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CANADINE SYNTHASE FROM *THALICTRUM TUBEROSUM* CELL CULTURES CATALYSES THE FORMATION OF THE METHYLENEDIOXY BRIDGE IN BERBERINE SYNTHESIS

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Key Word Index—*Thalictrum tuberosum*; Ranunculaceae; protoberberine alkaloids; canadine synthase; cytochrome P-450; biosynthesis.

Abstract—An enzyme system catalysing the formation of the methylenedioxy bridge at ring A of (*S*)-canadine [=(*S*)-tetrahydroberberine] from (*S*)-tetrahydrocolumbamine has been detected in microsomal preparations from different Ranunculaceae and Berberidaceae cell cultures. The cytochrome P-450 enzyme complex has been partly characterized from a protoberberine alkaloid producing *Thalictrum tuberosum* L. cell line. The enzyme complex consisting of a microsomal associated oxidase with a cytochrome P-450 reductase has a pH optimum at pH 8.5 and a temperature optimum of 40°. The apparent K_m values are 33 μM for NADPH and 11.5 μM for tetrahydrocolumbamine.

INTRODUCTION

A considerable number of natural products contain methylenedioxy groups in their structure. *In vivo* experiments employing [^{14}C]-labelled precursors demonstrated that the methylenedioxy groups, found especially in benzylisoquinoline alkaloids, are derived by cyclization of an *ortho*-methoxyphenol to furnish the methylenedioxy ring [1–3]. The first report of an enzyme reaction catalysing the methylenedioxy formation appeared [4] when two highly stereo- and regiospecific microsomal cytochrome P-450-, NADPH-, and O_2-dependent enzymes were discovered and partly characterized. These enzymes were responsible for the sequential introduction of two methylenedioxy groups from the substrate, (*S*)-scoulerine, via (*S*)-cheilanthifoline to (*S*)-stylopine. This system was further characterized [5] and more recently shown to consist of two separable cytochrome P-450 enzymes catalysing each of the methylenedioxy group formations first in the D-ring, leading from (*S*)-scoulerine to (*S*)-cheilanthifoline and subsequently in the A-ring of this molecule, leading from (*S*)-cheilanthifoline to (*S*)-stylopine [L. Kammerer and M. H. Zenk, unpublished results]. Due to the fact that cytochrome P-450-dependent monooxygenases are involved in the methylenedioxy bridge formation, we assume that this group is initiated via hydroxylation of the attached methoxy group. This step would generate a hemiformal, possibly representing the crucial intermediate in the reaction mechanism [3].

In a previous attempt to develop an assay system for the analysis of the methylenedioxy bridge formation in the conversion of columbamine to berberine in *Berberis* cell cultures, the expected release of one third of the tritium atoms residing in the [3-*O*-C^{3}H$_3$]-group of columbamine was used by Rueffer and Zenk [6] as a criterion to measure the activity of the suspected 'berberine synthase'. Activity of an Fe^{2+}-containing cytosolic enzyme was detected, showing at pH 8.9 the typical protoporphyrine absorption at 408 nm and an unusual high temperature optimum at 70°. The enzyme was isolated and purified to homogeneity but the suspected 'berberine synthase' turned out, however, to be a peroxidase. This enzyme was studied using [3-*O*-^{14}CH$_3$]columbamine as substrate [7]. The peroxidase did not form a methylenedioxy bridge, thus not yielding berberine, but rather catalysed the demethylation of the [3-*O*-CH$_3$]-group of columbamine [7]. The yield of radioactivity released into the medium was exactly 30%, which was previously interpreted [6] as the loss of one third of the [^{3}H]-atoms in the methyl group of columbamine during the transition to the methylenedioxy group. The claimed [6] 'berberine synthase' is, therefore, incorrect as shown by Bauer *et al.* [7]; the originally observed enzyme activity was an artifact.

Both *Berberis* and *Coptis* cell cultures [8, 9] have been used in the past to clarify the biosynthesis of berberine at the enzymic level [10, 11]. The last step in berberine biosynthesis, which still remains to be clarified, is the formation of the methylenedioxy group in the transition of (*S*)-tetrahydrocolumbamine to (*S*)-canadine.

Since both cell cultures from the families Berberidaceae and Ranunculaceae produce profuse amounts of berberine, cell cultures of both families were used for the detection of an enzyme or enzyme system possibly catalysing this remaining biosynthetic step. The metabolic

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route leading from (*S*)-tetrahydrocolumbamine to berberine has not yet been investigated at the enzymic level and is the subject of this study. It is shown that, similarly to the stylopine forming system of *Eschscholtzia* [5], the methylenedioxy bridge formation of berberine is catalysed by a specific cytochrome P-450-dependent enzyme.

RESULTS AND DISCUSSION

The substrate used for the detection of the enzyme catalysing the formation of the methylenedioxy group of canadine from tetrahydrocolumbamine was synthesized enzymically by incubating 2,3-dihydroxy-9,10-dimethoxyprotoberberine with [$^{14}CH_3$]*S*-adenosyl-L-methionine and commercially available catechol-*O*-methyltransferase. In the presence of O_2, [3-*O*-$^{14}CH_3$] columbamine was formed (80% yield) and subsequently reduced with $NaBH_4$. A new assay system was developed in which the [^{14}C]-labelled, TLC-purified substrate was incubated together with various protein fractions isolated from a range of cell cultures. The expected labelled products (canadine or berberine) were separated from the substrate by TLC and were subsequently quantitated by TLC-radioscanning.

Incubation of (*R,S*)-[3-*O*-$^{14}CH_3$]tetrahydrocolumbamine with crude protein extracts from different Ranunculaceae and *Berberis* cell cultures did not lead to the formation of compounds with methylenedioxy bridges. However, when microsomal preparations were isolated from different species of Berberidaceae and Ranunculaceae and incubated with either [3-*O*-$^{14}CH_3$]tetrahydrocolumbamine or [3-*O*-$^{14}CH_3$]columbamine, a clear but differential formation of berberine could be detected (Table 1) from tetrahydrocolumbamine. Columbamine did not serve as substrate for this reaction. The question remained why berberine was formed instead of the expected canadine. This was due to the activity of (*S*)-tetrahydroprotoberberine oxidase (STOX) which catalyses the oxidation of (*S*)-tetrahydroprotoberberines to the corresponding protoberberines [12]. This contaminating enzyme (STOX) was present in considerable quantities in the microsomal fraction oxidizing both the substrate tetrahydrocolumbamine and the product canadine to their corresponding protoberberines, i.e. columbamine and berberine. This enzyme could not be selectively inhibited nor could the vesicles in which the STOX enzyme is contained [13] be separated from the microsomal preparations by differential centrifugation. Comparison of the enzyme activities showed that the highest transformation of [$^{14}CH_3$]tetrahydrocolumbamine to berberine was found in a protoberberine-containing, yellow-coloured cell line of *Thalictrum tuberosum* (Table 1), which was used for further experiments. The reaction product of the transformation had been identified only by TLC-radioscanning and, therefore, the identity of the product had to be rigorously established. For the identification of the reaction product, twenty standard incubation assays were combined, the radioactively labelled product (13.3×10^6 dpm μmol^{-1}) was reduced with $NaBH_4$ to (*R,S*)-canadine (11.7×10^6 dpm μmol^{-1}), and diluted with unlabelled (*R,S*)-canadine resulting in a specific activity of 2.2×10^4 dpm μmol^{-1}. This compound was recrystallized three times from MeOH. The specific activity remained constant at 2.0×10^4 dpm μmol^{-1}. This experiment clearly identified the reaction product of the [^{14}C]tetrahydrocolumbamine incubation as berberine which had been formed via canadine by oxidation with STOX that contaminated the microsomal fraction.

The reaction was dependent on NADPH. Replacement of NADPH with NADH showed a reaction rate of only *ca* 21%. A synergistic effect achieved by adding both NADPH and NADH, which had been observed previously in cytochrome P-450-catalysed reactions [14], was not observed here. Replacement of NADPH with NAD, NADP, FAD or FMN showed no reaction. The enzyme was inactive in the absence of O_2, as shown by the removal of O_2 by a glucose oxidase/catalase system [15]. The formation of the methylenedioxy bridge in the presence of O_2 and NADPH could be strongly inhibited by carbon monoxide. The inhibition could be partially reversed by illumination with white light (Table 2). Further analysis showed that predominantly blue light (wavelength 438 nm) was responsible for this reversal (data not

Table 1. Occurrence of canadine synthase in plant cell cultures

Tissue culture	Enzyme activity6 (pkat l^{-1} medium)
Berberis aristata DC.	2.6
B. crataegina DC.	5.5
B. taliensis Schneid.	0.9
B. stolonifera Koehne & E. Wolf	2.6
B. henryana Schneid.	0.7
B. notabilis Schneid.	0
Coptis japonica Mak.	1.6
Thalictrum glaucum Desf.	2.1
T. glabrum K. Koch	4.4
T. macrocarpum Gren.	0.7
T. tuberosum L.	
Yellow cell line	10.4
White cell line	5.5

The values were determined with tetrahydrocolumbamine as substrate measured under standard assay conditions. Activity was not detected when columbamine was used as substrate.

Table 2. Effect of carbon monoxide and light on microsomal bound canadine synthase activity from *T. tuberosum* cell cultures under standard incubation conditions

Assay condition	Relative enzyme activity (%)	Product formed (nmol)
Air	100	73
N_2/O_2 (9:1) (light)	100	73
N_2/O_2 (9:1) (dark)	98	53
CO/O_2 (9:1) (dark)	8.5	6
CO/O_2 (9:1) (white light)	55.5	39

Table 3. Effect of inhibitors of cytochrome P-450 enzymes on microsomal bound canadine synthase from *T. tuberosum* cell cultures

Inhibitor	Concentration (μM)	Product formed (pmol)	Inhibition (%)
None	0	99	0
Ketoconazole	300	0	100
	35	50	50
Prochloraz	300	0	100
	35	50	50
Tetcyclacis	35	0	100
Metyrapone	300	58	41
Propiconazole	300	72	27
Tropolone	300	58	41
Triadimefone	300	62	37
Plumbagin	35	0	100
Juglone	0.35	50	50
	3.5	15	85
Menadione	0.35	48	52
	3.5	0	100
Cytochrome *c*	17	50	50
	170	0	100

The standard incubation assays containing 0.5 pkat of the enzyme were incubated with the inhibitor concentrations shown in the table.

shown). A second indication for the involvement of a cytochrome P-450 enzyme in the formation of the methylenedioxy bridges of canadine and berberine was the inhibition of the reaction by typical cytochrome P-450 inhibitors (Table 3). Inhibitors such as ketoconazole, tetcyclacis and the naphthoquinones, plumbagin, menadione and juglone, which interact with heme group [16], strongly inhibited the formation of the methylenedioxy bridge under the conditions given in Table 3. Strong inhibition was also observed by addition of cytochrome *c* which extracts electrons formed by the NADPH–cytochrome *c*-reductase from the enzyme complex (Table 3). The content of cytochrome P-450 in the microsomal preparations was determined by the method of Omura and Sato [17] to be 163 pmol mg^{-1}, which falls within the normal range determined for higher plants [18]. The enzyme system transforming tetrahydrocolumbamine to canadine has been named canadine synthase. Canadine is the preferred name over tetrahydroprotoberberine (which includes canadine) because tetrahydroberberine tends to be confused with the tetrahydro*proto*berberines and a specific oxidase like STOX (tetrahydroprotoberberine oxidase [12]) is readily confused with tetrahydroberberine oxidase [19].

The physical characteristics of canadine synthase were as follows. The optimal reaction rate was observed at pH 8.5 in glycine–NaOH buffer (Fig. 1) at 40°. The apparent K_m values for NADPH and (*R,S*)-tetrahydrocolumbamine were determined to be 33 and 11 μM, respectively (Fig. 2). The enzyme was highly substrate specific and would not accept tetrahydroprotoberberine alkaloids (differing from tetrahydrocolumbamine in ring

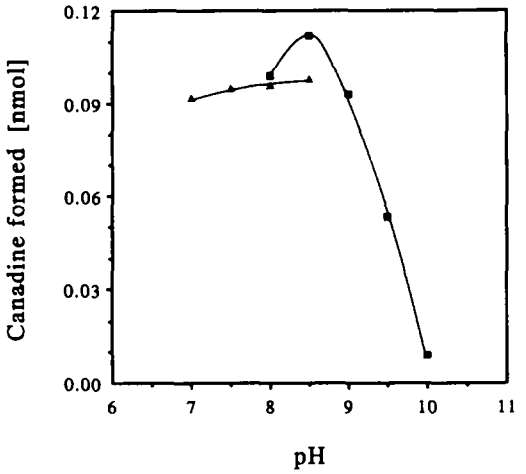

Fig. 1. pH-Dependence of the canadine synthase. The reaction was measured using the standard incubation assay with 0.09 pkat of the enzyme (0.65 pkat mg^{-1} protein) and 50 μmol of Tris–HCl buffer, pH 7–8.5, (▲ -▲) or glycine–NaOH buffer, pH 8.0–10, (■—■).

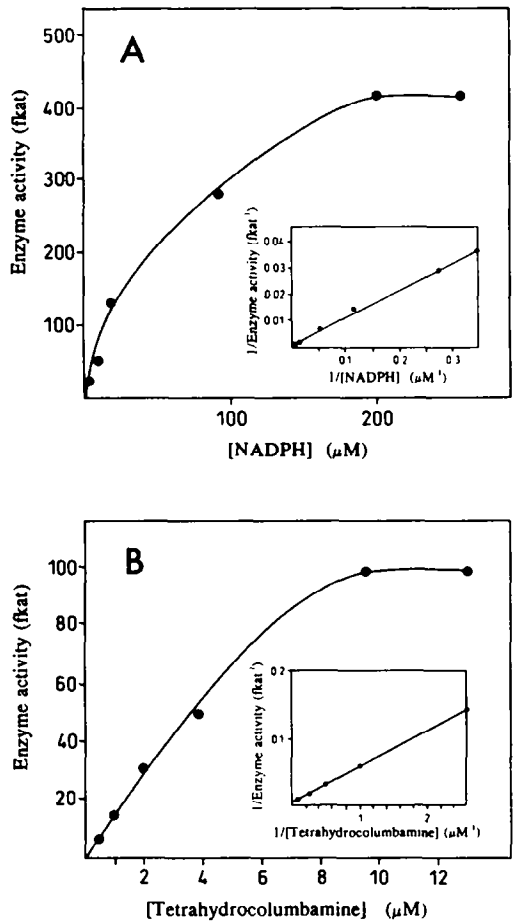

Fig. 2. The effect of NADPH (A) and tetrahydrocolumbamine (B) concentration on the microsomal bound canadine synthase activity. The insert depicts the double reciprocal plot. Standard incubation assays with varying NADPH or tetrahydrocolumbamine concentrations and 0.1 pkat of the enzyme were used.

Table 4. Substrate specificity of microsomal bound canadine synthase from *T. tuberosum* cell cultures under standard assay conditions

Substrate	Product formed (pmol)
(*R,S*)-Tetrahydrocolumbamine	160
(*S*)-Tetrahydrocolumbamine	155
(*R*)-Tetrahydrocolumbamine	0
Columbamine	0
(*R,S*)-Tetrahydrojatrorrhizine	0
(*R,S*)-Cheilanthifoline	0
(*R,S*)-Nandinine	0
(*R,S*)-Tetrahydrogroenlandicine	0
(*R,S*)-Tetrahydrothalifaurine	0

For determination of the substrate specificity 0.5 nmol of the corresponding alkaloid were added to the standard assay.

A or ring D substitution) as substrate (Table 4). In addition, the protoberberine alkaloid, columbamine, was not transformed. This again demonstrates that the pathway from tetrahydrocolumbamine to berberine proceeds through canadine, not through columbamine as previously suggested [6, 10] for *Berberis*. Of a wide variety of C-ring reduced protoberberine substrates, only tetrahydrocolumbamine was transformed. Moreover, like other enzymes in the protoberberine pathway [10], the canadine synthase showed strong stereospecificity, accepting only (*S*)-tetrahydrocolumbamine as substrate but not its (*R*)-congener (Table 4).

Canadine synthase is a unique microsomal bound cytochrome P-450 enzyme which occurs in berberine-producing cell cultures within the Berberidaceae and Ranunculaceae. The enzyme accepts exclusively (*S*)-tetrahydrocolumbamine as substrate and converts it to (*S*)-canadine. Although the canadine formed in the microsomal preparations was immediately oxidized to berberine by an (*S*)-tetrahydroprotoberberine oxidase contamination in this subcellular fraction, the stereochemical configuration of the canadine was without doubt the *S*-epimer due to the stereospecificity of STOX [12]. Had the canadine that was formed been the *R*-epimer, it would have accumulated during the assay.

Canadine synthase catalyses the penultimate step in berberine biosynthesis (Fig. 3). The final step in the pathway, the aromatization of ring C, is catalysed by (*S*)-tetrahydroprotoberberine oxidase [12] in the Berberidaceae and by a slightly different enzyme in the Ranunculaceae (i.e. *Coptis* [19] and *Thalictrum* [Galneder and Zenk, unpublished data]). In both families, the end product alkaloid is berberine. This investigation completes our knowledge of the biosynthesis of berberine starting from two molecules of the primary metabolite, L-tyrosine. A total of 13 enzymes are involved in the formation of this pharmacologically valuable alkaloid.

EXPERIMENTAL

Plant material. All cell culture were provided by the cell culture laboratory of this department. The cells were grown in 1 l flasks containing 250 ml Linsmaier and Skoog medium [20] over a period of 7 days at 23° on a gyratory shaker (100 rpm) at 750 lux. For partial characterization of the enzyme, a protoberberine producing cell line of *Thalictrum tuberosum* was used. Inoculated with 35 g fr. wt the 1 l conical flasks were harvested after one week by suction filtration yielding 230 g fr. wt corresponding to 6.7 g dry wtl^{-1} medium. The cells were deep frozen with liquid nitrogen and used immediately.

Radiochemicals. (*R,S*)-[3-*O*-$^{14}CH_3$]Tetrahydrocolumbamine was synthesized from 2,3-dihydroxy-9,10-dimethoxyprotoberberine by incubation with [$^{14}CH_3$]-*S*-adenosyl-L-methionine (SAM) and catechol-*O*-methyltransferase (Sigma). The columbamine formed was reduced with $NaBH_4$ and the product purified by TLC (Polygram Sil G/UV_{254}, Macherey and Nagel, toluene–EtOAc–diethylamine, 7:2:1 R_f 0.5). The isolated (*R,S*)-[3-*O*-$^{14}CH_3$]tetrahydrocolumbamine had a specific activity of 56 μCi μmol^{-1}. Due to the lack of regiospecificity of the porcine catechol-*O*-methyltransferase, (*R,S*)-[2-*O*-$^{14}CH_3$]tetrahydrojatrorrhizine (R_f 0.4) was formed to approximately 10% as a by product of the enzymic synthesis. (*R,S*)-[3-*O*-$^{14}CH_3$]Cheilanthifoline, (*R,S*)-[2-*O*-$^{14}CH_3$]tetrahydrogroenlandicine and (*S*)-[10-*O*-$^{14}CH_3$]nandinine were synthesized according to ref. [4]. [$^{14}CH_3$]Methionine was purchased from Amersham (U.K.) and bioconverted to radiolabelled SAM by the action of SAM-synthetase isolated from *Escherichia coli* EWH47 according to ref. [21].

Standard enzyme assay. (*R,S*)-[3-*O*-$^{14}CH_3$]Tetrahydrocolumbamine (0.5 nmol, 40000 cpm), 10 nmol

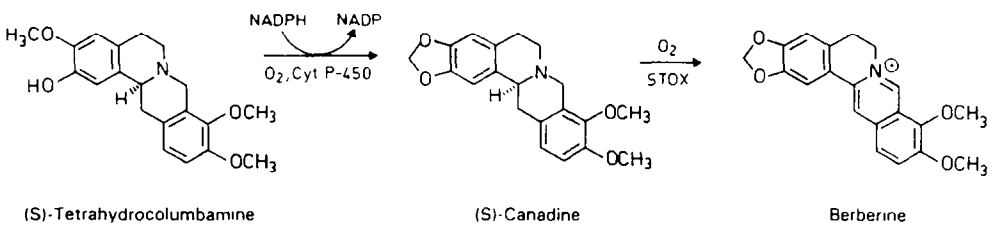

Fig. 3. Reaction scheme of the final two steps in the biosynthesis of the protoberberine alkaloid, berberine. The first step leading from tetrahydrocolumbamine to canadine is catalysed by the cytochrome P-450 enzyme, canadine synthase; the second step leading from canadine to berberine is catalysed by (*S*)-tetrahydroprotoberberine oxidase (STOX).

NADPH, 50 μmol glycine–NaOH buffer, pH 8.5, and 0.1–0.8 mg protein were incubated in a total volume of 300 μl for 30 min at 40°. Assays with boiled enzyme served as control. The incubation was terminated by the addition of 200 μl EtOAc, shaken mechanically for 10 min and the phases sepd by centrifugation at 10 000 *g* for 3 min at room temperature. The EtOAc layer was subjected to TLC (Polygram Sil G/UV_{254}, Macherey and Nagel) using cyclohexane–diethylamine (9:1) as solvent system (columbamine R_f 0.0, tetrahydrocolumbamine R_f 0.1, berberine R_f 0.5, canadine R_f 0.8). The chromatograms were scanned and quantitated for radioactivity by a TLC linear analyser (Berthold). Since in these tissue cultures the (*S*)-tetrahydroprotoberberine oxidase (STOX) [12] is very active and could not be separated from the microsomal frs either by sucrose density centrifugation or by gel filtration on Sephacryl-S-1000 (Pharmacia), the product of the reaction was not canadine, but rather berberine.

Preparation of microsomal fractions. Deep-frozen cells, typcially 100 g fr. wt, were thawed with the double amount (w/v) 0.1 M Tricine–NaOH buffer, pH 7.5, containing 1 mM EDTA and 5 mM thioglycolic acid in an ice-cold mortar and stirred with a pestle for about 20 min. After filtration through cheesecloth the crude extract was centrifuged at 10 000 *g* for 10 min at 4°. The supernatant was passed through an XAD-2 column (2.5 × 15 cm, Serva) that had been equilibrated with the above buffer. The microsomes and vesicles of the alkaloid-free column run-through were harvested by centrifugation at 50 000 *g* for 30 min at 4°. Addition of $MgCl_2$, as described earlier [22], did not result in higher enzyme activities.

Product identification. For product identification, 20 standard assays were incubated for 30 min, combined and passed over an XAD-2 column (Serva). The alkaloids were eluted with MeOH, concentrated under a stream of N_2 and resolved by TLC (Polygram, Macherey and Nagel, solvent system: cyclohexane–diethylamine, 9:1). After determination of the specific activity of berberine (13.3×10^6 dpm μmol^{-1}), this enzymic product was reduced with $NaBH_4$ to (*R,S*)-canadine and chromatographed again in the same solvent system. The specific activity was determined for a second time (11.7×10^6 dpm μmol^{-1}). In addition, the product was diluted with unlabelled canadine (180 mg = 532 μmol) and recrystallized × 3 times from MeOH. The specific activity was determined after each crystallization step.

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