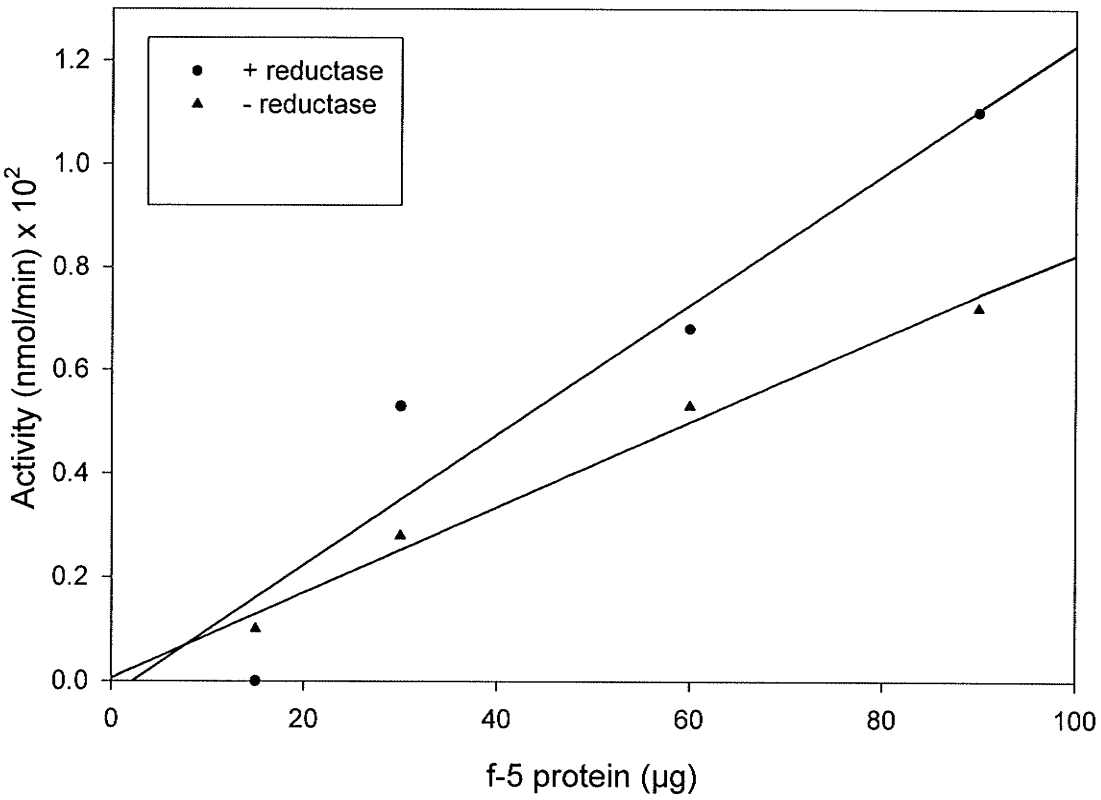


Figure 19. Effect of f-5 protein in bioconversion solution containing f-3 (190 µg) and EC-2 (25 µg) protein. The data are from Table 15.

Table 16. The effect of f-3 protein on the bioconversion rate – improved study.

20x-solution volume (µL)	1.25	2.5	5	10
concentration factor	0.25x	0.5x	1x	2x
protein (µg)	60	120	240	480
activity[a] (nmol/min) x 10^2				
+ EC-2 (25µg)	0.82	1.6	3.4	6.9
+ EC-2 (50µg)	1.5	2.6	5.8	12

[a]Bioconversion solutions contained NADPH (0.7 mM), $MgCl_2$ (3.3 mM), ferredoxin-NADP+ reductase (0.2 u/mL), compactin (0.21 mM), and f-5 protein (180 µg). These data are plotted in Figure 20.

Table 17. The effect of f-5 protein on the bioconversion rate – improved study.

50x-solution volume (µl)	1.25	2.5	5	10
concentration factor	0.625x	1.25x	2.5x	5x
protein (µg)	50	100	200	400
activity[a] (nmol/min) x 10^2				
+ EC-2 (25µg)	0.90	1.2	1.5	1.5
+ EC-2 (50µg)	1.2	1.8	2.4	2.5

[a]Bioconversion solutions contained NADPH (0.7 mM), $MgCl_2$ (3.3 mM), ferredoxin-NADP+ reductase (0.2 u/mL), compactin (0.21 mM), and f-3 protein (210 µg). These data are plotted in Figure 21.

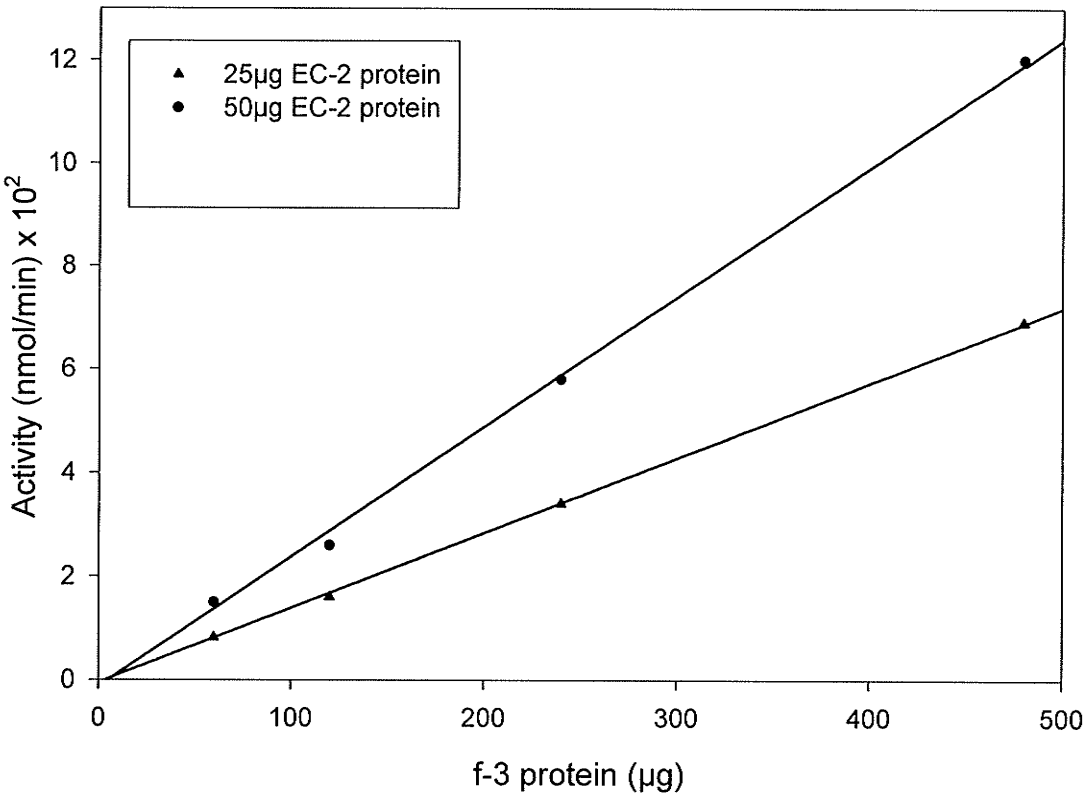


Figure 20. Effect of f-3 protein in bioconversion solution containing f-5 (180 µg) and EC-2 protein (25 and 50 µg). The data are from Table 16.

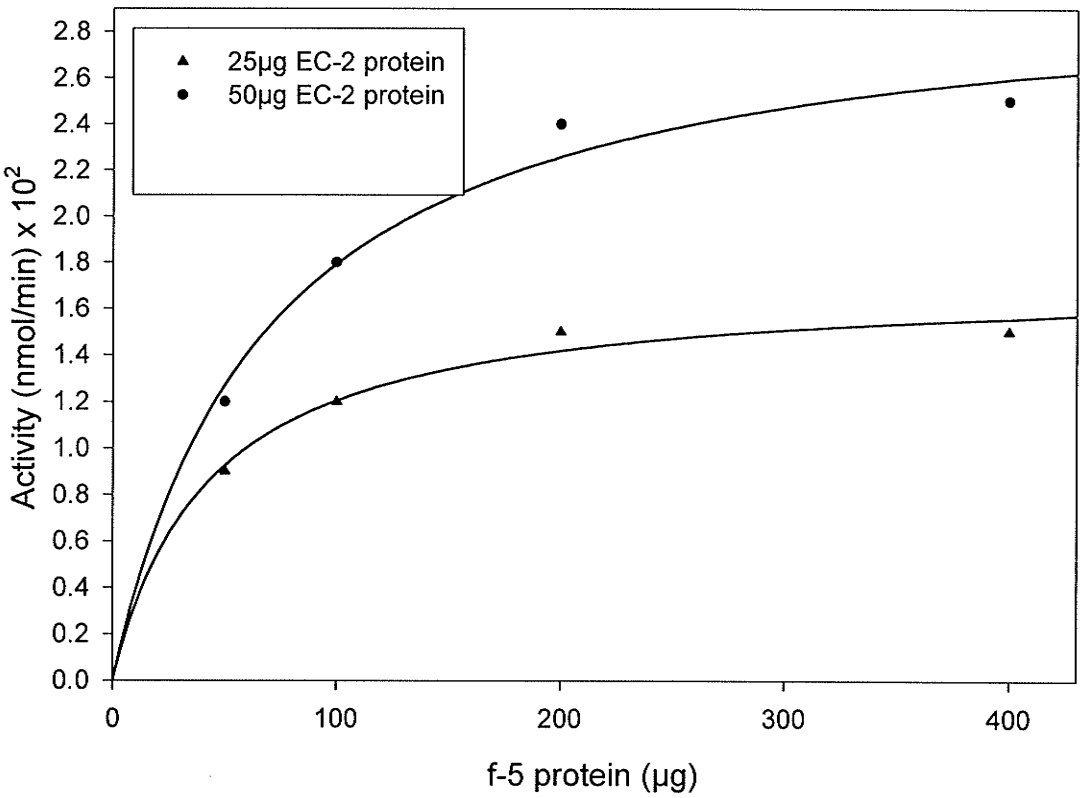


Figure 21. Effect of f-5 protein in bioconversion solution containing f-3 (210 µg) and EC-2 (25 and 50 µg) protein. The data are from Table 17.

The monooxygenase system was resolved by DEAE-IEC into components *A*, *B*, and *C* that up to now have been identified with the active fractions f-3, f-5, and EC-2, respectively. To further identify the components, discrete assays were defined from the above experiments to include the necessary proteins. Component *A* and *B* assays were to be performed in 90 µL of test solution to which is added the protein of EC-2 (~25 µg), f-5 (~200 µg) for *A*, and f-3 (~200 µg) for *B*, with the total volume, including the NADPH, $MgCl_2$, ferredoxin-NADP+ reductase, and compactin, of 108.8 µL. The assay for component *C* required much EC-1 protein (>500 µg), so instead of adding EC-1 protein to a test solution, a 5-fold concentrate of EC-1 was to be prepared (90 µL) and the test solution (10 µL) added to it.

Distribution of Components *A* and *B*

The distribution of *A* and *B* from chromatography was determined using the described assays. *Buffer-2*, cell extract protein (71 mg) was fractionated by DEAE-IEC and collected in 70 x 1-mL fractions. Fractions 51 – 70 were pooled to make EC-2 solution, which was then concentrated 50-fold by UF. Concentrates of f-3 and f-5, 20 and 50-fold, respectively, were prepared from fractions that were collected a week earlier and stored at –80°C. The bioconversion assays were tested in every third fraction from 22 – 40 for *A* and 31 – 49 for *B* (Fig. 22). A negative control set without f-3 and f-5 protein was performed in every third fraction from 25 – 46 to determine the activity from

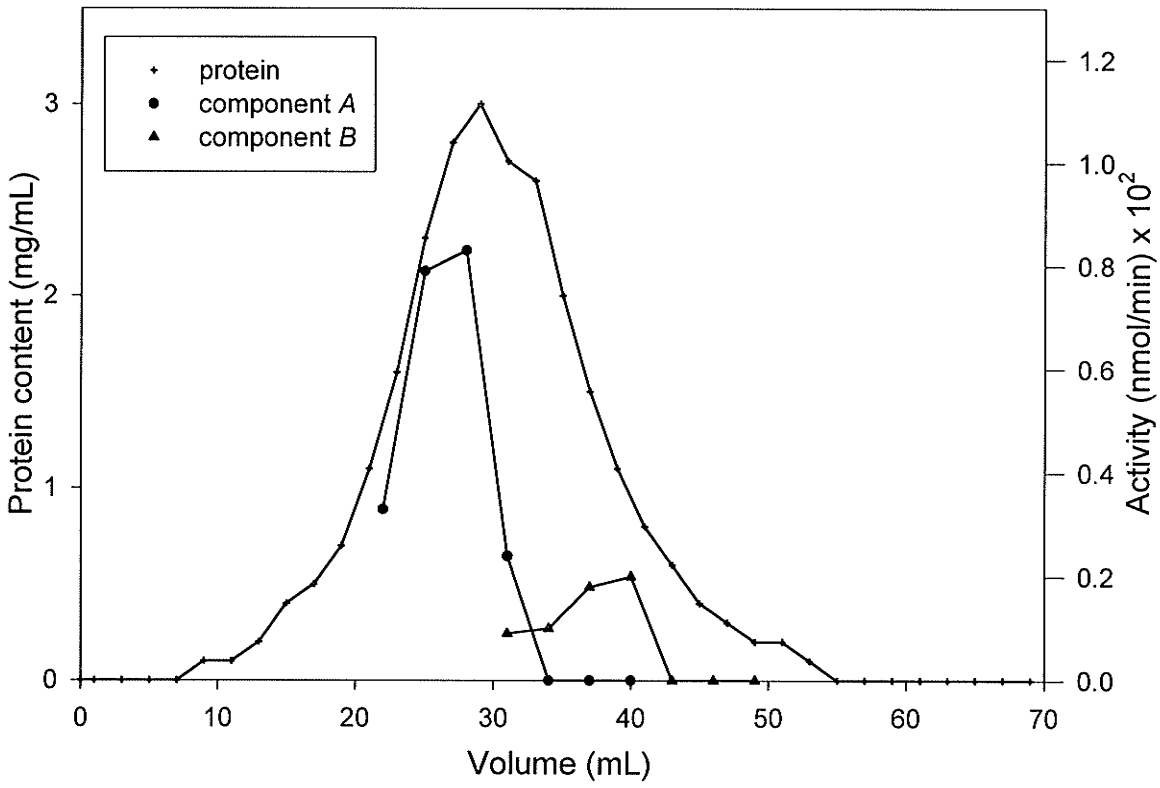


Figure 22. Distribution of the hydroxylase components *A* and *B* in DEAE-IEC fractions. The reaction solutions included the protein of EC-2 (25 μg), f-5 (225 μg) for *A*, and f-3 (270 μg) for *B*. Some of these fractions were analyzed by SDS-PAGE in Figures 23 and 24.

component overlap; no pravastatin was formed. Every third fraction from 19 – 43 was also analyzed by SDS-PAGE (Fig's 23 and 24). The profile in Figure 22 showed that the components do indeed overlap, as was originally proposed from the bioconversion data of an earlier run (Table 11). In this case, however, the amounts of the components that co-eluted were probably too small to hydroxylate compactin.

Left over fractions and concentrates were frozen by liquid nitrogen and placed at –80°C. After approximately 4.5 months of storage, the bioconversion reaction was repeated using the concentrates and several of the active fractions (Table 18). The separated hydroxylase components were relatively stable in *buffer-2* at –80°C.

Isolation of Component *C*

A SDS-PAGE gel (Fig. 24) from the previous experiment showed a distinct band at approximately 50 kDa for the protein of EC-2. This is typical for a CYP enzyme or a flavin-oxidoreductase. It was decided that little effort would be needed to purify a small amount of this protein. Cell extract protein (210 mg) in *buffer-3* was fractionated on a DEAE-Sephacel column (2.5 x 10.0 cm) and collected in 7 x 35-mL fractions. Fractions 6 and 7 were tested for component *C* and as expected from Table 11, most of the activity was found in fraction 7; almost 90% of their combined activities. Surprisingly though, when run on

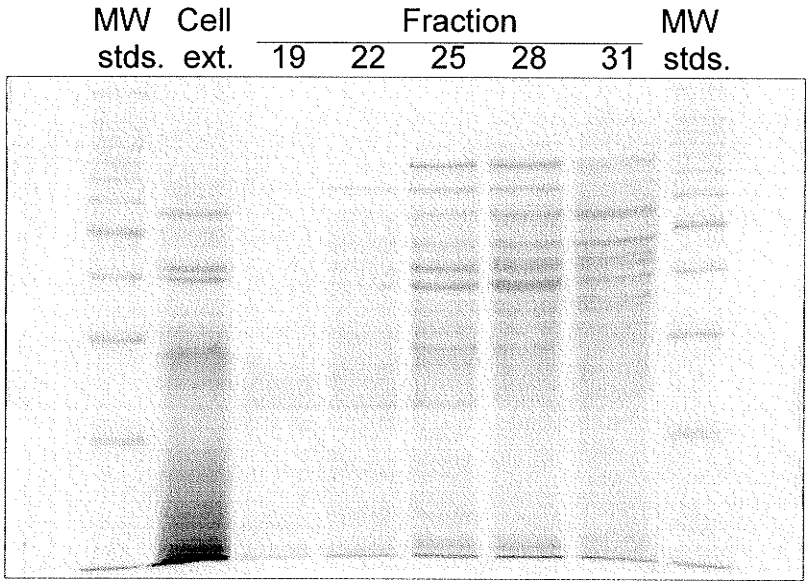


Figure 23. SDS-PAGE gel (1 of 2) of the fractions from Figure 22. Electrophoresis was carried out at 130 V on mini-gels containing 12% acrylamide and the protein bands developed with CBB-R250 stain. The molecular weight standard mixture consists of 12 bands from 10 to 120 kDa in 10 kDa increments, plus a 200 kDa band; the 10 kDa band is at the dye front.

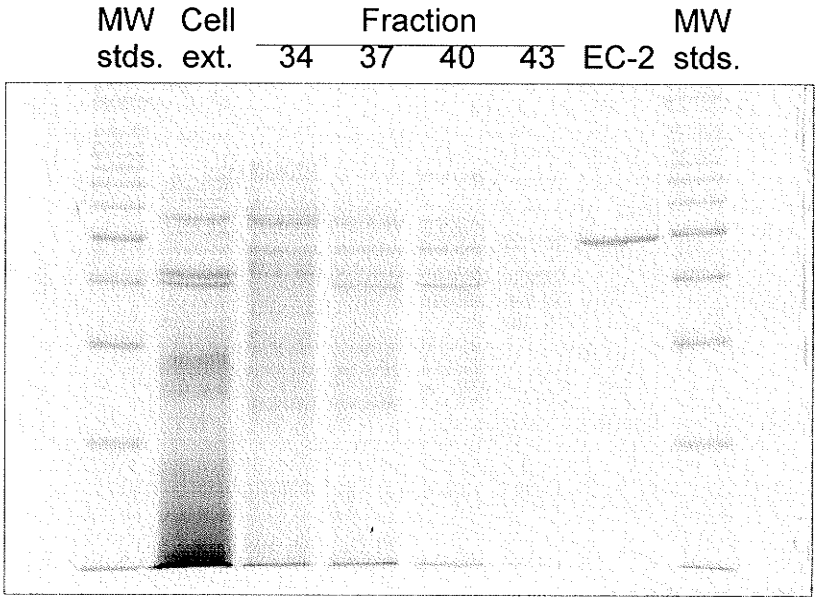


Figure 24. SDS-PAGE gel (2 of 2) of the fractions from Figure 22.

Table 18. Stability of the hydroxylase components in column fractions and concentrate solutions.

Hydroxylase component	Fraction	Activity (nmol/min) x 10^2	
		fresh	aged[a]
A	25	0.80	0.65
	28	0.83	0.69
B	37	0.18	0.18
	40	0.20	0.18

[a]Column fractions and concentrates of f-3, f-5, and EC-2 were stored at –80°C for 4.5 months and the bioconversion repeated.

SDS-PAGE it was fraction 6 that contained the 50 kDa protein (Fig. 25). Fraction 7 (30 mL) was desalted and reconstituted in 3 mL of fresh *buffer-3*. A portion of this (2.5 mL, ~0.5 mg protein) was re-chromatographed on a small column of DEAE-Sephacel (0.5 x 6.2 cm) and collected in 26 x 0.5 mL fractions. Based on OD280 readings, the bioconversion was tested in fractions 13 – 23 (Fig. 26). Correspondingly, a SDS-PAGE gel of fractions 15 and 16 revealed an intense, homogenous band at approximately 23 kDa (Fig. 27). Fractions 15 and 16 each contained approximately 50 µg of protein and were pooled. Component *C* of the monooxygenase system was purified from cell extract by DEAE-IEC (Fig. 28) and is a small protein with a molecular weight of approximately 23 kDa by SDS-PAGE.

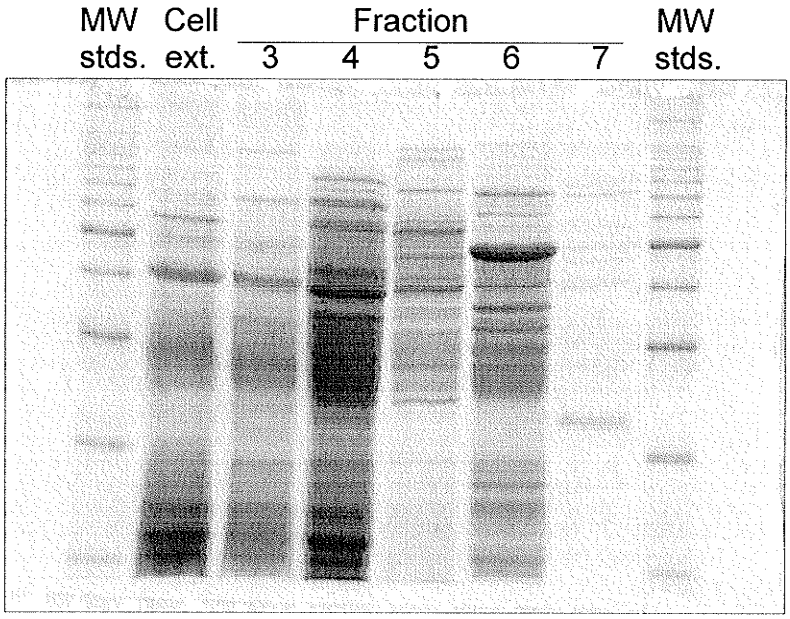


Figure 25. SDS-PAGE gel of the fractions from chromatographed cell extract. Electrophoresis was carried out at 130 V on mini-gels containing 12% acrylamide and the protein bands developed with CBB-R250 stain. The 10 kDa molecular weight standard is at the dye front.

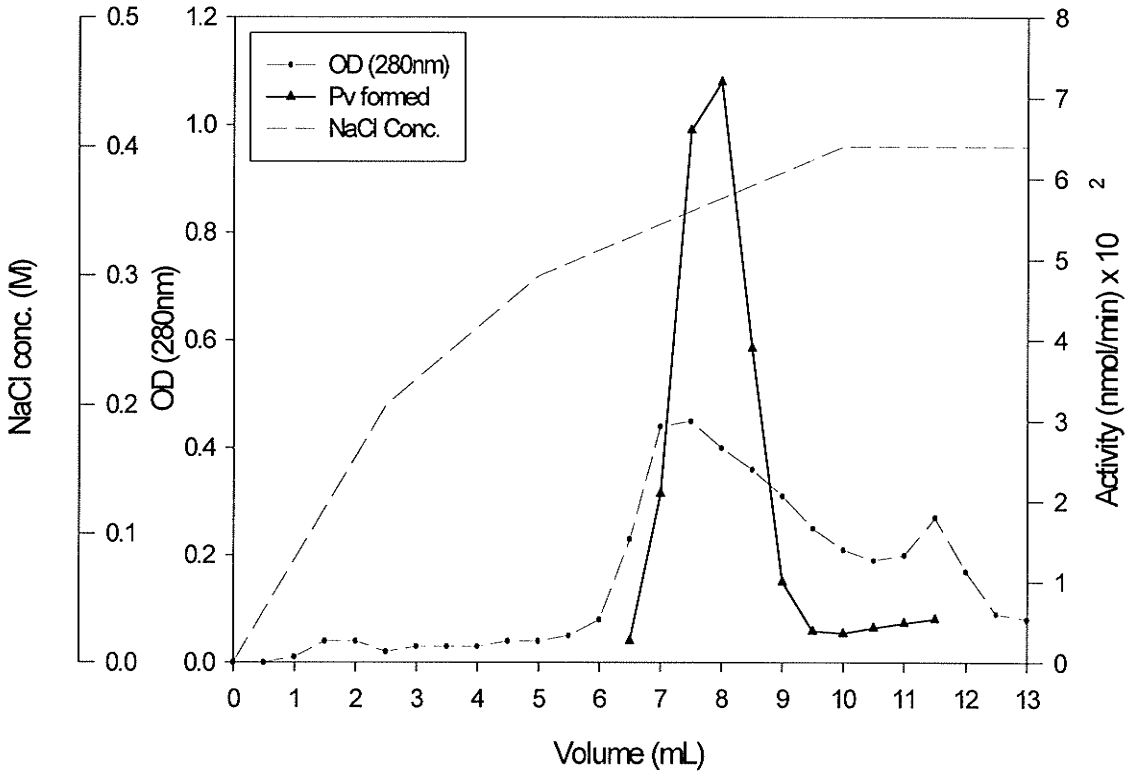


Figure 26. Purification of the hydroxylase component *C* by DEAE-IEC. Fraction 7 protein (Fig. 25, 0.5 mg) was loaded onto a column of DEAE-Sephacel resin an eluted with sodium chloride/buffer. The bioconversion solutions contained 810 µg of *A* + *B* protein, NADPH (0.7 mM), $MgCl_2$ (3.3 mM), ferredoxin-NADP+ reductase (0.2 u/mL) and compactin (0.21 mM). Active fractions were analyzed by SDS-PAGE in Figure 27.

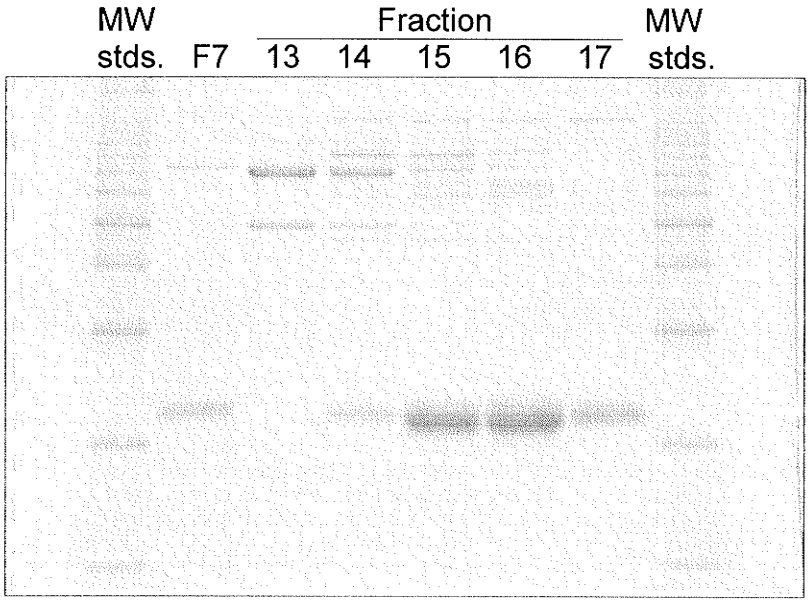


Figure 27. SDS-PAGE gel of the active fractions from Figure 26. Electrophoresis was carried out at 130 V on a mini-gel containing 12% acrylamide and the protein bands developed with CBB-R250 stain. The 10 kDa molecular weight standard is at the dye front.

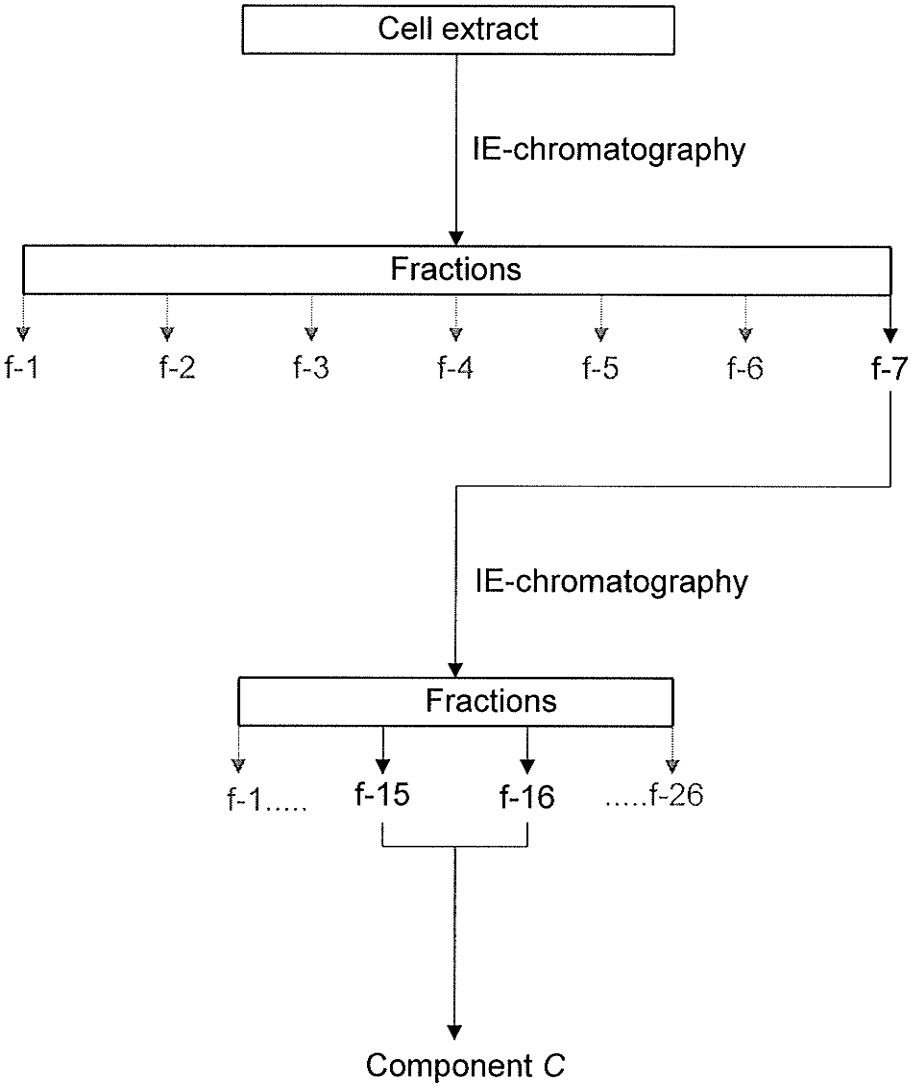


Figure 28. Purification scheme for the hydroxylase component *C*.

IDENTIFICATION OF A HYROXYLASE COMPONENT

Hydroxylase component *C* had an unexpected molecular weight by SDS-PAGE. It was too small for a reductase or terminal oxidase and too large to be a ferredoxin or an effector protein. A portion of the combined solutions containing 6 µg of the unknown protein was extensively diafiltered with *buffer-11* and the mass determined by nano-ESI-MS. The native weight of 11,876 Da indicated a probable ferredoxin.

Two approaches were taken to identify this small protein. Being from a lower organism, there was a good chance that the amino-terminus was not blocked. Component *C* protein (6 µg) was electrophoresed and blotted onto PVDF membrane for N-terminal sequencing. Peptide mass mapping by MALDI-MS, a powerful method of identifying proteins from a sequence database, was also used. A stained gel band from SDS-PAGE was treated with trypsin and the extracted peptides analyzed by MALDI-MS.

Convincingly, both approaches indicated that the small protein was a ferredoxin. The primary sequence of the amino-terminus was homologous with ferredoxins from various sources, especially species of *Streptomyces* (Table 19). Twenty-three of the 24 residues identified matched a ferredoxin from *S. erythraeus*. A fragment from the trypsin digest with a *m/z* of 1710 matched the mass of a peptide from a ferredoxin of *S. griseus* (SWISS-PROT P13279). Tandem mass spectrometry (MS/MS) was performed to sequence the peptide

from the digest. Since MS/MS data was not available for the matched ferredoxin, a theoretical fragmentation pattern was created using ProMac. The MS/MS spectrum of the digest peptide was a good match to an internal segment (aa 70 – 86) of the ferredoxin with two conservative replacements (Fig. 29).

Amino acid analysis was performed on protein that was blotted to PVDF. The amino acid composition also showed similarities to the ferredoxins from species of *Streptomyces* (Table 20).

Table 19. BLAST comparison of the amino-terminus sequence of the hydroxylase component *C* with ferredoxins of *Streptomyces* species.

Protein	Sequence
component *C*[a]	T-Y-V-I-A-Q-P-X-V-D-V-L-D-K-A-X-I-E-E-X-P-V-D-X-I-Y-E-G
S. erythraeus	T-Y-V-I-A-E-P-C-V-D-V-L-D-K-A-C-I-E-E-C-P-V-D-C-I-Y-E-G
S. griseus	T-Y-V-I-A-Q-P-C-V-D-V-K-D-K-A-C-I-E-E-C-P-V-D-C-I-Y-E-G

[a]As determined by 28 cycles of Edman degradation. The unassigned positions (X) may be Cys-propionamide formed by the reaction of cysteine with the unpolymerized acrylamide during SDS-PAGE.

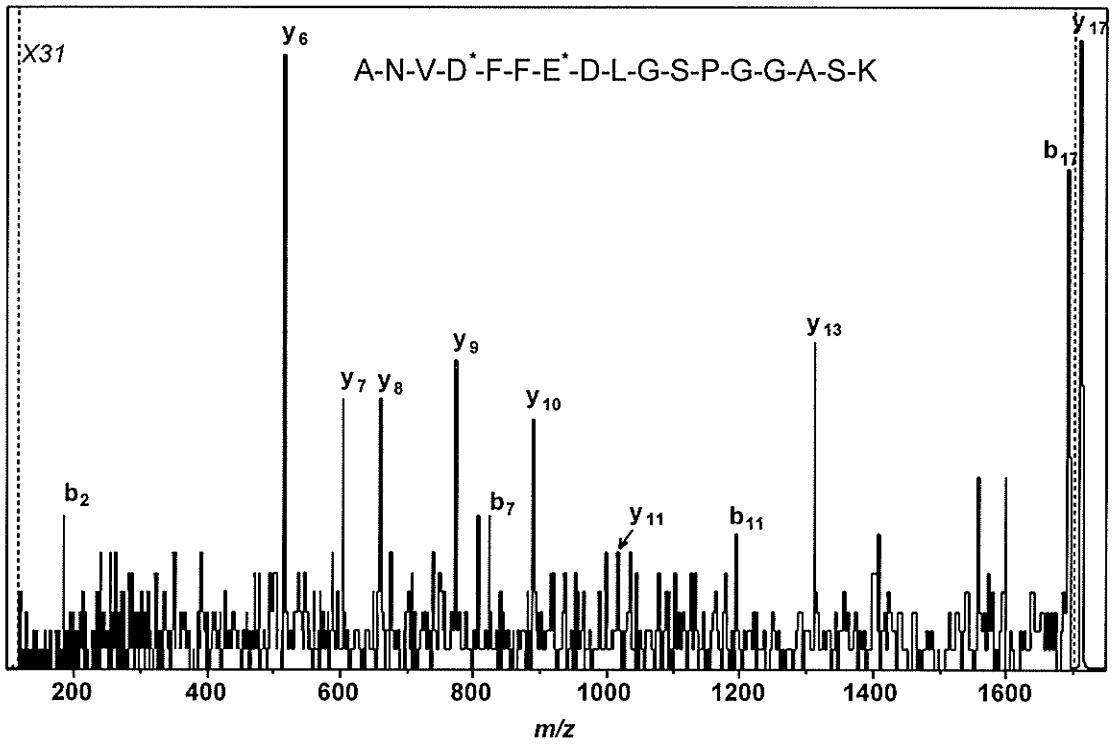


Figure 29. MALDI-MS/MS spectrum of a peptide with m/z 1710 from the trypsin digest of component *C* protein and the derived sequence. The primary sequence matches an internal segment of a ferredoxin from *S. griseus*, only the marked residues are switched in order. Ions at 1556 and 1593 may be from other fragmentation.

Table 20. Amino acid composition of the hydroxylase component *C*. Comparison with ferredoxins from *Streptomyces* sp. in the SWISS-PROT database.

Amino acid	Mole %	Residues[a] / molecule	*S. erythraeus* Residues[b] / molecule	*S. griseus* Residues[c] / molecule
Asx + Asn	9.1	9.9	13	13
Thr	0.9	1.0	2	2
Ser	1.7	1.8	3	3
Glu + Gln	19.7	21.4	13	14
Pro + Cys	13.3	14.5	18	18
Gly	7.4	8.0	8	7
Ala	8.8	9.6	9	8
Val	12.6	13.7	13	9
Met	0.8	0.8	1	0
Ile	6.6	7.2	5	6
Leu	6.5	7.1	4	5
Tyr	4.3	4.7	6	6
Phe	5.0	5.4	2	4
His	1.3	1.4	1	2
Lys	1.6	1.7	4	5
Arg	0.5	0.5	2	2
Trp	nd[d]	nd[d]	1	1
	Total	108.7	105	105

[a]Molecular weight: 11, 876 Da; recovered 1.651 µg of protein (0.1390 nmol).
[b]Molecular weight: 11,407 Da, SWISS-PROT: P24496.
[c]Molecular weight: 11,663 Da, SWISS-PROT: P13279.
[d]Not determined.

DISCUSSION

Typical Bacterial Monooxygenase

Compactin is converted to pravastatin by many organisms of the genera *Streptomyces, Nocardia, Mucor, Rhizopus,* and *Actinomucor* (Terhahara and Tanaka, 1985). Up until now, however, the eukaryotic-like cytochrome P450 (CYP) monooxygenase system from *S. carbophilus* is the only compactin hydroxylase system to be described. This soluble, two-component system contains two CYP isozymes (Matsuoka *et al.*, 1989) and a flavin-oxidoreductase that binds NADH as the co-reductant (Serizawa and Matsuoka, 1991). The compactin hydroxylase of *Actinomadura madurae* identified here is a typical bacterial monooxygenase that includes a ferredoxin to transfer the electrons provided by NADPH or NADH, preferably the former.

From the findings of this study, it appears that three components comprise the system. One component (*C*) with a molecular weight of 11,876 Da has been purified and is homologous to the ferredoxins from several species of *Streptomyces*. Another component is very likely a flavin-oxidoreductase. Even though this component was not isolated, this claim is substantiated by the dramatic effect of spinach ferredoxin-NADP+ reductase on the bioconversion activity of the enzyme preparations. There are also few other possibilities to consider. Interestingly though, the exogenous reductase did not substitute for either component *A* or *B* in the independent assays. That is, no compactin was

formed by solutions of f-3 (*A*) and f-5 (*B*) protein that contained spinach ferredoxin-NADP+ reductase and the endogenous ferredoxin (*C*). This unexpected and titillating discovery indicates a possible fourth component. The terminal oxygenase is by far the most important part of any monooxygenase system. Unfortunately, the hydroxylase of *A. madurae* remains unidentified for the information obtained in this study is inconclusive. Most members of the CYP super-family share special attributes that set them apart from other oxygenases, but these are lacking here. Yet, the developed bioconversion reaction mixture for cell extract is typical of CYP assays.

Terminal Oxygenase

Inhibition by carbon monoxide is one of the best ways to distinguish CYP enzymes from non-heme oxygenases. Carbon monoxide binds reversibly and exclusively to the ferrous form of CYP and prevents the binding of dioxygen for the reaction cycle. Both the *S. carbophilus* isozymes exhibit some sensitivity (Matsuoka and Shunichi, 1993). A cell extract of *A. madurae* contained a small amount of CYP, which is expected because these enzymes are ubiquitous. Carbon monoxide, however, under reducing conditions did not affect the activity of this extract. Of course this does not totally exclude a CYP enzyme for it is well known that they do vary in sensitivity. There is also the possibility that too little heme-CO complex formed under the aerobic conditions to detect a sensitivity. The method of Omura and Sato (1964) specifies the addition of carbon monoxide

before sodium dithionite to form the complex under aerobic conditions; order of addition did not make a difference here.

Apparently, CYP enzymes are readily converted to an inactive "P420 form" during their purification, especially when detergents are used. This is caused by the displacement of the characteristic thiolate anion ligand with an imidazole group of a histidine residue (Omura, 1999). To stabilize against rearrangement, glycerol is added at a high concentration to the buffers used for purification. This is well practiced in the literature, but did not make a difference here for the first round of purification. Granted, a formal stability comparison in buffer with and without glycerol has not been performed.

Perhaps the best evidence for involvement of a CYP enzyme in compactin hydroxylation is the ability of spinach ferredoxin-NADP+ reductase and $MgCl_2$ to enhance the bioconversion activity. Spinach ferredoxin-NADP+ reductase is used extensively for the assays of bacterial CYP monooxygenases. Even the CYP isozymes of the eukaryotic-like monooxygenase from *S. carbophilus* can receive electrons from it (Matsuoka, 1989). Here, the supplied reductase increases the activity by 2 – 3 fold. Magnesium chloride at 3.4 mM increases the bioconversion in cell extract by as much as 50%. It has long been recognized that Mg^{2+} and other metal ions, mono- and divalent, can stimulate the activity of CYP monooxygenases. One theory suggests that these ions increase the efficiency of the electron transfers by modulating the interaction of the

components (Hanukoglu *et al.*, 1980). The $P450_{cam}$ of *Pseudomonas putida* actually binds K+ along with camphor in its reaction cycle. Potassium is thought to promote the binding of camphor and enhance the reactivity of the heme center, but it is not absolutely necessary for function (Westlake, 1999). This is interesting, for it may shed some light on the identity of the small component that is lost when cell extract is filtered through a membrane with a MWCO of 10 kDa.

It is noteworthy that the researchers of non-heme systems rarely perform comparisons with CYP enzymes and when a comparison is made, the emphasis is usually on spectral properties or the number of components involved. Only after much searching was a non-heme enzyme found that had been tested with spinach ferredoxin-NADP+ reductase. The reductase was fully competent to serve as a redox center for the non-heme, toluene-4-monooxygenase of *P. medocina*, (Pikus *et al.*, 1996). A study using metal ions other than Fe^{2+} with non-heme monooxygenases could not be found. This is quite surprising since the two groups of oxygenases have similar components and, in some cases, mechanisms of catalysis.

Expression

Actinomadura madurae is a gram-positive, filamentous bacterium that under the described growth conditions expresses a compactin hydroxylase without exposure to compactin. Nor does compactin increase the enzyme content when added at a later stage of growth. If anything, it may reduce the

amount of enzyme formed by suppressing the growth, as was observed when the induction with compactin was tested. The amount of enzyme produced by the organism increases steadily with growth and then drops off sharply when the stationary phase is reached, perhaps the result of cell senescence and lysis or the loss of an inductant. Most bacterial monooxygenases are expressed *de novo* in response to chemical stimuli. This is the case with the compactin hydroxylase of *S. carbophilus*, a distant relative of *A. madurae*, which is expressed in an appreciable amount only after the organism is exposed to compactin or phenobarbital (Watanabe and Serizawa, 1998). Hence, the discovery here of a constitutive monooxygenase was unexpected. It is also possible that a metabolite or a chemical present in the growth medium induces the system. The highly reactive $P450_{soy}$ of *S. griseus* is expressed only when soybean flour is used in the grown medium; soy oil cannot be used as a substitute. Genistein, a highly conjugated isoflavonoid of soy flour, is the chemical responsible for the induction (Sariaslani and Kunz, 1986).

Assays

Hydroxylase activity coupled with HPLC analysis detected the monooxygenase during the purification. Experiments to reconstitute the bioconverting activity of cell extract, the crude source of enzyme, provided the basic reaction conditions to start the purification. Cell extract alone could not hydroxylate compactin. In the absence of a defined co-reductant and an electron transfer system, ascorbic acid and hydrogen peroxide when tested failed to

produce a detectable level of pravastatin. In retrospect, hydrogen peroxide is bound by ferric enzyme and therefore should have been tested in the absence of ascorbic acid. Also, the turn over with hydrogen peroxide is normally performed on purified enzyme without the other hydroxylase components.

The bioconversion by cell extract initially required spinach ferredoxin-NADP+ reductase in addition to NADH or NADPH, but later, the reductase was added only to increase the amount of pravastatin formed. Including spinach ferredoxin with the exogenous reductase did not increase the activity. This indicates that spinach ferredoxin may not be able to provide the electrons to the terminal oxygenase. Although the ferredoxins from sources other than spinach, like *Azotobacter vinelandii* and *Clostridium pasteurianum*, can accept electrons from ferredoxin-NADP+ reductase (Trower *et al.*, 1990), the oxygenases can be specific (Subramanian *et al.*, 1985). Often the ferredoxins from other monooxygenases are tested to reconstitute a system along with those commercially available, like spinach and *Clostridium*.

Non-enzymatic reductants had an adverse effect on the activity of cell extract. Ascorbic acid reduced the activity of extract by 30% when tested at 12 mM with only NADPH present. Similarly, in a different experiment sodium dithionite at 4.6 mM reduced the activity by 70%. A possible explanation for this is that, under the reducing conditions, the enzyme is unable to alternate between

the valence states required for the catalytic cycle. CYP enzymes must be ferric in the end to bind a substrate for the next cycle.

The simple and reproducible assay for cell extract quickly became inadequate when the preliminary purification revealed more than one component. Adjusting the assay for three separate components required much effort, but was well worth it in the end. For the three independent assays in practice are sensitive and provide more than adequate pravastatin to be detected by HPLC. One of the assays made it possible to isolate the endogenous ferredoxin. The other assays will undoubtedly be useful in isolating the remaining components.

Purification

Ion-exchange chromatography successfully resolved the hydroxylase components and, as anticipated, the ultrafiltration rate of hydroxylase solution improved after the first column. Components *A* and *B* eluted with much of the extract protein and will require more work to purify. The endogenous ferredoxin, on the other hand, separated well and required little effort to purify, although a different protein was initially sought for component *C*. It was fortunate that the activity moved away from this 50 kDa protein in the final column performed with extract protein. The purified ferredoxin shows a molecular weight of 23 kDa by SDS-PAGE, almost twice that obtained by mass spectrometry. This seemed odd at first, but a review of the literature revealed that this is typical for these small

iron-sulfur proteins and they may not be monomeric in their native form (Trower *et al.*, 1990; O'Keefe *et al.* 1991).

Cometabolism or Detoxification

Cultures of both *A. madurae* and *S. carbophilus* when exposed to compactin cause the accumulation of pravastatin in the growth medium. It is estimated that the solubility of pravastatin in water is 100-fold that of compactin (Daniewski *et al.*, 1992). Thus, the physiological significance of the compactin hydroxylase in each organism would seem to be detoxification. The inductive effect of compactin and the high inhibitory concentration of pravastatin in comparison to compactin support this in the case of *S. carbophilus* (Matsuoka *et al.*, 1989). Compactin does appear to have an adverse effect on the growth of *A. madurae*, but it does not induce the monooxygenase system. So whether or not the system is constitutive or induced by another chemical, the conversion here is truly fortuitous.

Looking to the various databases for a possible function for the hydroxylase of *A. madurae* turned up very little information. Only two monooxygenases, both CYP systems, were found in the *Actinomadura* genus, one in each *A. hibisca* and *A. verrucosospora*. In both organisms, the hydroxylase is encoded as part of a polyketide synthase gene cluster.

Bioconversion

The reaction catalyzed by the monooxygenase of *A. madurae* is not stereospecific. Hydroxylation at the 6-carbon occurs α and β to the hexahydronaphthalene moiety, but only the 6β-pravastatin is of commercial interest. From culture, cell extract, and the reconstituted system, the amount of 6α-product that formed was around 10% of the 6β-pravastatin content. This is in good agreement with the isomer ratio reported for the CYP monooxygenase of *S. carbophilus* by Matsuoka *et al.* (1989), who claim the enzyme has a loose recognition for the compactin substrate. Furthermore, the product distribution may indicate different bond energies for the respective hydrogen atoms that are abstracted to insert the atom of oxygen.

Compactin is metabolized by a culture of *A. madurae* to products other than pravastatin. In cases where the organism had consumed all of the compactin, the pravastatin formed accounted for as little as 60% of the substrate invested and extending the incubation did not change the level of pravastatin. The other products are unknown. The HPLC test method detects the diene chromophore of compactin and pravastatin by the absorbance at 238 nm. Therefore, a metabolite without the conjugated double bonds would be undetected here.

Conclusion

Pravastatin was first isolated from dog urine by Daniewski (1992) while developing compactin as a therapeutic agent for the treatment of hyper-cholesterolemia. Knowing that CYP monooxygenases likely caused the conversion and that these enzymes are ubiquitous in nature, microorganisms were tested to carry out this reaction. Many soil actinomycetes and fungi are capable of the bioconversion (Terhahara and Tanaka, 1985), but only one monooxygenase has been isolated so far and it is a CYP system as expected. The actinomycete system from this study is a typical bacterial monooxygenase that may contain a non-heme terminal oxygenase.

The approach taken to study the compactin hydroxylase of *A. madurae* was successful. Together, the developed assays and purification scheme clearly demonstrated that the system contains at least three components. However, with the closure of this investigation comes the realization that there is much work remaining. Left to isolate is the terminal oxygenase as well as the oxidoreductase and perhaps a cofactorless protein to complete the system. Fortunately, though, much of the groundwork has been laid should the interest arise to continue the work. Procedures are in place to prepare and detect the monooxygenase, although it would be beneficial to determine if the system can be induced. Also, perhaps with some luck a commercially available ferredoxin will substitute in the system.

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