PURIFICATION AND CHARACTERIZATION OF A CYTOCHROME P450 ENZYME FROM PIG LIVER, CATALYZING THE PHENOL OXIDATIVE COUPLING OF (R)-RETICULINE TO SALUTARIDINE, THE CRITICAL STEP IN MORPHINE BIOSYNTHESIS°

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Abstract - The cytochrome P450 enzyme catalyzing the conversion of (R)-reticuline to salutaridine in the presence of cytochrome c reductase, NADPH and O_2 was purified to homogeneity from pig liver and shown to be a highly regio- and stereoselective enzyme. This finding supports the view that the occurrence of morphine in mammals is of endogenous and not of dietary origin.

Introduction

There is a large and convincing body of evidence that the opium alkaloids, morphine and codeine, do exist in mammalian tissue.[1-9] The question remains, however, whether these analgesic

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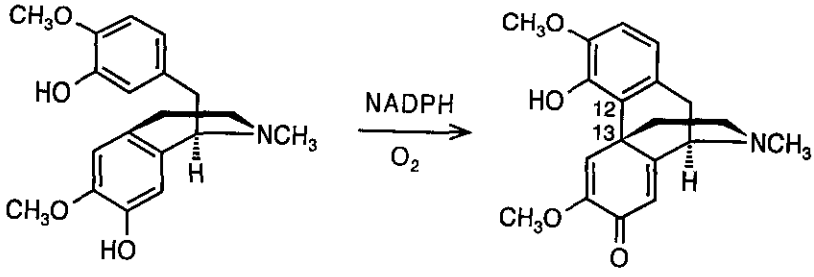
°Dedicated to Professor Rolf Huisgen on the occasion of his 75th birthday.

compounds are of dietary origin or are synthesized *de novo*.[5, 6] Only biosynthetic experiments can answer the question whether mammalian enzymes are capable of synthesizing the morphine carbon skeleton. Preliminary experiments showed that *in vivo*[10] established[11] precursors of the opium alkaloid pathway can be transformed to morphine and that rat liver homogenates can transform racemic reticuline to salutaridine,[12] the crucial reaction in morphine biosynthesis. These latter experiments have been recently criticized,[13] but it has also been demonstrated that in mammalian liver microsomes a regio- and stereoselective cytochrome P450 enzyme is present which performs this reaction leading from (R)-reticuline **(1)** to the tetracyclic morphine precursor salutaridine[13] **(2)** (Figure 1). This paper reports on the purification and characterization of this enzyme.



(R)-Reticuline (1) Salutaridine (2)

Figure 1: Intramolecular phenol coupling of (R)-reticuline to salutaridine catalyzed by a cytochrome P450 enzyme.

Results

In order to choose a suitable starting tissue and to increase the probability of success of purifying the target enzyme to homogeneity, microsomal preparations from livers of pig, rat, mouse, sheep, beef and humans were assayed by our previously published[13] salutaridine synthase activity procedure and compared for the ability to convert the radiolabelled substrate **(1)** to **2**. As can be seen in Table 1, all of the liver preparations possessed salutaridine synthase activity with pig liver showing the highest conversion rate. This organ was, therefore, chosen for further experiments.

Liver microsomes	Cytochrome P450 content (nmol x mg^{-1})	Salutaridine synthase activity (pmol x sec^{-1} x mg^{-1})
Pig	0.5	3
Beef	0.6	2
Mouse	0.5	0.7
Sheep	0.8	0.4
Rat	1.1	0.1
Human	0.07	0.005

Table 1. Cytochrome P450 content and salutaridine synthase activity of microsomes from different mammalian livers. Assays were carried out as described under "Experimental".

Catalytically active cytochrome P450 complexes consist of a membrane associated cytochrome P450 oxidase and an NADPH-cytochrome P450 reductase. The cytochrome P450 oxidase can only be obtained in a catalytically active form if the reductase and, possibly, membrane lipids are reconstituted to form the active complex. In a first step, therefore, the NADPH-cytochrome c reductase was purified by biospecific affinity chromatography following a published procedure for pig liver.[14] In our hands, the reductase yielded a 99-fold purified preparation with 32% yield and a specific activity of 0.24 µmol x sec^{-1} x mg^{-1}.

The purification of the cytochrome P450 oxidase is summarized in Table 2. The results of each stage of the purification procedure starting from frozen microsomes is shown. The enzyme was solubilized in the presence of EDTA, dithiothreitol, glycerol and sodium cholate. The supernatant from this step was fractionated further with ammonium sulfate and stepwise purified by chromatography on fast flow (ff) aminohexyl Sepharose 4B, hydroxylapatite, carboxymethyl Sepharose ff, S-Sepharose and chelating Sepharose ff.

Step	Total protein (mg)	Total P450 (nmol)	Specific content (nmol P450 x mg^{-1})	Salutaridine synthase activity (pmol x sec^{-1} x mg^{-1})
Solubilized microsomes	2378	1394	0.6	0.1
Ammonium sulfate fract.	918	723	0.8	0.15
Aminohexyl Sepharose 4B	177	325	1.8	0.2
Hydroxylapatite	49.5	154	3.1	0.3
CM-Sepharose ff	10.9	37.4	3.4	1.9
S-Sepharose ff	1.4	7.1	5.1	3
Chelating Sepharose ff	0.042	0.84	20	8.1

Table 2. Purification of the cytochrome P450 isoenzyme, catalyzing the conversion of **1** to **2**. Assays were carried out as described under "Experimental".

At each step, salutaridine synthase activity was measured by a reconstitution assay. Levels of synthase activity and cytochrome P450 content were parallel, within experimental error, at each step of the purification procedure indicating that the majority of the microsomal salutaridine synthase activity resided in the protein isolated. Figure 2A shows the elution profile of low spin cytochrome P450 and other chromophors with an absorption at 417 nm and salutaridine synthase activity at an ion exchange chromatography step on S-Sepharose ff. The final purification step was performed on chelating Sepharose ff[15] (Figure 2B) which yielded the most highly purified enzyme fraction with a specific cytochrome P450 content of 20 nmol x mg^{-1}. The enzyme yield in that final step dropped by a factor of 10 with respect to the previous step, but yielded a homogeneous preparation.

SDS-PAGE of the active fraction eluted from the chelating Sepharose ff showed a single major band which is shown in Figure 3. The molecular weight of the purified enzyme was calculated from the SDS-PAGE gel to be 50 kD. The homogeneity of this protein was confirmed by amino acid sequence analysis (Edman degradation) which gave no indication of protein impurities present in this final preparation.

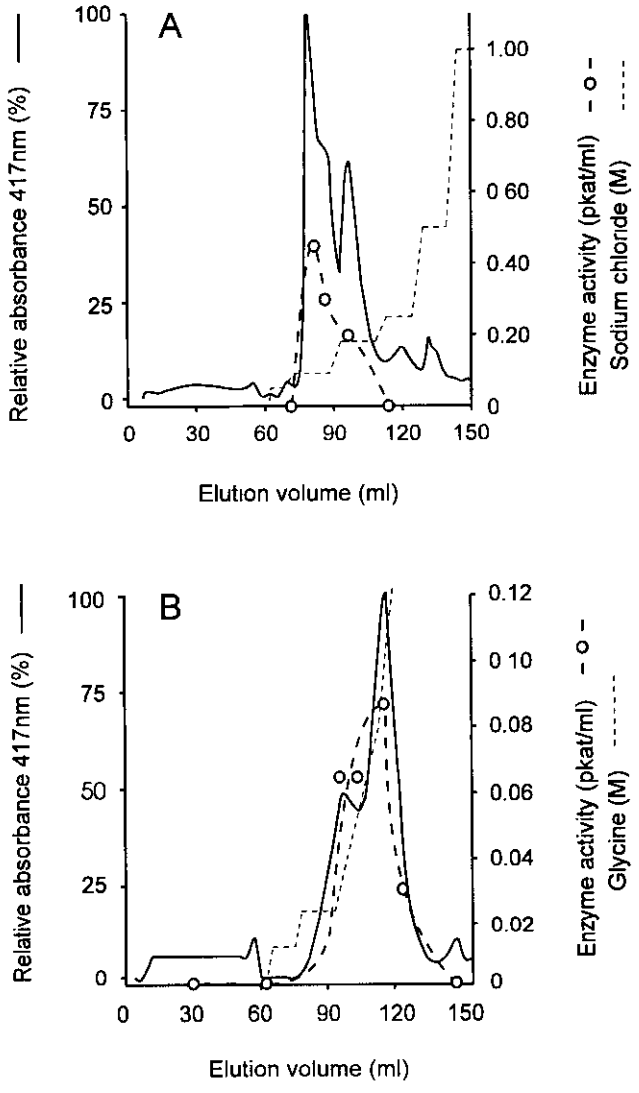


Figure 2: The last two steps of the purification of the cytochrome P450 enzyme catalyzing the conversion of **1** to **2**. (A) Elution profile of the S-Sepharose ff column (1 cm x 8.8 cm). The column was eluted with a step-gradient from 0 to 1 M NaCl. Salutaridine synthase activity was associated with cytochrome P450 eluting with 90 - 180 mM NaCl; flow rate 1 ml x min^{-1}. (B) Elution from Ni^{2+}-loaded chelating Sepharose ff column (1 cm x 10.7 cm). Elution of cytochrome P450 was performed with a step-gradient going from 0 to 200 mM glycine. Electrophoretically homogeneous enzyme eluted with 24 to 80 mM glycine; flow rate 1 ml x min^{-1}. The assay of enzyme activity during the last two purification steps was carried out as described under "Experimental".

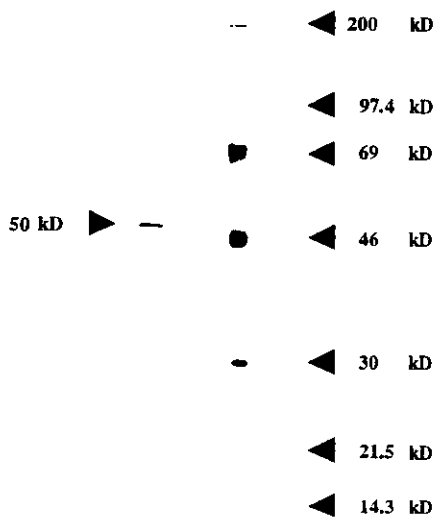


Figure 3: Purity of cytochrome P450 enzyme producing **2** from **1** after the final purification step. SDS-PAGE pattern of 0.5 μg protein. Protein bands were visualized by silver staining. Molecular weight standards were Rainbow Markers (Amersham).

The absorption spectrum of the cytochrome P450 salutaridine synthase is presented in Figure 4. The absorption maximum for the dithionite-reduced CO-complex occurred at 450 nm while the oxidized form exhibited Soret absorption around 417 nm. The salutaridine forming cytochrome P450 enzyme was strictly dependent on the presence of O_2. This was demonstrated by addition of an oxygen consuming glucose oxidase/catalase system into the reaction mixture which resulted in a significant loss of substrate conversion (Table 3).

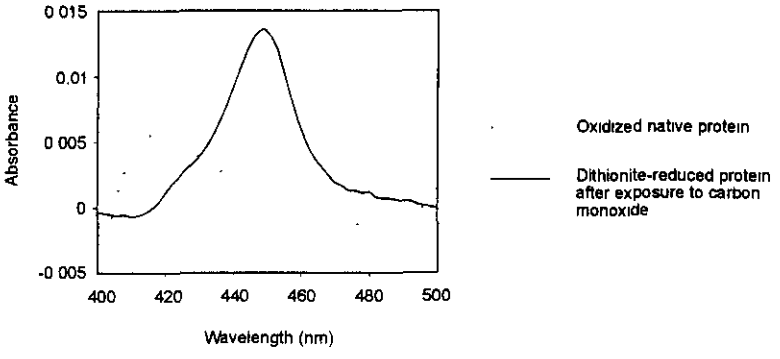


Figure 4: Absorption spectra of homogeneous cytochrome P450 containing 20 nmol x mg^{-1}. Spectra were recorded as described under "Experimental".

Assay conditions	% activity[a]
Complete	100
Only cytochrome P450	0
Only NADPH-cytochrome c reductase	0
Without NADPH	0
Without oxygen[b]	16

Table 3. Reconstitution of salutaridine synthase activity. The complete reconstitution system consisted of 130 pmol cytochrome P450 (specific content: 2.3 nmol x mg^{-1}), 20 µg NADPH-cytochrome c reductase (specific activity: 0.24 µmol x sec^{-1} x mg^{-1}) and 0.7 mM dilauroyl-L-α-phosphatidylcholine. The detergent-free enzyme solution was incubated in the presence of 3 µM (R)-[N-$^{14}CH_3$]reticuline (1 nmol, 12 x 10^4 dpm), 1 mM NADPH and 100 mM tricine buffer pH 7.75 for 60 min at 37°C. [a] 100% activity represents 0.9 pmol x sec^{-1} x mg^{-1}. [b] Assays were carried out by adding glucose oxidase (15 units), catalase (10 units) and glucose (5 mM).

Both catalase and superoxide dismutase did not affect the enzyme activity. This also excludes the participation of hydrogen peroxide or superoxide anions in the conversion of **1** to **2**. The kinetic properties of the reconstituted homogeneous cytochrome P450 enzyme were determined for **1** as substrate. From the Lineweaver-Burk diagram shown in Figure 5, a Michaelis-constant of 3 µM and a V_{max} value of 16.7 pmol x sec^{-1} x mg^{-1} protein were determined.

The substrate specifity of the reconstituted enzyme was analyzed by the standard radiochemical assay employing [N-$^{14}CH_3$] substrates or, in the case of unlabelled precursors, by hplc analysis.[16] The **2** forming enzyme complex was found to be surprisingly substrate specific. Of the differently substituted tetrahydrobenzylisoquinoline substrates with (R)-configuration tested, only **1** was transformed to **2**. No transformation could be detected with (R)-protosinomenine and (R)-norreticuline. A transformation was detectable with (R/S)-orientaline. The metabolite formed, however, is not a **2** analogue. (R/S)-Isoorientaline, (S)-protosinomenine, (S)-norreticuline and (S)-reticuline were not transformed by this enzyme (Table 4).

The product of the reaction with **1** was purified by tlc and subjected to ms analysis. The mass spectrum (EI mode) of the product is shown in Figure 6. As it can be seen in this figure, the mass

spectrum is in all aspects identical to that of synthetic **2**. Incubation of the unknown product with the highly specific and stereoselective salutaridine: NADPH 7-oxidoreductase from *Papaver somniferum*[17] only yielded salutaridinol, whereas, chemical reduction with BH_4^- yielded two products co-chromatographing with salutaridinol and 7-epi-salutaridinol.[18] It has therefore been unequivocally demonstrated that the reconstituted homogeneous cytochrome P450 enzyme produces salutaridine **(2)** from the substrate **(1)**. This transformation is highly stereo- and regioselective.

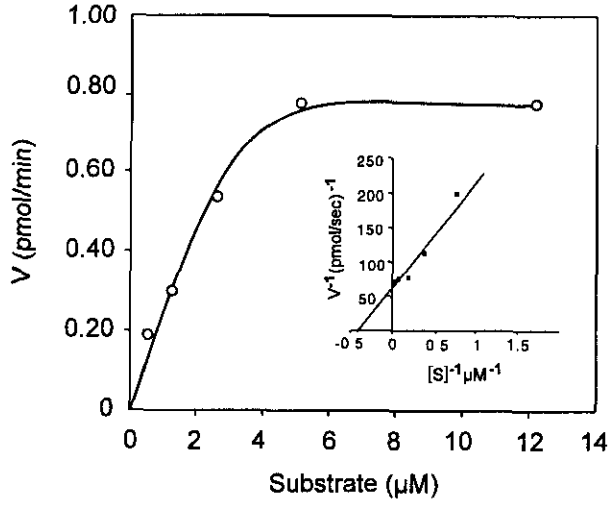
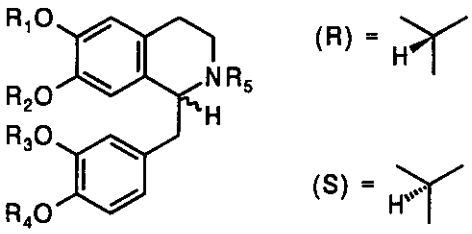


Figure 5: Salutaridine synthase activity of the homogeneous cytochrome P450 enzyme as a function of (R)-reticuline concentration. Enzyme activity was assayed as described under "Experimental".

Finally, the occurrence of this enzyme activity was studied in the different organ tissues of *Sus scrofa f. domestica* L. As shown in Table 5, the cytochrome P450 salutaridine synthase activity is found only in liver and kidney tissue of the pig but does not occur in heart, lung, spleen, maw or brain tissue. This result is clearcut and if morphine and morphine derivatives do occur in the pig brain, then one has to assume that in the light of the results presented in this paper, at least the crucial step in morphinan biosynthesis, the phenol coupling of **1** to yield **2**, takes place only in liver and kidney tissue and that **2** is either further converted into morphine in liver or during or after transport into the brain. Morphine may then be deposited in the brain in a free form or conjugated to sulfate or glucuronic acid.[19,20]



Substrate	Substitution	Reaction product	Specific activity pmol x sec^{-1} x mg^{-1}
(R)-Reticuline	R_1=R_4=CH_3, R_2=R_3=H, R_5=CH_3	Salutaridine	3.7
(S)-Reticuline	R_1=R_4=CH_3, R_2=R_3=H, R_5=CH_3	-	0
(R)-Norreticuline	R_1=R_4=CH_3, R_2=R_3=H, R_5=H	-	0
(S)-Norreticuline	R_1=R_4=CH_3, R_2=R_3=H, R_5=H	-	0
(R)-Protosinomenine	R_1=R_3=H, R_2=R_4=CH_3, R_5=CH_3	-	0
(S)-Protosinomenine	R_1=R_3=H, R_2=R_4=CH_3, R_5=CH_3	-	0
(R/S)-Orientaline	R_1=R_3=CH_3, R_2=R_4=H, R_5=CH_3	n.d.*	16
(R/S)-Isoorientaline	R_1=R_4=H, R_2=R_3=CH_3, R_5=CH_3	-	0

Table 4. Substrate specifity of the **2** forming enzyme, purified to homogeneity. * = not determined, but the product was not a **2** analogue.

Microsomes	Cytochrome P450 (nmol x mg^{-1})	Salutaridine synthase activity (pmol x sec^{-1} x mg^{-1})	Cytochrome c reductase activity (nmol x sec^{-1} x mg^{-1})
Liver	0.5	2.6	2
Brain	0	0	0.8
Heart	0	0	0.13
Lung	0	0	1.5
Kidney	2.4	0.3	2.5
Maw	0	0	0.17
Spleen	0	0	0.37

Table 5. Cytochrome P450 content, salutaridine synthase and NADPH-cytochrome c reductase activity in different tissues of *Sus scrofa f. domestica* L. Assays were carried out as described under "Experimental".

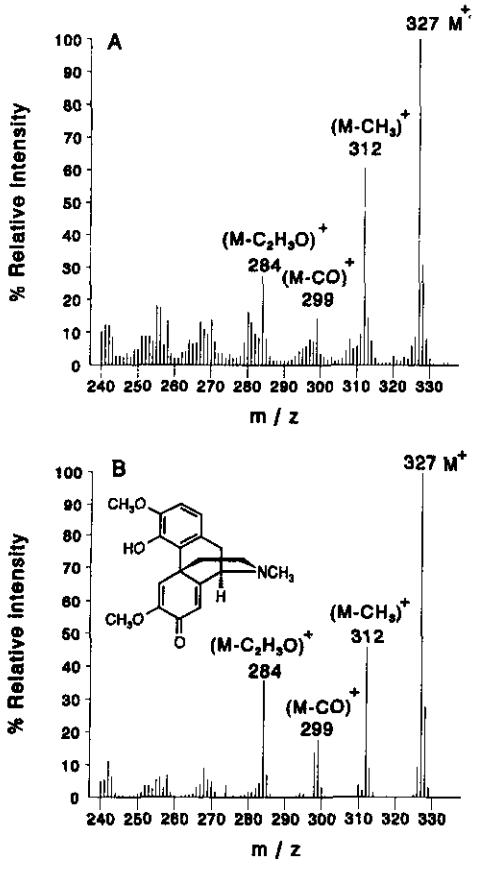


Figure 6: Electron ionization mass spectra of putative salutaridine **(2)** enzymatically synthesized from (R)-reticuline **(1)** (A). Reference compound **(2)** (B).

Discussion

In their investigation, Weitz *et al.*[12] have for the first time claimed that racemic [^{3}H]reticuline is converted by rat liver homogenates to [^{3}H]salutaridine. These results have, however, been criticized for several reasons.[13] Previously it had been shown that the oxidative coupling of **1** to **2** in the poppy plant occurs *via* a microsomal cytochrome P450 enzyme.[16,21] The same type of enzyme was found also to occur in microsomes of liver tissue[13] from various mammalian species. In a similar type of experiment, Kametani *et al.*[22] had previously tried to transform (R,S)-reticuline by incubation with rat liver microsomes. In these cases, however, only (-)-coreximine, (-)-scoulerine,

isoboldine and (-)-pallidine were formed but not **2**. This discrepancy cannot be explained in light of recent experiments.[12,13] In the present study, the pig liver cytochrome P450 responsible for the conversion of **1** to **2** was purified to homogeneity using reconstituted systems. The properties of the enzyme, its absorption at 450 nm of the reduced CO-complex, its dependence on O_2 and NADPH have demonstrated that it is this cytochrome P450 enzyme responsible for the production of the morphine precursor **(2)** in mammalian tissue. Thus it has been firmly established that **2** can be formed from its precursor **(1)** in mammalian, including human, livers. The amino terminus of this cytochrome P450 enzyme has been sequenced (data not shown) and isolation of the cDNA encoding this enzyme is underway. This information will reveal, whether this liver cytochrome P450 is a new enzyme, which fact may be likely since known cytochrome P450 enzymes have not been shown before to catalyze the oxidative coupling of phenolic compounds. Research has to concentrate now on the biosynthetic pathway leading from the aromatic amino acids to **1**. A first attempt has already been made[23,24] and parallel the further fate of **2** in mammalian tissue. The fact that this crucial intermediate **(2)** in the morphinan biosynthetic pathway can be synthesized in all mammalian livers thus far analyzed supports the assumption[1-4, 7-10] that the occurrence of morphine in mammals is of endogenous and not of dietary origin.

Experimental

Mass spectra in the electron impact (EI) or chemical ionization (CI) mode were taken with a Finnigan MAT 44 SSQ700 mass spectrometer. The radioactive measurements were obtained by liquid scintillation counting with a Beckman (model LS 6000 TA) liquid scintillation counter and by a radioactivity scanner Berthold LP 2832. High pressure liquid chromatography was carried out with Merck-Hitachi L-6200, L-4200, AS-4000, D-2500 instruments, equipped with a Nucleosil column RP18, 10 µm, 4 mm x 25 cm (Bischoff). Column chromatography was done with a Pharmacia LC- and FPLC-system.

Enzyme isolation.

Pig liver NADPH-cytochrome P450 reductase was purified according to standard procedures.[14] The first steps in the purification of the **2** synthesizing cytochrome P450 followed the procedures given by Sono *et al.*[25] Pig liver was obtained from a local slaughter house. Microsomes were prepared according to standard procedures.[25] Sixty-three ml microsomal material (40 mg x ml^{-1} protein; 20.6 nmol x ml^{-1} cyt. P450) containing a salutaridine synthase activity of 1.5 pmol x sec^{-1} x mg^{-1} protein were added to 790 ml solubilization buffer (100 mM KPO_4^{2-} pH 7.4; EDTA 0.1 mM, dithiothreitol 1 mM, sodium cholate 0.6% (w/v)). The mixture was stirred for 1 h and subsequently centrifuged at 105 x 10^3 g. The supernatant was subjected to ammonium sulfate precipitation (45%). The precipitate was taken up in dialysis buffer (KPO_4^{2-} 10 mM pH 7.4; 0.1 mM EDTA, 1 mM DTT, 0.6% sodium cholate, 20% (v/v) glycerol) and dialyzed against the same buffer overnight. The dialysate was applied to an aminohexyl Sepharose 4 B-column (AH-Sepharose 4B 2.7 x 16 cm (equilibrated with dialysis buffer)). The column was washed with 2 column volumes of dialysis buffer and eluted with dialysis buffer which contained, in addition to sodium cholate, emulgen 913 (0.5% w/v). Fractions containing cytochrome P450 salutaridine synthase activity were pooled and applied to a hydroxylapatite column (1.5 x 25 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.4), containing 20% glycerol, 0.2% emulgen 913 and 1 mM DTT. The column was washed with 1.5 volumes of this buffer and eluted with a linear gradient of 300 ml each of starting buffer and 300 mM potassium phosphate buffer (pH 7.4), containing 20% glycerol, 0.2% emulgen 913 and 1 mM DTT. Fractions which were enriched in salutaridine synthase activity were pooled, dialyzed *against CM-Sepharose-equilibration buffer and applied to a CM-Sepharose ff column (2 x 40 cm)* equilibrated with KPO_4^{2-} buffer (pH 6.8) 50 mM; 1 mM DTT, glycerol 20%, emulgen 913 0.2%. The column was washed with 1.5 column volumes of the same buffer and salutaridine synthase was eluted with a linear gradient of 300 ml each of equilibration buffer and 300 mM KPO_4^{2-} buffer pH 6.8 containing 20% glycerol, 0.2% emulgen and 1 mM DTT. Fractions showing the enzyme activity were collected, combined and diluted with one half volume 20 mM MOPS buffer (pH 7.0); EDTA 1 mM, emulgen 911 0.4%, glycerol 20%. The dilution was applied to S-Sepharose ff (1 cm x 8.8 cm) equilibrated with 20 mM MOPS buffer (pH 7.0); EDTA 1 mM; emulgen 911 0.4%, glycerol 20%. The column was eluted with a step gradient from 0 to 1 M NaCl in the above MOPS buffer. The enzyme containing fractions were pooled and subjected to metal chelate affinity chromatography with a

Ni^{2+} ligand[15] (column size 1 x 10.7 cm). The protein sample (8.7 ml) was diluted with 34.8 ml 50 mM KPO_4^{2-} buffer (pH 7.5), containing 0.5 M NaCl, emulgen 911 0.4%, glycerol 20%. A step gradient of 0 - 0.2 M glycine in the above buffer was applied and salutaridine synthase activity was eluted from the column and dropped immediately into test tubes containing 1 ml each of 50 mM KPO_4^{2-} buffer (pH 7.2); EDTA 2 mM, glycerol 20%. The enzyme eluted at 24-80 mM glycine. Cytochrome P450 obtained by this elution was found to be homogeneous. The purified cytochrome P450 was stored at -80°C for at least six months without considerable loss of activity.

The fractions obtained were monitored for salutaridine synthase activity in a reconstituted system. 20-500 pmol cytochrome P450 were added to highly purified cytochrome c reductase (2000-10000 pmol x sec^{-1}; specific content: 0.24 µmol x sec^{-1} mg^{-1}) and the solution was made to 0.7 mM in dilauroyl-L-α–phosphatidylcholine. The resulting solution was shock-frozen in liquid nitrogen, allowed to thaw on ice and was subsequently freed of detergent by passing through an Extractigel® column (0.5 x 1 cm; Pierce). The detergent-free enzyme solution was analyzed by incubation with 3 µM (R)-[N-$^{14}CH_3$]reticuline (1 nmol, 12 x 10^4 dpm) and 1 mM NADPH for 20-60 min at 37°C in a total volume of 300 µl. The incubation mixture was extracted with $CHCl_3$ (0.4 ml), the organic phase was evaporated and the residue subjected to tlc analysis after adding authentic compounds. Tlc was conducted on silicon gel polygram sil G/UV_{254} (Macherey and Nagel) plates developed in the following chromatographic system: $CHCl_3$/acetone/diethylamine (5:4:1) and subsequently scanned for radioactivity (R_f: reticuline: 0.5; salutaridine: 0.8). For preparative isolation of the reaction product formed, the standard incubation mixture was increased by a factor of 150, each assay containing 12 µM unlabelled **1**. After extraction with $CHCl_3$, **2** was purified by tlc.

Other procedures

SDS-PAGE was performed as described by Laemmli.[26] Protein bands were visualized by silver staining.[27] Protein was quantitated using bovine serum albumin as standard according to Smith *et al.*[28] Cytochrome P450 content was determined from the carbon monoxide difference spectrum according to the method of Omura and Sato.[29]

Materials

Tetrahydrobenzylisoquinoline alkaloids were synthesized as indicated separated into their optical antipodes by the α-methoxybenzyl isocyanate method.[30] Salutaridine and all radioactive alkaloids were synthesized according to the methods given.[31] All column chromatography material was purchased from Pharmacia except for hydroxylapatite (bio-gel HTP), this was purchased from Bio-Rad Laboratories and emulgen 913 and 911 was kindly supplied by the KAO Corporation, Tokyo. Sodium cholate, glucose oxidase and the lipid component were from Sigma, catalase was obtained from Boehringer Mannheim. Other chemicals and biochemicals were of highest quality commercially available.

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