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An (*R*)-specific *N*-methyltransferase involved in human morphine biosynthesis

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ABSTRACT

The biosynthesis of morphine, a stereochemically complex alkaloid, has been shown to occur in plants and animals. A search in the human genome for methyltransferases capable of catalyzing the N-methylation of benzylisoquinoline alkaloids, as biosynthetic precursors of morphine, yielded two enzymes, PNMT (EC 2.1.1.28) and NMT (EC 2.1.1.49). Introduction of an N-terminal poly-histidine tag enabled purification of both proteins by immobilized metal affinity chromatography. Recombinant PNMT and NMT were characterized for their catalytic activity towards four benzylisoquinolines: tetrahydropapaveroline (THP), 6-*O*-methyl-THP, 4′-*O*-methyl-THP and norreticuline. Human PNMT accepted none of the offered alkaloids and was only active with its established substrate, phenylethanolamine. The second enzyme, human NMT, converted all four benzylisoquinolines, however, with a strict preference for (*R*)-configured morphine precursors. Determination of kinetic parameters of NMT for the four (*R*)-configured benzylisoquinoline alkaloids by LC–MS/MS revealed (*R*)-norreticuline to be the best substrate with an even higher catalytic activity as compared to the previously reported natural substrate tryptamine. In addition, isolation of the morphine precursor salutaridine from urine of mice injected (i.p.) with (*R*)-THP provides new evidence that the initial steps of morphine biosynthesis in mammals occur stereochemically and sequentially differently than in plants and suggests an involvement of the herein characterized (*R*)-specific NMT.

Introduction

Methyltransferases are ubiquitous enzymes with wide ranging functional roles. Among others, they are involved in the synthesis of small molecules such as antibiotics in microorganisms [1], neurotransmitters in animals [2] or alkaloids in plants [3]. The alkaloid morphine, a highly chemically condensed benzylisoquinoline (BIQ)[1] with five asymmetric centers (Fig. 1), requires up to three methylations that all occur *in planta* exclusively on (*S*)-configured substrates. It is notable however, that only the 9-(*R*)-configured morphine is highly pharmacologically active, while the 9-(*S*)-configured morphine is without biological activity [4].

In the biosynthetic formation of morphine, it has to take into consideration that morphine and its close precursors are (*R*)-configured at carbon atom 9. In plants, the vast multitude of isoquinoline alkaloids found are (*S*)-configured [5–7], except for very rare alkaloid members that are converted from (*S*) to (*R*) during their biosynthetic course [8]. This conversion takes place as shown in Fig. 2. (*S*)-Reticuline is dehydrogenated to form the quarternary tetrahydrobenzylisoquinoline alkaloid 1,2-dehydroreticuline [8,9] and subsequently stereoselectively reduced at the expense of NADPH to yield (*R*)-reticuline, an irreversible reaction [10].

Morphine occurs in substantial amounts only in one single plant species, in *Papaver somniferum* the opium poppy, while in certain mammals it seems to occur widespread but in very small quantities [11–15]. Recently, the capability of living animals to form morphine *de novo* was elucidated [16]. The biosynthesis of morphine in mammals has been suggested to start with tetrahydropapaveroline (THP), a simple BIQ alkaloid, yielding the first morphinan alkaloid salutaridine by four enzymatic reactions: two O-methylation reactions at C6, C4′, one N-methylation and one phenol coupling reaction. However, it is not known if these initial steps occur with (*S*)-configured substrates as has been shown for the plant pathway.

It has been found in mammals that catechol *O*-methyltransferase methylates simple BIQ alkaloids predominantly at the hydroxyl group at position C6 [17]. Cashaw et al. [18] described also the detection of 4′-*O*-methyl-THP after intracerebroventricular administration of THP into rats. We reported recently on two human cytochrome P450 (CYP) enzymes, CYP 2D6 and CYP 3A4, as catalysts of the oxidative C–C phenol-coupling reaction of (*R*)-reticuline to salutaridine [19]. A mammalian enzyme capable of catalyzing the N-methylation of BIQ alkaloids to yield (*R*)-reticuline has

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[1] *Abbreviations used:* BIQ, benzylisoquinoline; CYP, cytochrome P450; NMT, amine *N*-methyltransferase; PNMT, phenylethanolamine *N*-methyltransferase; THP, tetrahydropapaveroline.

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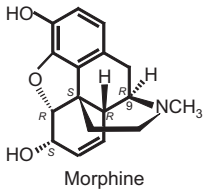

Fig. 1. Molecular structure and absolute configuration of morphine.

not yet been reported. The present study was thus directed towards the identification and characterization of such a human *N*-methyltransferase.

The N-methylation of tetrahydroisoquinoline alkaloids, which are structurally similar to BIQ alkaloids, has been previously shown for the synthesis of *N*-methyl-(*R*)-salsolinol from (*R*)-salsolinol in humans [20–23]. In addition, two rabbit and bovine amine methyltransferases, A and B, have been isolated [24,25]. Distinct pH optima and catalytic activities were determined for both isoforms and upon comparison, isoform A was characterized by a basic pH optimum with a greater catalytic activity for tetrahydroisoquinoline alkaloids [24,25]. Information on the encoding gene sequences and on catalytic properties towards BIQ alkaloids of these *N*-methyltransferases, however, were missing.

Our search in the database of the human genome resulted in two promising candidates for an enzyme involved in the N-methylation of BIQ alkaloids: phenylethanolamine *N*-methyltransferase (EC 2.1.1.28, PNMT) and indolethylamine *N*-methyltransferase (EC 2.1.1.49, NMT). While PNMT is involved in the final step of the biosynthesis of the neurotransmitter adrenaline from noradrenaline [2,26,27], a functional role for NMT has not yet been assigned [28,29]. Both human *N*-methyltransferase-encoding cDNAs, *PNMT* and *NMT*, were heterologously expressed in *Escherichia coli* in order to determine the catalytic activity of the recombinant enzymes with BIQ alkaloids.

Material and methods

Alkaloids

Alkaloidal substrates, except (*R*)- and (*S*)-THP, were from our laboratory collection. (*R*)- and (*S*)-THP were synthesized according to [30] from (*R*)- and (*S*)-norreticuline, respectively. The reaction product obtained from [30] was dried for further purification under a stream of nitrogen, resuspended in 50% methanol containing 0.2% acetic acid and subjected to HPLC equipped with a L-7100 HPLC pump, L-7200 autosampler and L-7450 diode array detector (Merck Hitachi). Separation of (400 µl) samples was achieved using a Hibar Pre-Packed column RT250-25 (Merck, 7 µm, LiChrosorb RP-18). The mobile phase total flow was set to 8 ml/min with binary gradient elution, using 0.1% TFA (solvent A) and acetonitrile (solvent B). The gradient started with 10% B and was increased to 30% B over 60 min. Fractions containing THP were pooled, concentrated, shock-frozen in liquid nitrogen and lyophilized. THP trifluoroacetate was obtained in 77% yield and purified further *via* thin layer chromatography (TLC) in butanol/water/acetic acid (4:1:1). The UV-absorbing band corresponding to THP was eluted with methanol and filtered through a 0.2 µm filter to remove remaining silica residue. After excess methanol was evaporated, THP was reconstituted in 24% HBr and subsequently lyophilized. THP hydrobromide was obtained in 64% yield.

Cloning into pET28a and heterologous expression of his-tagged PNMT and NMT cDNA

Stab cultures of PNMT and NMT were obtained from the American Type Culture Collection (clone MGC-26382) and Deutsches Ressourcenzentrum für Genomforschung (clone BC106902.1), respectively. Plasmid DNA was prepared according to the protocol of QIAprep Spin Miniprep Kit (Qiagen). A standard PCR-reaction was used to introduce *Nde*I and *BamH*I restriction sites for PNMT or *Nhe*I and *Xho*I restriction sites for NMT at the 5′- and 3′-end of each cDNA inserted into pCR BluntII-TOPO. The corresponding primers are shown in Table 1. The cDNA for PNMT or NMT was inserted into pET28a (Novagen) followed by transformation into *E. coli* Plus kJ (Expression Technologies Inc). This cloning strategy resulted in recombinant proteins containing a His-tag sequence located at the N-terminus of the complete amino acid sequence of PNMT (Met1-Leu282, N-terminal His-tag sequence MGSSHHHHHHSSGLVPRGSHM, 33 kDa) and of NMT (Met1-Phe263, N-terminal His-tag sequence MGSSHHHHHHSSGLVPRGSHMAS, 31 kDa). Expression of cDNA was induced by adding isopropyl β-D-1-thiogalactopyranoside (final concentration 1 mM). After incubation for 16 h at 28 °C, the one-liter-culture was centrifuged and the bacterial pellet was resuspended in 50 mM Tris/HCl, pH 7.5, containing 500 mM NaCl, 10% (v/v) glycerol, 2.5 mM imidazole and 5 mM β-mercaptoethanol (His-tag buffer). Addition of 1 mg/ml lysozyme was followed by a 40 min-incubation at 4 °C. Cells were disrupted by sonication and centrifuged. The supernatant was incubated with Talon resin (Clontech) for 40 min at 4 °C. Unbound protein was discarded, protein bound to the resin was transferred to a disposable gravity column (Clontech) and left to settle for 30 min. Bound protein was eluted with His-tag buffer containing 150 mM imidazole. Eluted His-tag protein was desalted on a PD10 column (GE Healthcare) with 20 mM Tris/HCl pH 7.5 containing 150 mM NaCl, 10% (v/v) glycerol and 5 mM β-mercaptoethanol.

Thrombin digestion of His-tagged protein

All steps were conducted at 4 °C. Digestion was started by adding 0.5 µl of thrombin solution (Sigma Aldrich, 1 unit/ml) to 1 mg of His-tagged protein (1/6400 the amount of NMT by weight, [31]). The reaction mixture was transferred into the dialysis membrane (VISKING Typ 1 7/8 ss, Roth, cat. 5358.1, molecular weight cut-off of 14,000 kDa) that had been previously washed with 1 l MilliQ water for 2 × 30 min. After dialysis for 2 × 12 h in 1 l of 20 mM Tris/HCl pH 7.5 containing 150 mM NaCl, 10% (v/v) glycerol and 5 mM β-mercaptoethanol, the dialyzed sample was transferred into an Eppendorf tube and a resin mixture consisting of 100 µl Talon (Clontech) and 80 µl Benzamidine Sepharose (GE Healthcare) was added to the dialyzed sample. After an incubation time of

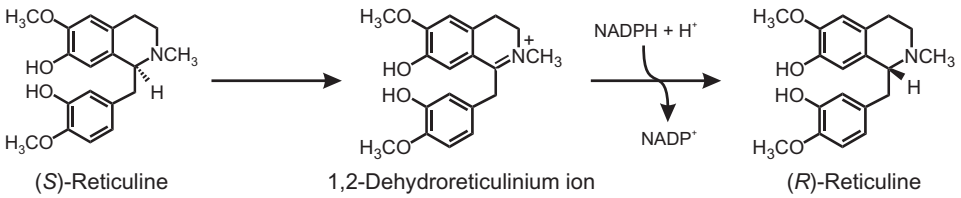

Fig. 2. (*S*)-Reticuline is converted to (*R*)-reticuline *via* formation of 1,2-dehydroreticuline during the biosynthesis of morphine in plants.