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ENZYMATIC FORMATION OF PROTOPINES BY A MICROSOMAL CYTOCHROME P-450 SYSTEM OF CORYDALIS VAGINANS

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Abstract: A microsomal cytochrome P-450-NADPH dependent enzyme which hydroxylates stereo- and regiospecifically carbon atom 14 of (S)-cis-N-methyltetrahydroprotoberberines has been discovered in a number of plant cell cultures originating from species containing protopine alkaloids; the monooxygenase was solubilized, partially purified (100-fold) and characterized.

The protopines are one of the most widely distributed groups of alkaloids in the plant kingdom [1], characterized by the presence of a ketone group at C-14. Feeding experiments with differentiated plants and callus cultures using ^{14}C- [2] or ^{13}C-labelled [3] tetrahydroprotoberberines or their N-methyl derivatives have shown that these compounds are the immediate precursors of protopines. During the oxygenation process it was demonstrated that the hydrogen atom at C-14 of a tetrahydroprotoberberine such as stylopine was completely lost [2]. The fact that N-methylation of tetrahydroprotoberberines occurs prior to oxygenation was clearly shown by in vivo [3] feeding and enzyme [4] experiments. As part of our investigation involving the elucidation of reaction sequences in isoquinoline biosynthesis, we searched for the enzyme which catalyzes the hydroxylation of N-methyltetrahydroprotoberberines.

A number of cytochrome P-450 dependent hydroxylating systems have been previously found to occur in microsomal preparations of higher plants as reviewed in [5]. Microsomes from 4-day-old cultured suspension cells of Corydalis vaginans were prepared by standard methods [6]. The $MgCl_2$ pelleted microsomal fraction was resuspended in 0.1 M tricine buffer pH 8.5 containing 50 mM $MgCl_2$ and 5 mM thioglycolic acid. The protein (10 - 100 µg) was

incubated in the presence of 200 mM tricine buffer pH 8.5, 200 µM NADPH and 2 µM (S)-[8,14-^{3}H]-cis-N-methylcanadine or the correspondingly labelled stylopine (30 000 dpm; prepared enzymatically [4]) in a total volume of 250 µl for 20 min at 30°C. The enzyme reaction was monitored by measuring the release of tritium into the aqueous phase of the incubation mixture [7]. As a control assays with heat denatured protein were used. Enzymatically catalyzed removal of tritium was observed in microsomal preparations from cell suspension cultures of the Fumariaceae and Papaveraceae (Table I) but neither in those of the Berberidaceae or Ranunculaceae nor in cell cultures which do not produce isoquinoline alkaloids like Catharanthus roseus or Nicotiana tabacum.

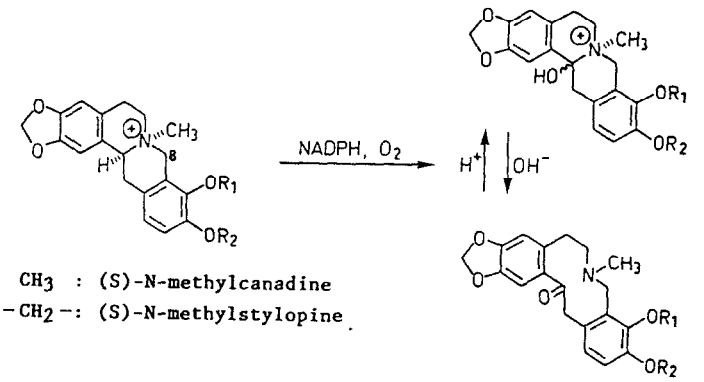
TABLE I. Survey of distribution of hydroxylation activity in different cell cultures

Plant family	Cell culture	Enzyme activity (pkat/g dry weight)
Fumariaceae	Corydalis ophiocarpa	52
	Corydalis vaginans	52
	Dicentra spectabilis	79
	Fumaria capreolata	84
	Fumaria officinalis	260
	Fumaria parviflora	137
Papaveraceae	Bocconia cordata	117
	Eschscholtzia californica	73
	Glaucium flavum	69
	Papaver somniferum	44

The product of the reaction was unequivocally identified as the protopine molecule, allocryptopine, by a large scale (600-fold) incubation containing 60 mg protein from C. vaginans in the presence of (S)-cis-N-methylcanadine. The incubation mixture was freeze dried, chromatographed (TLC), and the newly formed compound isolated. The mass spectrum of the unknown product showed m/e M^{+} at 369 (4.6%); 341 (4.6%); 297 (4.6%); 268 (16.3%); 206 (27.9%); 164 (100%); 163 (20.9%); 149 (16.3%). The mass spectrum was in every aspect identical to that of allocryptopine. Exactly the theoretical value of 33% of the radioactivity of the substrate molecule

was removed during this transition. The enzyme catalyzes the hydroxylation of the (S)-cis-N-methylderivatives of canadine (100%), stylopine (80%), tetrahydrothalifendine (30%), tetrahydropalmatine (19%), and corydalmine (6%). The (R)-cis/trans-N-methylcanadine epimeric mixture was completely inactive which fact indicated that synthetic (R,S)-cis/trans-N-methyltetrahydroprotoberberines can be interchangeably used with (S)-cis-N-methyltetrahydroprotoberberines in subsequent work.

The enzyme complex was solubilized from microsomal membranes by sodium cholate and the proteins purified further on an affinity column containing Procion red H3B as the ligand. In this manner, the enzyme complex was purified 100-fold with a 35% overall yield. The final preparation had a specific activity of 425 pkat/mg protein. The optimum of the reaction was at pH 8.5 and 30°C with half maximal activity at pH 6.5 and 9.7, as well as 10 and 45°C. In the absence of molecular oxygen no reaction was observed. Replacement of NADPH with NADH resulted in only 33% of the original activity. NAD and NADP were both ineffective. The K_M was 12.5 µM for cis-N-methylcanadine and 62.5 µM for NADPH. The UV spectrum of the $Na_2S_2O_4$ reduced enzyme (tricine buffer pH 8.5) showed peaks at 420, 450 and 480 nm suggesting a cytochrome P-450 enzyme. Characteristic absorption changes occurred in the presence of carbon monoxide which, in spite of the presence of O_2, rendered the enzyme inactive. The carbon monoxide inhibition was reversed in light. The enzyme complex was inhibited neither by CN^- nor by EDTA. The concentration for 50% inhibition of the enzyme activity was determined for the typical inhibitors of cytochrome P-450: ketoconazole (1.5 µM), metyrapone (2 µM), prochloraz (6 µM), ancymidol (12 µM), and cytochrome c (40 µM). Collectively, these properties of the enzyme clearly show that the hydroxylase is a cytochrome P-450 containing enzyme complex. The enzyme responsible for the formation of this important metabolite is termed: (S)-cis-N-methyltetrahydroprotoberberine-14-hydroxylase. It is the first monooxygenase discovered in benzylisoquinoline alkaloid metabolism. The protopine alkaloids, of which about twenty differently substituted members are known, arise biosynthetically by action of these hydroxylases with possibly differing substrate specificities. Protopine alkaloids can be further converted to benzazepine and benzophenanthridine alkaloids as has been shown by in vivo experiments [3,8-10]. It is our continued interest to clarify the biosynthesis of these alkaloids at the enzyme level.



$R_1 = R_2 = CH_3$: (S)-N-methylcanadine
$R_1 + R_2 = -CH_2-$: (S)-N-methylstylopine

$R_1 = R_2 = CH_3$: allocryptopine
$R_1 + R_2 = -CH_2-$: protopine

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