Characterization of a novel N-methyltransferase (NMT) from *Catharanthus roseus* plants

Detection of NMT and other enzymes of the indole alkaloid biosynthetic pathway in different cell suspension culture systems

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Abstract

Young leaves from *Catharanthus roseus* plants contain a novel N-methyltransferase which transfers the methyl group from S-adenosyl-L-methionine specifically to position 1 of (2R, 3R)-2,3-dihydro-3-hydroxytabersonine, producing the N-methylated product. The enzyme shows a high degree of specificity toward substrates containing a reduced double bond at position 2,3 of tabersonine derivatives but the more substituted N-desmethyl-deacetylvindoline did not act as a substrate. The enzyme catalyses the third last step in vindorosine and vindoline biosynthesis, and is associated with chlorophyll-containing fractions in partially purified enzyme preparations. The lack of vindoline accumulation in cell suspension cultures is correlated with the lack of expression of this enzyme activity as well as that of an acetyltransferase which catalyses the last step in vindoline biosynthesis. Neither fungal elicitor treatment of cell line #615 nor transfer to alkaloid production medium resulted in expression of these two enzyme activities, nor was either enzyme activity detected in phototrophic or hormone autotrophic cultures. Cell lines #200, 615-767 and 916 could not be induced to produce DAT or NMT enzyme activities.

Introduction

It was recently proposed (Fahn et al 1985a,b) that the biosynthesis of the aspidosperma alkaloid, vindoline, proceeds from the less functionalized alkaloid, tabersonine, by a sequence consisting of three consecutive hydroxylations, followed by N-methylation, O-methylation and O-acetylation. This proposal was based on results obtained from substrate specificity studies performed with an acetyl CoA-dependent O-acetyltransferase (Fahn et al 1985a) and on demonstration of an S-adenosyl-L-methionine O-methyltransferase activity (Fahn et al 1985b). The enzyme-catalysed acetylation of deacetylvindoline was also suggested to be the last step in vindoline synthesis by DeLuca et al (1986). Further studies performed with *Catharanthus roseus* seedlings (Balsevich et al 1986, DeLuca et al 1986) suggested that the route from tabersonine to vindoline was not as proposed by Fahn et al (1985a,b), but rather proceeded by the sequence aromatic hydroxylation, O-methylation, hydration of 2,3-double bond, N(1)-methylation, hydroxylation at C-4, and 4-O-acetylation (Fig. 1). In accordance with this latter proposal, we report the isolation of a specific alkaloid N-methyltransferase which catalyses the third last step in vindoline biosynthesis.

Material and Methods:

Plant Material. *Catharanthus roseus* (L.) G. Don cv. Little Delicata plants were grown from seed and were maintained in the greenhouse.

Chemicals. S-adenosyl-L-(methyl-14C) methionine (2.18 GBq/mmol) and (1-14C) acetyl-coenzyme A (2.04 GBq/mmol) were purchased from Amersham. (2R,3S)-2,3-dihydrotabersonine was prepared from tabersonine (hydrochloride salt) by reduction with sodium cyanoborohydride in a mixture of acetic acid and methanol. (2R,3S)-2,3-dihydro-3-hydroxytabersonine was prepared from tabersonine (hydrochloride salt) by reduction with sodium cyanoborohydride in a mixture of acetic acid and methanol. (2R,3R)-2,3-dihydro-3-hydroxytabersonine was prepared from tabersonine by oxidation with m-chloroperbenzoic acid according to the procedure of Calabi et al (1982). (2R,3R)-2,3-dihydro-3-hydroxy-N(1)-methyl-tabersonine was prepared from tabersonine by the procedure of Hugel et al (1981), and N-desmethyl-deacetylvindoline was prepared from N-desmethylvindoline by reaction with 1.1 equivalents of sodium methoxide in methanol at 50°C. Other alkaloids were from our reference collection. N-desmethylvindoline was a gift of Dr. Lajos Szabo, Budapest; O-desmethylvindoline and O-desmethyl-dihydrovindoline were gifts from Dr. John Rossazza, University of Iowa. Sephadex G-100 was from Pharmacia fine chemicals and all other chemicals were...
The substrate-free supernatants were assayed for DAT, yielding a spectrum identical to that obtained from a centrifuge, a clear supernatant was obtained. Homogenates were transferred to Eppendorf tubes and, described previously (Kurz and Constabel 1982). Enzyme preparation or were extracted for alkaloids as two day intervals and were kept frozen until transferred to alkaloid production medium (Zenk et al. 1977). Cells were harvested in liquid nitrogen at 14 days. At this point, cells were sub-cultured and were taken to dryness by evaporation for radioactivity. The methylated product was confirmed by co-chromatography with authentic preparative silica gel G TLC plate using ethyl acetate. The identity of the methylated product was produced in sufficient quantity for chemical analysis by repeating many assays with prolonged incubation times of 1 hr. In this case, ethyl acetate extracts were pooled and were taken to dryness by evaporation under vacuum. The residue was chromatographed on a preparative silica gel 6 TLC plate using ethyl acetate. The identity of the methylated product was confirmed by co-chromatography with authentic 2,3-dihydro-3-hydroxytabersonine-N-methyltransferase (NMT) assay contained: pH 7.4, 3.4 M deacetylvindoline, 4.4 M (1-C) -acetyl-coenzyme A (.05 M) and enzyme in a total of 100 &l 0.1 M Tris-HCl, pH 8. The mixture was incubated for 5 min at 30°C and the reaction was terminated by the addition of 50 &l of 1 N NaOH. The acetylated alkaloid was extracted by adding 250 &l of chloroform and shaking for 2 min. The organic and aqueous phases were separated by 1 min centrifugation in an Eppendorf centrifuge. 100 &l of the organic phase were transferred to scintillation vials and counted for radioactivity. The S-adenosyl-L-methionine: S-adenosyl-L-(methyl-C)methionine was used in the presence of 2,3-dihydro-3-hydroxytabersonine and S-adenosyl-L-methionine, the corresponding N-methylated alkaloid was produced, whereas no product was found in the absence of either substrate or enzyme. The product was analyzed by gas chromatography: mass spectrometry and was identified as the N-methylated derivative of 2,3-dihydro-3-hydroxytabersonine. When radiolabelled S-adenosyl-L-(methyl-C)methionine was used in assays with the alkaloid substrate and enzyme, TLC and autoradiography of the labelled product resulted in only one radioactive spot coincident with authentic 2,3-dihydro-3-hydroxy-N-methyltabersonine.

Protein estimation. Protein was determined according to the method of Bradford (1976) using the Bio-Rad protein reagent and bovine serum albumin as standard protein.

Chlorophyll content. Total chlorophyll was determined as described by Arnon (1949).

RESULTS

Biosynthesis of N-methylated products in cell-free preparations. When leaf tissue was extracted (as described in Material and Methods), it was subsequently applied directly to a Sephadex-G25 column. The eluate was utilized without further treatment in order to develop an enzyme assay for NMT. When this preparation was incubated in the presence of 2,3-dihydro-3-hydroxytabersonine and S-adenosyl-L-methionine, the corresponding N-methylated alkaloid was produced, whereas no product was found in the absence of either substrate or enzyme. The product was analyzed by gas chromatography: mass spectrometry and was identified as the N-methylated derivative of 2,3-dihydro-3-hydroxytabersonine. When radiolabelled S-adenosyl-L-(methyl-C)methionine was used in assays with the alkaloid substrate and enzyme, TLC and autoradiography of the labelled product resulted in only one radioactive spot coincident with authentic 2,3-dihydro-3-hydroxy-N-methyltabersonine.

Sephadex G-100 gel permeation chromatography. Crude leaf extracts were centrifuged at 3000 g for 10 min in order to eliminate large cellular debris. Higher centrifugation speeds were not chosen since this resulted in lower recoveries of NMT in the supernatant. The centrifuged supernatant was applied directly to a pre-equilibrated Sephadex-G100 gel filtration column (as described in methods). The fractions which eluted from this column were assayed for NMT, SS, and TDC enzyme activities (Fig. 2). The NMT activity co-chromatographed with the chlorophyll peak and this enzyme activity was completely separated from DAT, SS and TDC enzyme activities. When fractions containing NMT enzyme activity were centrifuged (10,000 xg for 15 min), a clear supernatant and a chlorophyll-containing

of analytical grade.

Enzyme preparation. Shoot tips were harvested (ca. 15 g) and were homogenized in 40 ml of 0.1 M TRIS-HCl buffer (pH 8, 28 mM 2-mercaptoethanol, and 5 mM EDTA) using an Ultra-Turrax equipped with an S25N head. This homogenate was filtered through 6 layers of a nylon mesh (50 micron) and the filtrate was centrifuged at 3000 xg for 10 min in a Sorvall RC-8 centrifuge equipped with an SS-34 head. The supernatant was applied directly to a Sephadex G-100 (medium) gel filtration column pre-equilibrated with 100 mM Tris-HCl (pH 8.0 and 28 mM 2-mercaptoethanol). Fractions were collected and assayed for enzyme activities.

Enzyme assays. Acetyl coenzyme A: deacetylvindoline O-acetyltransferase (DAT) was assayed as described previously (Delucia et al 1985). The assay mixture consisted of 100 &l, pH 8, 3.4 M deacetylvindoline, 4.4 M (1-C)-acetyl-coenzyme A (.05 M) and enzyme in a total of 100 &l 0.1 M Tris-HCl, pH 8. The mixture was incubated for 5 min at 30°C and the reaction was terminated by the addition of 50 &l of 1 N NaOH. The acetylated alkaloid was extracted by adding 250 &l of ethyl acetate and shaking for 2 min. The organic and aqueous phases were separated by 1 min centrifugation in an Eppendorf centrifuge. 100 &l of the organic phase were transferred to scintillation vials and counted for radioactivity.

The S-adenosyl-L-methionine: 16-methoxy-2, 3-dihydro-3-hydroxy-tabersonine-N-methyltransferase (NMT) assay contained: pH 7.4, 3,4 M deacetylvindoline, 8.8 M (1-C) -acetyl-coenzyme A (.05 M) and enzyme in a total of 200 &l 0.1 M Tris-HCl, pH 8. The assay lasted 30 min at 30°C and was processed as described for the acetyltransferase activity. The organic phase contained the product and was counted for radioactivity. The methylated product was produced in sufficient quantity for chemical analysis by repeating many assays with prolonged incubation times of 1 hr. In this case, ethyl acetate extracts were pooled and were taken to dryness by evaporation under vacuum. The residue was chromatographed on a preparative silica gel 6 TLC plate using ethyl acetate. The identity of the methylated product was confirmed by co-chromatography with authentic 2,3-dihydro-3-hydroxy-tabersonine and S-adenosyl-L-methionine, the corresponding N-methylated alkaloid was produced, whereas no product was found in the absence of either substrate or enzyme. The product was analyzed by gas chromatography: mass spectrometry and was identified as the N-methylated derivative of 2,3-dihydro-3-hydroxytabersonine. When radiolabelled S-adenosyl-L-(methyl-C)methionine was used in assays with the alkaloid substrate and enzyme, TLC and autoradiography of the labelled product resulted in only one radioactive spot coincident with authentic 2,3-dihydro-3-hydroxy-N-methyltabersonine.

Sephadex G-100 gel permeation chromatography. Crude leaf extracts were centrifuged at 3000 g for 10 min in order to eliminate large cellular debris. Higher centrifugation speeds were not chosen since this resulted in lower recoveries of NMT in the supernatant. The centrifuged supernatant was applied directly to a pre-equilibrated Sephadex-G100 gel filtration column (as described in methods). The fractions which eluted from this column were assayed for DAT, NMT, SS and TDC enzyme activities. Cell lines #200, 615A and 916 (Kurz 1984) were also investigated for these enzyme activities.

Elicitor treatment of cell line #615. Treatment of cell line #615 with an autoclaved preparation of Pythium aphanidermatum fungal extracts (Eilert et al 1987a) resulted in the induction of SS and TDC followed by the accumulation of indole alkaloids including tabersonine. This cell line was grown for 14 days on B5 medium, and stationary phase cells were transferred to fresh B5 medium. After four days of growth in this medium, cells were treated with fungal elicitor and samples were harvested at time 0, 2, 4, 8, 12, 16, 24, 36 and 48 h of elicitor treatment. Harvested cells were frozen in liquid nitrogen and were maintained frozen until extraction. Cells were processed as described in the previous section (Growth of tissue cultures).

Growth of hormone autotrophic cell line #615H and photoautotrophic cell line #555G. Cell line #615H was grown for 24 days on B5 medium in the absence of hormones (OB5 medium) as described by Eilert et al (1987b). Photoautotrophic cell line #555G was grown for 6 weeks under conditions described previously (Tyler et al 1986).

Protein estimation. Protein was determined according to the method of Bradford (1976) using the Bio-Rad protein reagent and bovine serum albumin as standard protein.