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NICOTINE *N*-DEMETHYLASE IN CELL-FREE PREPARATIONS FROM TOBACCO CELL CULTURES

DONG-YUN HAO and MICHAEL M. YEOMAN

Institute of Cell and Molecular Biology, The University of Edinburgh, Daniel Rutherford Building, Mayfield Road, Edinburgh EH9 3JH, U.K.

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco cell culture; *N*-demethylation; nicotine *N*-demethylase.

Abstract—The activity of the enzyme(s) catalysing the bioconversion of nicotine to nornicotine has been demonstrated, for the first time, in cell-free preparations from tobacco cell cultures. Using ^{14}C-assay, it has been shown that a maximal specific activity of *ca* 0.2 pkat mg protein^{-1} is present exclusively in the supernatant fraction after centrifugation at 8 800 *g*. The enzyme has a pH optimum between 9.0 and 9.5, and a temperature optimum between 25 and 30°, while the V_{max} and the apparent K_m are 7.6×10^{-2} pkat and 7.4 μM of ^{14}C-nicotine, respectively. The decline in enzyme activity, following the removal of some endogenous co-factors and co-enzymes by dialysis of the crude enzyme preparation, can be restored and indeed enhanced by the addition of NADPH, suggesting that this enzyme may be NADPH dependent.

INTRODUCTION

The *N*-demethylation of nicotine (**1**) to nornicotine (**2**), a natural reaction in the tobacco plant [1], has long been assumed to be catalysed enzymatically. This is despite the lack of direct evidence for the existence of a *N*-demethylase in tobacco. As early as 1955, Griffith, Valleau and Stokes [2] showed, in a breeding study with *Nicotiana tabacum*, that the *N*-demethylation which takes place during the curing of leaves is controlled by a single dominant gene and, predicted the presence of this enzyme. In addition, Leete and Chedekel [3] presumed, in their hypothesis, a mechanism of this *N*-demethylation involving an asymmetric molecule. In an earlier investigation also using tobacco cell suspension cultures [4], we have suggested that the preferential production of (+)- or (−)-nornicotine from (−)-nicotine is likely to be enzyme-controlled, although our results did not support the Leete and Chedekel hypothesis [3].

Much earlier, Bose *et al.* (1956) [5] reported that crude homogenates of the leaves of *N. tabacum* were capable of converting nicotine to nornicotine and, 10 years later, Schröter [6] also showed that nicotine could be demethylated by extracts of *N. alata*. In addition, James reported [7] that leaf homogenates of *N. tabacum* could convert nicotine into nornicotine in the presence of glycine and ethanolamine, providing some evidence for the existence of a transmethylase. However, none of these reports included any details of the enzyme catalysing this reaction. It is only very recently that nicotine *N*-demethylase in cell-free preparations from tobacco cell cultures has been demonstrated in this laboratory [8]. At almost the same time, an enzyme catalysing a similar reaction in an isolated microsomal preparation from *N. otophora* was reported by Chelvarajan *et al.* [9]. In this paper, we describe the occurrence and partial characterisation of nicotine *N*-demethylase in cell-free preparations from cell suspension cultures of *N. tabacum* L cv. Wisconsin-38 [10].

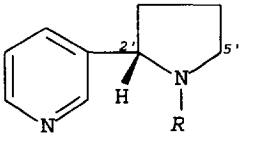
RESULTS AND DISCUSSION

Occurrence of nicotine N-*demethylase and development of an* in vitro *enzyme assay*

Nicotine *N*-demethylase activity was assayed in cell-free preparations from a 10-day-old cell suspension cultures of *N. tabacum* using ^{14}C-nicotine as the substrate. In preliminary experiments, no enzyme activity was detected in crude extracts, prepared with 0.1 M phosphate buffer (pH 7.5) and supplemented with a variety of additives such as mercaptoethanol, DTT, EDTA, sucrose, ascorbate, glycine, $MgSO_4$ and $MnSO_4$ in various combinations. However, activity was detected at pH 9.0 when 0.1 M Tris–HCl or 0.1 M glycine–NaOH buffer, supplemented with 3 mM DTT

1: $R = CH_3$

2: $R = H$



and 5 mM EDTA, was used. The highest activity was found with 0.1 M Tris–HCl buffer at pH 9.0. The *N*-demethylation product, i.e. nornicotine, was determined by co-elution with authentic non-radioactive nornicotine as a carrier (see Experimental). The product of this reaction had also previously been confirmed by HPLC and GC-MS (see ref. [4]). After taking into account the impurities of the ^{14}C-nicotine substrate and the minor reaction side-products, this reaction was established as stoicheiometrical. Since none of the additives used, such as DMSO, PVP, PMSF, Triton ×100, CHAPS, deoxycholate and NaCl, improved enzyme activity over a 30 min incubation course, only 3 mM DTT and 5 mM EDTA were subsequently used in the assay.

Centrifugation of the crude homogenate at 8800 *g* increases the amount of soluble protein in the supernatant reaching a maximum value at 10 min. However, maximal enzyme activity was achieved after centrifugation for only 1 min. This increase in enzyme activity in the supernatant may be due to the removal of some competitive proteins or inhibitors which are associated with the cell debris. In contrast, prolonged centrifugation appears to lead to the denaturation of the enzymes. Therefore, the freshly prepared enzyme homogenates were employed in all subsequent assays.

Most of the enzyme activity (0.14 pkat mg protein^{-1}) is present in the supernatant fraction with only a small amount of activity in the pellet (Table 1). The activity present in the supernatant is nine times greater than the control, in which the enzyme was denatured by heating, and superior to the crude homogenate. This preliminary study resulted in the adoption of the radioactive assay procedure described in the Experimental.

Characterisation of nicotine N-*demethylase*

Using the radioactive assay procedure, nicotine *N*-demethylase was characterised in the supernatant fraction from centrifugation of the crude cell-free homogenate. The results show that nicotine *N*-demethylation increases rapidly for *ca* the first 30 min of the reaction at 30° and pH 9.0. Thereafter, the reaction gradually tends to become constant and remains so until the end of the 90 min incubation. A similar linear pattern was reported by Chelvarajan *et al.* [9] for the same reaction in a microsomal fraction of tobacco leaves, although a non-linear phase for up to 1 hr was observed for *p*-chloro-*N*-methylaniline *N*-demethylase in avocado pear [11]. In addition, a linear relationship exists between enzyme concentration and enzyme activity, but only between 0.11 and 0.22 mg of protein per 0.5 ml of enzyme solution. Accordingly, kinetic studies were carried out using a protein concentration of 0.2–0.4 mg protein per ml enzyme solution.

The results also show that the pH optimum for this catalytic reaction lies between 9.0 and 9.5, and the temperature optimum lies between 25° and 30°. The temp optimum is within a range common for a large number of plant enzymes [12, 13] and, is the same as that reported for nicotine *N*-demethylase activity in isolated microsomes from leaf tissue of *N. otophora* [9]. However, the rather high pH optimum is unusual for a plant enzyme, although putrescine *N*-methyltransferase from tobacco roots displays a pH optimum between 8.0 and 9.0 [14]. In contrast, a much lower pH optimum of 7.0–7.5 was reported for a similar enzyme in an isolated microsomal preparation from leaf tissue of *N. otophora* [9], for aminopyrine *N*-demethylase from microsomes of Jerusalem artichoke (pH 7.5) [15] and for *p*-chloro-*N*-methylaniline *N*-demethylase in avocado pear (pH 7.6–8.0) [11]. The reason for such a high pH optimum for nicotine *N*-demethylase from tobacco cell cultures in this study is at present unclear. It would appear that the pH optimum of *N*-demethylation depends on the specificity and source of the enzyme, despite the fact that the enzyme(s) appears to catalyse the same reaction. Perhaps, this high pH optimum is related to the endogenous level of alkaloids present in the cells.

Effect of the substrate concentration on reaction velocity

The kinetic properties of nicotine *N*-demethylase were studied when the enzyme was assayed for 30 min, at 30° and pH 9.0, and the amount of ^{14}C-nicotine substrate was varied from 0 to 15.2 μM per reaction tube. The resulting Michaelis-Menten kinetic plot shows a clear relationship between substrate concentration and reaction velocity. Using a statistical computing package (Minitab), the regression of the reciprocals of the two sets of kinetic data resulted in a linear plot

Table 1. Distribution of nicotine *N*-demethylase activity in a crude homogenate

Fraction	Yield of protein* (mg ml^{-1})	Specific activity (pkat × 10^{-3} mg protein^{-1})
Control†	0.4±0.1	15±3.0
Crude homogenate	0.4±0.1	99±15
Supernatant	0.4±0.1	139±6
Pellet	0.04±0.1	2.7±1.1

*Yield of protein is presented without subtraction of the control.

†Control was the crude homogenate without centrifugation and denatured by heating at 100° for 5 min.

with a coefficiency of determination of 99.2%, suggesting that the kinetic data fulfil the criteria of a Lineweaver-Burk plot. From these data, the K_m and V_{max} were estimated to be 7.4 μM and 7.6×10^{-2} pkat (equivalent to 0.38 pmol min^{-1} mg $protein^{-1}$) respectively. Previous studies using isolated microsomes from leaf tissue of *N. otophora* [9] show a higher K_m (51 μM) and V_{max} (24 pmol min^{-1} mg $protein^{-1}$) for nicotine *N*-demethylase than those reported here. This may be due to the fact that a much higher concentration of enzyme (2 mg $protein^{-1}$) was used in their study than this study (0.4 mg $protein^{-1}$). Indeed, their assay was performed using a buffer supplemented with NADH and NADPH, a co-enzyme known to stimulate enzyme activity (this will be discussed next). Conclusively, these kinetic data, together with the characteristics of the enzyme obtained in this study, suggest that this reaction is an enzymatic *N*-demethylation rather than autocatalysis.

Requirements for co-factors and co-enzymes

The addition of 0.5 mM ATP, 1 mM NAD or 1 mM NADP to the assay mixture did not affect enzyme activity, whilst 1 mM NADH slightly decreased enzyme activity (Table 2). However, NADPH (1 mM) increased the enzyme activity. In crude homogenates without these additives where co-enzyme was quite active, it is likely that there are several co-factors aiding enzyme activity.

When an enzyme preparation dialysed for 24 hr against the preparation buffer was used, the results largely confirmed the preliminary results with the crude homogenate. In order to distinguish between the denaturation effects on the enzyme caused by storage in the cold room for 24 hr from that of dialysis, however, an experimental control (S) without dialysis [the same as the fresh enzyme preparations (FS)] was maintained beside the dialysis breaker under the same conditions and assayed 24 hr later together with the dialysed sample (DS). It can be seen from Table 2 that storage of FS for 24 hr resulted in a marked reduction in enzyme activity to near 0.1 pkat mg $protein^{-1}$ (see S), compared with 0.2 pkat mg $protein^{-1}$ in FS, suggesting that denaturation of enzyme occurred during the 24 hr storage in the cold. An even lower activity was detected in the 24 hr-dialysed enzyme preparation (DS). This was probably due to a combination of enzyme denaturation during dialysis, together with the removal of some small molecules necessary for enzyme activity. However, the addition of co-factors or co-enzymes to the dialysed soln (DS) restored enzyme activity in all cases but to different extents. Although the addition of ATP, NADH and NADP restored enzyme activity to the S level, no marked increase in enzyme activity was detected. It would appear that the enzyme is unlikely to be ATP, NAD and NADP dependent, but these co-factors or co-enzymes may affect enzyme activity via other pathway(s), such as the pyridine nucleotide cycle, which are associated with this reaction. In contrast, the addition of the reduced form NADH to DS not only restored enzyme activity caused by dialysis, but also enhanced the activity to the FS level. A marked stimulation of enzyme activity was observed only when NADPH was added to DS. These results suggest that nicotine *N*-demethylation requires NADPH, but probably also NADH.

The requirement for the reduced form of pyridine nucleotide has also been observed with other *N*-demethylases from a variety of plant sources, including *p*-chloro-*N*-methylaniline *N*-demethylase [11], aminopyrine *N*-demethylase [15], chlorotoluron *N*-demethylase [16] and nicotine *N*-demethylase [9]. This is particularly interesting in view of the fact that NADPH dependence for a secondary metabolic reaction is one of the criteria used to establish the involvement of cytochrome P_{450} [17]. Indeed, the association of nicotine *N*-demethylation with cytochrome P_{450} has recently been suggested [9]. The present results are also con-

Table 2. Effect of co-factors and co-enzymes on enzyme activity

		Specific activity (pkat $\times 10^{-3}$ mg $protein^{-1}$)	
Addition*	Concn (mM)	Crude enzyme prepn	Dialysed enzyme prepn
Control	—	15±2	10±2
None (FS)	—	207±21	212±23
S	—	n/a	95±2
DS	—	n/a	54±2
ATP	0.5	246±27	93±15
NADPH	1.0	286±38	603±29
NADH	1.0	171±8	189±19
NADP	1.0	206±5	99±9
NAD	1.0	207±24	81±9

*All additives were added to the reaction mixture at the same time as the substrate; None (FS): the fresh enzyme preparation, S: the same as FS but stored at 4° for 24 hr without dialysis and DS: the same as FS but dialysed for 24 hr. Control was the FS for crude enzyme preparation and DS for dialysed enzyme preparation which were both denatured by heating at 100° for 5 min. n/a: not applicable. See Experimental and text for details.

sistent with the involvement of cytochrome P_{450} with nicotine *N*-demethylation to nornicotine.

However, the nature and mechanism of the enzymatic reaction are currently unclear. If cytochrome P_{450} is involved, this reaction is likely to be an oxidative demethylation rather than a transmethylation. In contrast to this, it has been reported [7] that leaf homogenates of *N. tabacum* can convert nicotine into nornicotine in the presence of methyl group acceptors, such as glycine and ethanolamine. However, the results of our preliminary experiments (data not shown), in which several possible methyl group acceptors were tested, did not favour a transmethylation.

EXPERIMENTAL

Plant material. Cell suspension cultures of *Nicotiana tobacum* L cv. Wisconsin-38 were grown in the dark at 25±2° in B_5 medium [18] supplemented with sucrose (30 g l^{-1}), NAA (0.15 mg l^{-1}), kinetic (0.2 mg l^{-1}) and ascorbic acid (5 mg l^{-1}) at pH 5.6. Subculture was carried out every 20 days, by the aseptic transfer of 1 g fr. wt. of cells to 50 ml of fresh medium in a 250 ml conical flask. These cultures were then incubated on a rotary shaker at a speed of 90 rpm. Ten-day-old cultures were selected for the preparation of enzyme extracts, because cells at this age have a reasonable amount of biomass and are active in the bioconversion of nicotine to nornicotine [19].

Preparation of cell-free extracts. All preparative steps were carried out in a cold room at 4°. After separation of cells from culture medium by filtration under red. pres., the 10-day-old cell cultures were homogenised with acid-washed sand in a pestle and mortar together with 0.1 M Tris–HCl buffer supplement with 3 mM DTT and 5 mM EDTA at a cell : buffer ratio of 1 : 1. The homogenate was then used immediately for the enzyme assays.

Dialysis of the enzyme preparation. The fresh enzyme preparations were dialysed for 24 hr against the same buffer used for enzyme preparation.

Enzyme assay. The activity of the enzyme(s) catalysing the *N*-demethylation of nicotine to nornicotine was determined by measuring the radioactivity of ^{14}C-nornicotine produced from added ^{14}C-nicotine substrate. The reaction was started by the addition of 10 μmol of [pyrrolidine-2′-^{14}C]-nicotine (*NEN*, Du Pont), corresponding to 3.8×10^{-3} μmol (0.02 μCi), to 0.5 ml of enzyme extract contained in a 1.5 ml centrifuge microtube. After incubation for 30 min at 30°, the reaction was terminated by the addition of 3 drops of concd NH_4OH and 1 ml of $CHCl_3$ to each of the reaction tubes. The tubes were then shaken for 5 min and the interface between the organic and aq. phase clarified by centrifugation for 1 min. The $CHCl_3$ phase was then collected by pipetting and the aq. phase re-extracted twice with equal vols of $CHCl_3$. The three $CHCl_3$ extracts were combined and evaporated in an air stream to give a final vol of 0.1 ml. The control for the assay was processed as the experimental treatment, but was heated at 100° for 5 min to denature the enzyme(s).

Conc $CHCl_3$ extract from each of the reaction tubes and control tubes (0.1 ml) was loaded onto a silica Gel-60F_{254} TLC plate, together with 0.2 g of cold authentic nicotine and nornicotine as carriers. The TLC plate was then eluted with a solvent system of $CHCl_3$–MeOH–20% NH_4OH (60 : 10 : 1) for 1.5 hr. After drying the TLC plate in a fume cupboard at room temp (*ca* 22°), each alkaloid spot was marked under UV light and then scraped off and counted. Counting was performed with a Beckman liquid scintillation counter after the addition of each alkaloid spot to a counting vial containing 5 ml of scintillant (1.0 l of toluene + 0.5 l of Triton × 100 + 10 g BuPBD). Routinely, each treatment was assayed in triplicate and expressed as the the mean±s.d. after correction for their scintillation blank and enzyme assay control.

Protein assay. Protein content was measured using the method of ref. [20]. A protein range from 0 to 50 μg was selected for all protein assays, since this is where the protein/dye binding response is linear.

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