A cytochrome P450 (P450) enzyme in porcine liver that catalyzed the phenol-coupling reaction of the substrate \((R)-\)reticuline to salutaridine was previously purified to homogeneity (Amann, T., Roos, P. H., Huh, H., and Zenk, M. H. (1995) \textit{Heterocycles} 40, 425–440). This reaction was found to be catalyzed by human P450s 2D6 and 3A4 in the presence of \((R)-\)reticuline and NADPH to yield not a single product, but rather \((-\)isoboldine, \((-\)corytuberine, \((+)\)pallidine, and salutaridine, the \textit{para-}

orthohedral enzymes established mechanism of precursor in the poppy plant and most likely also in mammals. \((S)-\)Reticuline, a substrate of both P450 enzymes, yielded the phenol-coupled alkaloids \((+)\)isoboldine, \((+)\)corytuberine, \((-\)pallidine, and sinoacutine; none of these serve as a morphine precursor. Catalytic efficiencies were similar for P450 2D6 and P450 3A4 in the presence of cytochrome \(b_5\) with \((R)-\)reticuline as substrate. The mechanism of phenol coupling is not yet established; however, we favor a single cycle of iron oxidation to yield salutaridine and the three other alkaloids from \((R)-\)reticuline. The total yield of salutaridine formed can supply the \(10 \text{nmol}\) concentration of morphine found in human neuroblastoma cell cultures and in brain tissues of mice.

Cytochrome P450 (P450) enzymes catalyze the most versatile chemical reactions in nature (1). There is, however, a discrepancy between the plant and the animal kingdoms with regard to the sheer number of these biocatalysts. Whereas in a single model plant, \textit{Arabidopsis thaliana}, there are to date 273 P450 proteins, in the human genome, only 57 of these proteins are present. Whereas plants and animals share a multitude of highly regio- and stereospecific O-demethylation reactions, more complex reactions such as phenol coupling are much more abundant in plants than in animals, especially in the alkaloid field (2–11). The proposal of Barton and Cohen (12) correlated the structure of specific plant alkaloids in terms of this reaction mechanism and gave mechanistic proposals of how these phenol-coupled products may possibly be biosynthesized in nature. The oxidation of phenols by one-electron transfer affords radicals, which, by radical pairing, form new C-C or C-O bonds either by intra- or intermolecular coupling. The first two examples that unequivocally demonstrated the formation of C-C and C-O bonds in a stereo- and regioselective manner in plant metabolism are catalyzed by specific P450-linked microsomal-bound plant enzymes (13). One of these enzymes was salutaridine synthase from \textit{Papaver somniferum} (opium poppy) (14). This synthase catalyzes the intramolecular formation of the critical C12-C13 carbon bridge and is a key enzyme in morphine biosynthesis.

The groups of Goldstein (15) and Spector (16) have published a number of reports over the past 25 years claiming that mammals are capable of synthesizing \textit{de novo} traces of endogenous morphine. However, no convincing experimental data have been presented regarding the enzymes. Phenol-coupling reactions in mammals are extremely rare, and the only example described thus far is the formation of thyroxine, by radical pairing, in humans. If the key step of morphine synthesis, the formation of phenol-coupled salutaridine from \((R)-\)reticuline, occurs in mammals then in analogy to plants, a P450 enzyme must be present (in mammals) to catalyze this reaction. In 1987, the first experiments were conducted in an attempt to discover the reaction by supplying uniformly labeled racemic \(^{[3]H}\)reticuline in the presence of rat microsomes and NADPH to examine whether \(^{[3]H}\)salutaridine can be formed under these conditions (15). A radioactive compound was formed in 1% yield and assumed to be the phenol-coupled product salutaridine. We later repeated this experiment using \((R)-[\text{\(-14\text{CH}_3\)}]\text{reticuline and NADPH-treated microsomes from pig, rat, cow, and sheep. We observed the formation of } [\text{\(-14\text{CH}_3\)}]\text{salutaridine with the correct stereochemistry at carbon 9 (17). The enzyme from porcine liver was subsequently purified to homogeneity and the reaction product was characterized by mass spectrometry and physical parameters to be a product of a P450 enzyme, which we provisionally named “salutaridine synthase” (18). The aim of this report is the identification of the homogenous porcine P450 enzyme, its equivalent in humans, and the mechanism of the phenol-coupling reaction in the formation of precursors of morphine.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**Alkaloids were from our departmental collection, and isoboldine, pallidine, and sinoacutine were gifts of Dr. André Cavé, University Paris-Sud, Dr. Shoei-Sheng Lee,
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National Taiwan University, and Dr. F. Bracher, Ludwig-Maximilians-University Munich. \(\alpha\)-Dilauroyl-sn-glycero-3-phosphocholine, bovine liver catalase, and bovine erythrocytes superoxide dismutase were obtained from Sigma.

Enzymes—Human P450 2D6 and rat NADPH-P450 reductase were prepared as described (19). Human P450 2D6 and human P450 3A4 (with or without cytochrome \(b_5\)) were purchased from BD Biosciences (Woburn, MA). Porcine salutaridine synthase was purified according to Ref. 18 and subjected to SDS-PAGE on a 10% (w/v) acrylamide gel. The protein band at \(\sim 50\) kDa (containing \(\sim 10\) \(\mu\)g of protein) was excised and eluted, and the N terminus was sequenced by Edman degradation using an Applied Biosystems Model 470 gas-phase sequencer at the Max Planck Institute for Biochemistry, Martinsried, Germany.

Enzyme Assays—Enzyme assays utilizing radioactive labeled substrate were conducted in a total volume of 140 \(\mu\)l containing 36 mM potassium phosphate buffer (pH 6.5), 20 pmol of P450 enzyme (BD Biosciences, microsomal preparation from baculovirus-infected insect cells coexpressing human NADPH-P450 reductase), 1,8 mM NADPH, 0.36 mM EDTA, and 0.2 \(\mu\)Ci (R)- or (S)-[\(\text{H}^4\)] reticuline (200,000–300,000 cpm, 6.75 nmol). The reaction mixture was incubated for 5 h at 37 \(^\circ\)C. For identification of products, the reaction mixture was separated by two-dimensional TLC (solvent 1: toluene/acetone/ethanol/water, 5:4:4:1 (v/v/v/v); solvent 2: CHCl3/acetone/diethylamine, 5:4:1 (v/v/v)), and radioactive bands were detected by phosphorimaging with a Typhoon 9410 (Molecular Dynamics). For plant-feeding experiments, potential precursor solutions were obtained by separation of the reaction mixture by TLC using the solvent mixture chloroform/acetone/diethylamine, 5:4:1 (v/v/v) and radioactive bands were detected by phosphorimaging with a Typhoon 9410 (Molecular Dynamics).

Sequencing of Porcine Salutaridine Synthase—The microsomal porcine liver enzyme catalyzing the formation of the phenol-coupling reaction from (R)-reticuline to salutaridine was purified to apparent electrophoretic homogeneity and subjected to Edman degradation. The N terminus yielded the amino acid sequence: Gly-Leu-Leu-Thr-Gly-Asp-Leu-Leu-diethylamine, 7:2:1 (v/v/v). Radioactive bands were detected by phosphorimaging.

LC-MS/MS Analysis—Enzyme activities were analyzed with a 4000 QTRAP LC-TIS-MS-MS system by monitoring the enhanced product ion (EPI) and multiple reaction monitoring (MRM) in the positive ionization mode. The system consisted of a CTC PAL autosampler (LEAP Technologies), a Shimadzu LC-20AD HPLC system, and a 4000 QTRAP mass spectrometer (Applied Biosystems). Separation of (10 \(\mu\)l) samples was achieved by using a Luna C18 octadecylsiline HPLC column (Phenomenex, 5 \(\mu\m), 150 mm \times 2 mm) combined with a C18 guard column (Phenomenex, 4 mm \times 2 mm). The mobile phase total flow was set to 0.5 ml/min with binary gradient elution, using solvent A (5% CH3OH, 5% CH3CN, 10 mM NH4HCO3, 45 mM NH4OH) and B (90% CH3CN, 10 mM NH4HCO3, 15 mM NH4OH) (all v/v). The gradient started with 100% A for 2 min and was increased to 100% B over 10 min. Elution was continued for 2 min at 100% B followed by a 5-min equilibration with starting condition. The following TIS source parameters were used: CUR 30, CAD high, IS 5000, TEM 500, EP 10, CXP 17. Compound-dependent parameters for the compounds of interest (collision energy, declustering potential, quantifier MRM transition, qualifier MRM transition, dwell time) are listed in Table 1. Identification of an analyte was based on retention times, the quantifier-to-qualifier MRM transition ratio, and comparison with the expected values for standards. Quantitation was performed by constructing standard curves for each analyte and integrating the peak area of the quantifier MRM transition with Analyst 1.4.1 (Applied Biosystems, MDS SCIEX Instruments).

RESULTS

Sequencing of Porcine Salutaridine Synthase—The microsomal porcine liver enzyme catalyzing the formation of the phenol-coupling reaction from (R)-reticuline to salutaridine was purified to apparent electrophoretic homogeneity and subjected to Edman degradation. The N terminus yielded the amino acid sequence: Gly-Leu-Leu-Thr-Gly-Asp-Leu-Gly-Ile-Leu-Ala-Leu-Ala-Met-Val-Ile-Phe-Leu-Leu-Asn-(Leu)-Val-Asp-Leu-Met-X-Arg.

Upon comparison of this peptide sequence to those present in the public databases, this is the exact N-terminal of pig P450 2D25, except for the missing N-terminal Met. Homology to human P450 2D6 was observed (56% identity). Consequently, human P450 2D6 was obtained from a commercial source (BD Biosciences) and found to indeed catalyze the phenol-coupling.

TABLE 1

| Analyte    | Collision energy | Declustering potential | Quantifier MRM transition | Qualifier MRM transition | Dwell time |
|------------|------------------|------------------------|---------------------------|--------------------------|------------|
| Reticuline | 35               | 45                     | 330 → 192                 | 330 → 137                | 50         |
| Corytuberine| 40               | 50                     | 328 → 265                 | 328 → 282                | 50         |
| Pallidine  | 40               | 50                     | 328 → 211                 | 328 → 237                | 50         |
| Salutaridine| 40              | 50                     | 328 → 211                 | 328 → 237                | 50         |
| Isoboldine | 40               | 50                     | 328 → 265                 | 328 → 237                | 50         |
| Thebaine   | 35               | 40                     | 312 → 251                 | 312 → 281                | 50         |
| Oripavine  | 35               | 40                     | 298 → 218                 | 298 → 249                | 50         |

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reaction from (R)-[N-14CH₃]reticuline to salutaridine in the presence of NADPH. However, close examination of the reaction products of the porcine and the commercially available human P450 2D6 enzymes by two-dimensional TLC revealed four metabolites in addition to unconsumed 14C-labeled (R)-reticuline (vide infra).

Identification of P450 2D6-catalyzed Reaction Products—Incubation of (R)-reticuline in the presence of NADPH-P450 reductase and P450 2D6 resulted in the formation of four metabolites that were not found in controls with heat-inactivated enzymes. These metabolites were subjected to MS after separation by HPLC. Comparison of the four compounds with standards of alkaloids that were expected to be formed according to the phenol-coupling reactions allowed the identification of the unknown compounds as salutaridine (para-ortho coupling), (+)-pallidine (para-para coupling), (−)-isoboldine (ortho-para coupling), and (−)-corytuberine (ortho-ortho coupling) (Fig. 1). The identification of these compounds is consistent with the previous findings by the incubation of rat liver microsomes in the presence of racemic reticuline (20, 21). However, the important morphine precursor salutaridine was missed during that investigation. Salutaridine is formed unequivocally during the phenol-coupling reaction and was identified by MS and retention time. Application of the para-ortho coupled product to 5-day-old poppy seedlings verified salutaridine by its incorporation into thebaine (Fig. 2), while the other three phenol-coupled products of the (R)-series were not incorporated into thebaine. Analogous incubation of NADPH-P450 reductase in the presence of P450 2D6 and (S)-reticuline again gave four phenol-coupled products of the (S)-series, which were sinoacutine, (−)-pallidine, (+)-isoboldine, and (+)-corytuberine and did not serve as precursors to morphine. The collision-induced dissociation (CID) spectra of salutaridine [M+H]+ 328 by EPI yielded the characteristic fragment ions m/z 297, 285, 265, 239, and 237 (22) that were also found in CID spectra of the para-ortho coupled product. The CID spectra of salutaridine standard and para-ortho coupled enzymatic product are shown (Fig. 3).

Human P450 2D6 is known to catalyze a multitude of reactions both with natural compounds and xenobiotics and is involved in the oxidation of about 30% of drugs used by humans (19, 23). Interestingly, human P450 2D6 is known to catalyze several reactions within the putative pathway leading from l-tyrosine to morphine. These reactions include the hydroxylation of tyramine to dopamine (23, 24), the demethylation of thebaine to oripavine (25), and the demethylation of codeine to...
morphine (26); all of these reactions can be catalyzed by a single enzyme-P450 2D6. Tyramine hydroxylation to dopamine plays only a minor role, if any, in the production of dopamine in humans; however, three P450 2D6-catalyzed reactions may play a role in the biosynthesis of endogenous morphine.

P450 3A4 has previously been shown to N-demethylate codeine to N-norcodeine (27). This similarity of P450 3A4 in accepting codeine as substrate prompted us to investigate whether P450 3A4 can catalyze the phenol coupling of (R)-reticuline. Indeed P450 3A4 showed the product pattern known for 2D6, i.e. the formation of salutaridine, (+)-pallidine, (-)-isoboldine, and (-)-cortyuberine from the precursor (R)-reticuline as determined by MS (Fig. 4). The morphine biosynthetic precursor salutaridine is indeed formed by P450 3A4 as well.
P450 3A4 was also tested to determine if it can catalyze the phenol-coupling reaction with the (S)-configuration of reticuline as substrate. Incubation of P450 3A4 with (S)-reticuline again yielded four products, sinoacutine, (−)-pallidine, (+)-isoboldine, and (±)-corytuberine, as determined by MS.

The four phenol-coupled alkaloids formed in the presence of human P450 2D6 and 10 μM (R)-reticuline were individually quantitated after an incubation time of 10 min, in which time product formation is in the linear range: (−)-corytuberine (4 nM), (−)-isoboldine (54 nM), (+)-pallidine (24 nM), and salutaridine (6 nM). With the (S)-configuration of reticuline as substrate under the same incubation condition the yields of phenol-coupled products were: (±)-corytuberine (1 nM), (−)-isoboldine (61 nM), (−)-pallidine (15 nM), and sinoacutine (3 nM). With P450 3A4 as catalyst, the same set of phenol-coupled alkaloids yielded the following with (−)-reticuline (7 nM), (−)-isoboldine (30 nM), (+)-pallidine (18 nM), and salutaridine (28 nM). With (S)-reticuline as substrate, under the same conditions as above and P450 3A4 as catalyst, the yields were: (−)-corytuberine (5 nM) (+)-isoboldine (15 nM), (−)-pallidine (7 nM), and sinoacutine (15 nM). Similar yields were obtained for the (R)− and (S)-series of phenol-coupled products suggesting that the two different P450s oxidize reticuline via radical formation without stereoselectivity. A pH profile for both P450 enzymes catalyzing the phenol coupling of (R)-reticuline to salutaridine is shown in supplemental Fig. S1. The relative distribution of the four phenol-coupled products was the same after 10 min and 120 min of incubation as well as at any pH between 5 and 9.

**DISCUSSION**

It has been shown that human P450 2D6 and P450 3A4 catalyze what appear to be radical-induced phenol-coupling reactions with both (R)− and (S)-reticuline, yielding four (R)−-configured and four (S)-configured phenol-coupled products. As shown in Fig. 5, a mechanism can be postulated that starts with the fully activated P450 ("compound I" form), which abstracts a hydrogen atom to create a phenoxy radical. The substrate then leads to immediate abstraction of a second phenolic hydroxyl to create a second phenoxy radical and can still produce a second phenol. The non−phenolic radical then abstracts another phenolic hydroxyl, yielding the final product salutaridine as one of the (R)-coupled alkaloids and the missing link in human morphine biosynthesis. The intramolecular C-C phenol-coupling reaction of (S)-reticuline to corytuberine catalyzed by CYP80G2 in the plant *Coptis japonica* is postulated to occur by a diradical mechanism (7). This postulate was based on a previously

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**FIGURE 5. Proposed mechanism of the oxidative phenol-coupling reaction in mammals.** The formation of salutaridine from (R)-reticuline is catalyzed by P450 2D6 and P450 3A4 and passes through a single cycle of iron oxidation.
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The proposed mechanism in the first report of a plant cytochrome P450 that catalyzes a phenol-coupling reaction, berbamunine synthase from Berberis stolonifera (2). The concept of a diradical mechanism in phenol-coupling reactions finds origin in the proposal of Barton and Cohen (12) in which radical pairing furnishes diphenyl ether or aryl-aryl bonds. A new C-C phenol-coupling reaction catalyzed by CYP121 of Mycobacterium tuberculosis has recently been reported (29). It is proposed therein that the intramolecular C-C coupling of the two tyrosyl side chains of cYY to cyclodipeptide proceeds via activation of the tyrosyl residues through a radical mechanism. A diradical mechanism can also be proposed herein; however, the mechanism proposed in Fig. 5 avoids the energetic instability of a formal diradical species.

Salutaridine has previously escaped detection using rat liver microsomes (20, 21), being a minor phenol-coupled alkaloid. Salutaridine is the para-ortho phenol-coupled product, which is the biosynthetic precursor of morphine both in the poppy plant and in humans and other mammals. This finding adds considerable credibility to the controversial discussion about whether endogenous morphine occurs in mammals. It should be pointed out that the occurrence of P450 2D6, not only in liver tissue but also in human brain, has been unequivocally proven (30). P450 2D6 occurs mainly in the neocortex, telencephalon, hippocampus, diencephalon, mesencephalon, cerebellum, and myelencephalon. The second P450 that catalyzes the phenol-coupling reactions with (R)-reticuline as substrate forming again four phenol-coupled products, including salutaridine-3A4– is found to occur in brain, mainly in pons and cervical cord (31) and in liver and small intestine (32).

The presence of endogenous morphine in brain of humans and mammals has been suggested by various researchers (16, 33) and in sterile human cell cultures, e.g. neuroblastoma cells SH-SY5Y (34–36). The possible occurrence of morphine in human and mammalian cells has been a matter of controversy for more than 30 years. A resolution of this problem can come only by the application of state-of-the-art analytical chemistry, biochemistry, and molecular biology, with the application of critical controls to exclude introduction of morphine from food sources to the animals and exclusion of laboratory contamination with morphine.

It has been suggested that human P450 2D6 might catalyze the transformation of (R)-reticuline to salutaridine (37). In a recent report, Hawkins and Smolke (38) tested whether P450 2D6 might catalyze the formation of salutaridine from (R)-reticuline in recombinant yeast. (R,S)-Norlaudanosoline was fed to the recombinant yeast strain which produced racemic (R,S)-reticuline. When P450 2D6 was introduced into the (R,S)-reticuline-producing yeast strain, a product was formed that had the MS fragment ions of m/z 297 and m/z 265 and was designated salutaridine (38). The yield of the morphine precursor was reported to be between 6 and 8%. The yield could not be further increased (38) by available techniques.

P450 2D6 (and P450 3A4) catalyze reactions that can be interpreted as a diradical mechanism or alternatively involving a single cycle of iron oxidation, vide supra (Fig. 5). This mechanism will lead to four final alkaloid products, each alkaloid in a different but consistent concentration. Only one of these products is the desired morphine precursor salutaridine, in 0.06% concentration. Use of racemic (R,S)-reticuline (via (R,S)-norlaudanosoline in the recombinant yeast system) leads to another four phenol-coupled products of the (S)-series, namely the alkaloids (+)-cortyubeine, (+)-isoboldine, sinoacutine, (−)-pallidine, all known natural products. This addition of the (S)-configured compounds would produce seven alkaloids in the recombinant system; only one product (salutaridine) is the morphine precursor. All eight phenol-coupled products derived from (R,S)-reticuline yield the fragment ions m/z 297 and 265 upon mass fragmentation (supplemental Figs. S6 and S7). Eight different structures in different concentrations will result in the recombinant yeast strain; the yield of salutaridine will be ~0.2%. Although the low and inherent concentration of salutaridine generated by P450 2D6 and P450 3A4 is low for a biotechnological production of morphine, it is expected to be sufficient to generate the ~10 mM concentration of morphine (via salutaridine) in human cells (35) and mouse cerebellum (36).

The phenol-coupling reaction of (R)-reticuline to salutaridine is the key reaction in the biosynthesis of morphine in plants and animals (Fig. 5). The enzyme in endogenous morphine biosynthesis in mammals (P450 2D6) is unique (Fig. 6) in that it catalyzes the 3-O-demethylation of codeine to morphine (26), the 3-O-demethylation of thebaine to oripavine (25), and the phenol coupling of (R)-reticuline to salutaridine (this work). A minor reaction, the oxidation of tyramine to dopamine that is also catalyzed by P450 2D6 (23, 24), does not seem to play a role in endogenous morphine biosynthesis (39). These different
reaction types, which are catalyzed by one enzyme in morphine biosynthesis, are rather unique in nature.

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