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1,2-Dehydroreticuline synthase, the branch point enzyme opening the morphinan biosynthetic pathway

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Dedicated to Wolfgang Steglich on the occasion of his 70th birthday.

Abstract

A synthase which oxidizes (*S*)-reticuline to 1,2-dehydroreticuline has been found to occur in seedlings of opium poppy (*Papaver somniferum* L.). Due to its instability, this enzyme could only be partly purified (ca. 5-fold enrichment). Partial characterization at this stage of purification showed that it does not need a redox cofactor and accepts both (*S*)-reticuline and (*S*)-norreticuline as substrates. [1-^{2}H, ^{13}C]-(*R,S*)-reticuline was enzymatically converted into [1-^{13}C]-dehydroreticuline, which has been identified by mass spectrometry. Release of the hydrogen atom in position C-1 of the isoquinoline alkaloid during the oxidative conversion, was exploited as a sensitive assay system for this enzyme. The enzyme has a pH optimum of 8.75, a temperature optimum of 37 °C and the apparent K_M value for the substrate reticuline was shown to be 117 μM. Moreover it could be demonstrated by sucrose density gradient centrifugation that the enzyme is located in vesicles of varying size. In combination with the previously discovered strictly stereoselective and NADPH dependent 1,2-dehydroreticuline reductase the detection of this enzyme, the 1,2-dehydroreticuline synthase, provides the necessary inversion of configuration and completes the pathway from two molecules of L-tyrosine via (*S*)-norcoclaurine to (*R*)-reticuline in opium poppy involving a total number of 11 enzymes.

Keywords: *Papaver somniferum*; Dehydroreticuline; Morphinan biosynthetic pathway; Dehydroreticuline synthase

1. Introduction

(*S*)-Reticuline is the common precursor for more than 2500 different benzylisoquinoline alkaloids found in higher plants. Curiously (*S*)-reticuline is also an intermediate in the biosynthetic pathway leading to the pharmaceutically highly valuable alkaloid morphine with (*R*)-configuration (Battersby et al., 1965a). However, this means that the (*S*)-configurated molecule has to be transformed enzymatically to its (*R*)-isomer in opium poppy (*Papaver somniferum*) (Battersby et al., 1965b). Salutaridine synthase, a cytochrome P-450 enzyme, catalyzing the carbon-carbon coupling of C-12 and C-13 exclusively accepts the (*R*)-configurated reticuline to yield salutaridine (Gerardy and Zenk, 1993), a precursor of morphine (Barton et al., 1965).

Previously it was demonstrated by labeling experiments with both (*S*)- and (*R*)-reticuline that tritium in the chiral position C-1 is lost to a large extent during the transformation to morphinan alkaloids in case of the [1-^{3}H]-(*S*)-enantiomer, whereas with [1-^{3}H]-(*R*)-reticuline as substrate up to 60% of the tritium label is retained (Battersby et al., 1965b). A reversible, facile redox system via 1,2-dehydroreticuline, suspected to occur endogenously in poppy plants, could account for the loss of tritium, thereby affecting inversion of configuration at C-1 (Brochmann-Hanssen, 1985). These experiments were repeated using 1-^{2}H, 1-^{13}C chiral reticulines and we concluded that the (*S*)-isomer is subject to complete inversion of configuration via oxidative attack at the asymmetric site followed by a stereospecific reduction. No susceptibility to oxidation was seen in application experiments using the (*R*)-isomer (Loeffler et al., 1990). 1,2-Dehydroreticuline is a naturally

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occurring alkaloid in *Papaver somniferum* and it has been shown to be incorporated with high yield into the morphinan alkaloids (Borkowski et al., 1978). Furthermore, a 1,2-dehydroreticuline reductase was discovered, purified and characterized from poppy seedlings which stereospecifically and irreversibly reduces 1,2-dehydroreticuline in the presence of NADPH to (*R*)-reticuline (De-Eknamkul and Zenk, 1992).

In summary, it is obvious that (*S*)-reticuline, which is formed via the (*S*)-norcoclaurine biosynthetic pathway (Stadler et al., 1987), is transformed first to 1,2-dehydroreticuline which in turn is stereospecifically reduced in the presence of NADPH to (*R*)-reticuline, thus completing the inversion of the configuration at C-1. Our aim was to search for the presumed dehydroreticuline synthase (a reticuline oxidase), which catalyzes the first step of the isomerization reaction of (*S*)-reticuline.

The present report describes the detection and characterization of the enzyme dehydroreticuline synthase, which opens the morphinan biosynthetic pathway in *Papaver somniferum*.

2. Results and discussion

2.1. The analytical method

Inversion of configuration from (*S*)- to (*R*)-reticuline involves a dehydrogenation in position 1 of the benzylisoquinoline alkaloid (Loeffler et al., 1990). This reaction allows a convenient and sensitive assay system based on a ^{3}H label in position C-1 of (*S*)-reticuline, which is eliminated during the enzymatic conversion. [1-^{3}H]-(*R*,*S*)-Reticuline was synthesized by reduction of 1,2-dehydroreticuline with NaB[^{3}H]$_4$ of high specific activity and subsequent purification of the alkaloid as described previously (Borkowski et al., 1978). The specific activity of the product was 2 mCi·μmol^{-1}, and the total yield was approximately 50%.

[1-^{3}H]-(*R*,*S*)-Norreticuline was synthesized analogously by reduction of 1,2-dehydronorreticuline with NaB[^{3}H]$_4$. In both cases, the assay was based on the quantification of the released tritium into the surrounding aqueous phase by scintillation counting after removal of residual alkaloids by absorption to dextran coated charcoal (Amann et al., 1984).

Incubation of [1-^{3}H]-(*R*,*S*)-reticuline or [1-^{3}H]-(*R*,*S*)-norreticuline without enzyme at a pH of 7.0–9.0 yielded a spontaneous loss of about 0.3% of the total ^{3}H activity into the surrounding water phase at time zero, reaching a level of approximately 0.6% after a time period of up to 1000 min. This spontaneous release of ^{3}H in the absence of a biocatalyst was subtracted at any given time in the subsequent experiments.

A protein extract was prepared from 8-day-old poppy seedlings after germination, since this system has already been successfully used in the detection and purification of 1,2-dehydroreticuline reductase (De-Eknamkul and Zenk, 1992). Incubation of both ^{3}H-labeled substrates with the redox factors FAD, FMN, NAD or NADP, either separately or in combination with each other (concentration 0.1 mM each) showed no significant release of tritium from the substrates into the water phase. However, when the protein was precipitated by ammonium sulfate (0–70% saturation), dialyzed and brought up to the original volume, substantial amount of ^{3}H was released from the substrates in the presence of these cofactors. Without addition of cofactors release of ^{3}H from both substrates was still catalyzed to the same extent by the enzyme extract (Fig. 1). Boiled enzyme preparations yielded the same tritium release from the labeled substrates as the control samples undergoing a small spontaneous oxidation.

Since the removal of the tritium label in position C-1 of both substrates was not dependent on soluble redox cofactors, it was very likely that the oxidative catalyst resided was linked to the protein.

2.2. Enzyme preparation and attempted purification

Using the described assay system, the purification of the enzyme catalyzing the oxidation of [1-^{3}H]-(*R*,*S*)-reticuline or [1-^{3}H]-(*R*,*S*)-norreticuline was attempted.

As mentioned above, the crude extract prepared from the poppy seedlings did not catalyze release of tritium from the substrates that was higher than the one of the controls without or boiled protein. Catalytic activity, however, was observed when the protein was precipitated by ammonium sulfate (0–70% saturation) and subsequently dialyzed. Apparently, low molecular weight inhibitors were removed during the process of precipitation and dialysis. The crude dialysate was loaded on top of a DEAE column and fractionated by

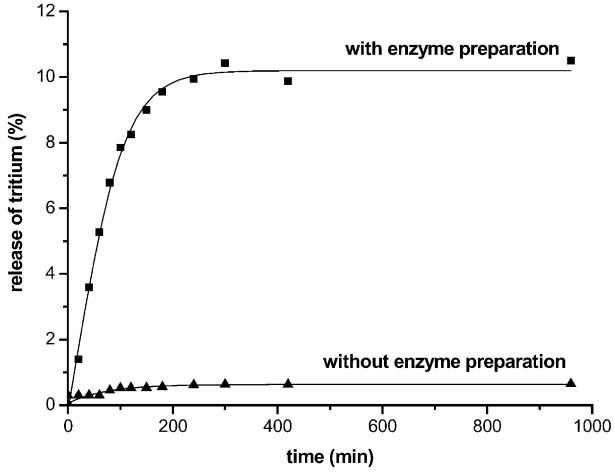

Fig. 1. Release of tritium from [1-^{3}H]-(*R*,*S*)-reticuline by an enzyme preparation from poppy seedlings obtained after $(NH_4)_2SO_4$ precipitation (70% saturation) and subsequent dialysis.

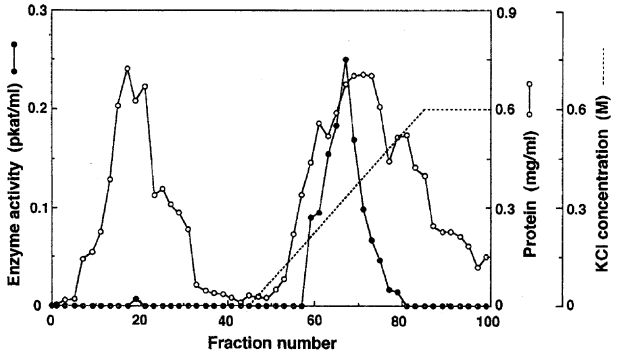

Fig. 2. DEAE Sephacel® anion exchange chromatography elution profile of the enzyme preparation obtained from poppy seedlings after $(NH_4)_2SO_4$ precipitation and dialysis. The enzyme under investigation eluted at concentration of 0.3 M KCl. Enzyme activity was measured using [1-^{3}H]-(*R,S*)-norreticuline as substrate.

applying a KCl concentration gradient. The desired enzyme activity eluted at 0.3 M KCl, and active fractions were combined (Fig. 2). With this purification step a 5-fold enrichment was achieved (Table 1). This fraction is referred to as DEAE pool. All other attempts to further purify this protein failed. Moreover, conventional methods to stabilize the enzyme were not successful and the inactivation of this protein proceeded very rapidly after DEAE purification. The enzyme was also sensitive to freezing.

2.3. Product identification

So far, only the release of tritium from the radioactively labeled alkaloids has been demonstrated. In order to gain further evidence for the reaction mechanism catalyzed by the protein in question, we used [1-^{2}H, 1-^{13}C]-(*S*)-reticuline which has previously been used successfully in incorporation experiments to elucidate the in vivo metabolism in poppy plants (Loeffler et al., 1990). The chiral, double labeled (*S*)-reticuline, whose optical purity was determined to be at least 98% ee, was incubated with the dialyzed enzyme preparation under standard conditions (see Experimental). After 15 h incubation, the reaction mixture was lyophilized, purified and subjected to LC-MS/MS. As shown in Fig. 3, unlabeled (*S*)-reticuline afforded a $[M+H]^+$ ion at *m/z* 330 (Fig. 3A) and synthetic dehydroreticuline displayed a $[M]^+$ ion at *m/z* 328 (Fig. 3B). The ESI-MS of [1-^{2}H, 1-^{13}C]-(*S*)-reticuline showed a $[M+H]^+$ ion at *m/z* 332 (Fig. 3C), while that of the reaction product showed a $[M]^+$ ion at *m/z* 329 (Fig. 3D). The main fragmentation pattern of the ^{13}C-labeled product versus the unlabeled synthetic dehydroreticuline standard showed the following fragments: *m/z* 329/328, 314/313, 313/312, 285/284, 268/267, 250/249, 205/204 and 191/190. Analysis of unlabeled and labeled dehydroreticuline by high resolution FT-ICR-MS showed a molecular mass of *m/z* 328.15498 and *m/z* 329.15814, respectively. The molecular mass of the labeled dehydroreticuline was in excellent agreement with the elemental composition of $^{12}C_{18}{}^{13}C_1H_{22}NO_4$. The significant enhancement of the peak at *m/z* 329.15814 indicated that the [^{13}C-1]-label was retained, while the ^{2}H-label in position C-1 was lost during the conversion of [1-^{2}H, 1-^{13}C]-reticuline into [1-^{13}C]-1,2-dehydroreticuline.

Hence, we concluded that (*S*)-reticuline was dehydrogenated in the presence of the poppy enzyme to yield 1,2-dehydroreticuline as shown in Fig. 4. The reaction mechanism may be either a single electron transfer or a concerted covalent catalysis. Additionally this result demonstrated that the standard assay conditions used for detection and quantitation of the enzyme activity using [1-^{3}H]-labeled (*R,S*)-reticuline or (*R,S*)-norreticuline as substrate was valid.

2.4. Enzyme properties

Because of the instability of the enzyme its catalytic properties could only be determined after 5-fold enrichment. At this stage of purification, NADPH, the compulsory cofactor for the 1,2-dehydroreticuline reductase (De-Eknamkul and Zenk, 1992), can be still adhearing to the crude protein fraction. In order to avoid any transformation of 1,2-dehydroreticuline, formed by the oxidase, by NADPH, to yield (*R*)-reticuline, the reticuline analog (*S*)-norreticuline, which does not naturally occur in *Papaver somniferum,* was used as substrate for the oxidase. This analogue is accepted by the oxidase, however, is completely inert—even in the presence of NADPH—towards the 1,2-dehydroreticuline reductase. This made (*S*)-norreticuline the preferred substrate at this stage of purification for the oxidase for further characterization. The pH optimum of the enzyme was found to be 8.75 both for [1-^{3}H]-(*R,S*)-reticuline and

Table 1
Summary of the attempted purification of 1,2-dehydroreticuline synthase from *Papaver somniferum* seedlings using [1-^{3}H]-(*R,S*)-norreticuline as a substrate

Purification step	Volume (ml)	Protein (mg)	Total act. (pkat)	Spec. act. (pkat mg^{-1})	Yield (%)	Purification (-fold)
Crude extract	470	616	0	0	100	–
$(NH_4)_2SO_4$ precipitation (0–70%)	50	247	1.83	0.0074	40.1	1
DEAE-Sephacel	35	61.3	2.10	0.0343	10.0	4.6

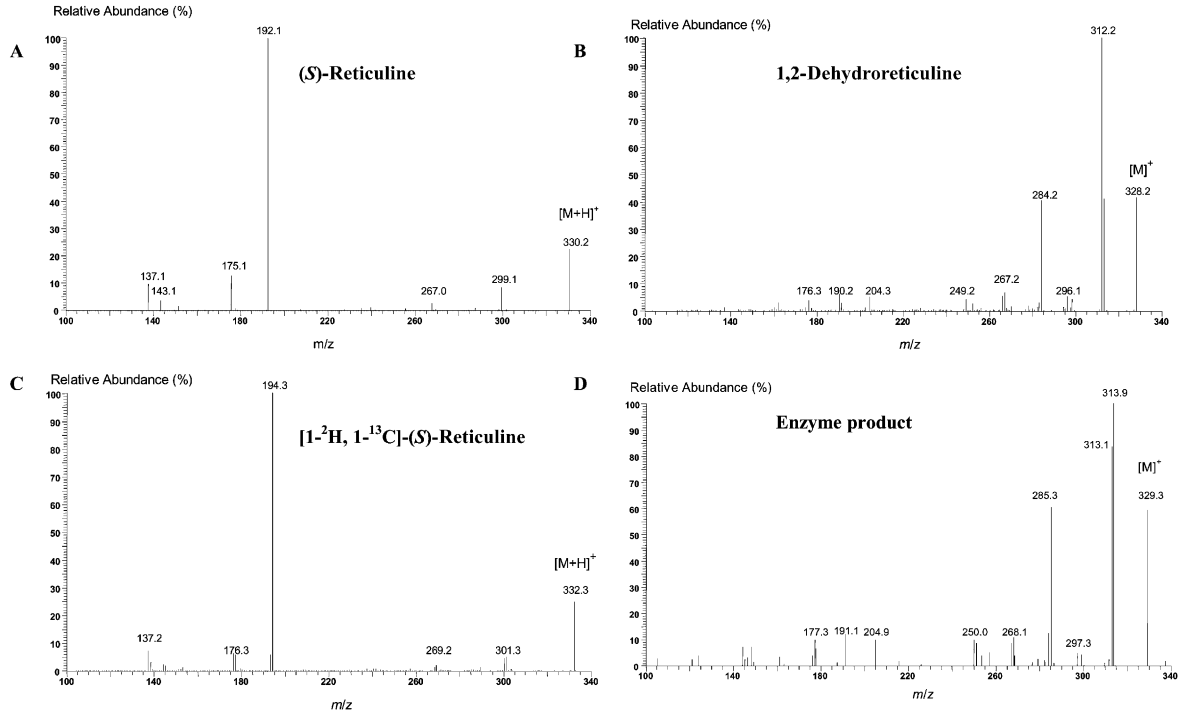

Fig. 3. CID mass spectrum of (*S*)-reticuline (A), CID mass spectrum of unlabeled synthetic 1,2-dehydroreticuline (B), CID mass spectrum of [1-^{2}H,-^{13}C]-(*S*)-reticuline (C), CID mass spectrum of the enzymatic reaction product using [1-^{2}H,-^{13}C]-(*S*)-reticuline as a substrate (D).

[1-^{3}H]-*(R,S)*-norreticuline. Half maximum activity occurred at a pH of 8 and 9.5. Under standard assay conditions, the formation of the dehydro species was linear up to 100 min and proportional to the protein concentration. The optimum temperature for catalytic activity was 37 °C, whereas at 27 °C and 55 °C only half maximum activity was found. The oxidase enzyme was active towards both tested 1-^{3}H-labeled substrates (*R,S*)-reticuline and (*R,S*)-norreticuline (ca. 95% of reticuline). The K_M value of this oxidase was 117 μmol · l^{-1}, determined with the substrate analogue norreticuline. At this stage of purification of the oxidase the substrate specificity was determined only by using the two substrates for which the 1-^{3}H-labeled compounds had been synthesized. Further substrate analogues will be tested as soon as labeled analogues become available.

In order to determine, whether this enzyme, oxidizing (*S*)-reticuline, is a cytosolic protein or occurs in some kind of organelles a sucrose density gradient centrifugation was performed. Poppy seedlings were homogenized in an isotonic buffer solution and the cell debris as well as intact cells removed by low spin centrifugation. Subsequent differential centrifugation of the supernatant at 1500 *g*, 2800 *g*, 4800 *g* and 9000 *g* yielded pellets, which were analyzed for the presence of the target enzyme using [1-^{3}H]-norreticuline as substrate. Substantial enzyme activity was found in the 4800 *g* and 9000 *g* pellets. These two pellets were placed separately on top of a sucrose step gradient consisting of layers of 25, 33, 38, 44 and 60% (w/v) sucrose. Table 2 shows the distribution of enzyme activity as determined by the tritium release method with the substrate [1-^{3}H]-norreticuline of the low spin (4800 *g*) and high spin (9000 *g*) pellet within the gradient. From these data it is obvious that this enzyme occurs in particles of different densities. While the particles of the 4800 *g* pellet harbouring the enzyme activity accumulate at the interface of 33% and 38% as well as 38% and 44% (w/v) sucrose concentration, particles of the 9000 *g* pellet are mainly found in the 60% (w/v) sucrose cushion. Clearly the 1,2-dehydroreticuline synthase is present in vesicles of varying

Table 2
Typical fractionation profile of a sucrose density step gradient of vesicles isolated from *P. somniferum* seedlings. 1,2-Dehydroreticuline synthase activity was determined by the tritium release method using [1-^{3}H]-(*R,S*)-norreticuline as substrate

Sucrose density gradient	Release of tritium from [1-^{3}H]-(*R,S*)-norreticuline	
	4800 *g* fraction pkat/ml	9000 *g* fraction pkat/ml
Extraction buffer	$1.0\pm\times10^{-2}$	$8.3\pm\times10^{-3}$
25% sucrose	$9.1\pm\times10^{-3}$	$4.0\pm\times10^{-3}$
33% sucrose	$3.7\pm\times10^{-2}$	$1.2\pm\times10^{-2}$
38% sucrose	$5.3\pm\times10^{-2}$	$1.3\pm\times10^{-2}$
44% sucrose	$6.6\pm\times10^{-2}$	$1.1\pm\times10^{-2}$
60% sucrose	$4.1\pm\times10^{-2}$	$9.7\pm\times10^{-2}$

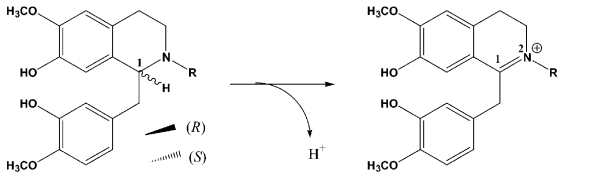

Fig. 4. Reaction catalysed by 1,2-dehydroreticuline synthase from (*S*)-reticuline to 1,2-dehydroreticuline.

size in *P. somniferum*, as has been shown for other benzylisoquinoline pathway enzymes in various other plant species, such as *Berberis* and *Eschscholtzia* (Amann et al., 1986). Hence, this is another example that enzymes of a complex pathway such as the morphine biosynthesis are localized in different cellular compartments.

Both, the (*S*)- and (*R*)-enantiomeric forms of reticuline have been firmly established in *Papaver somniferum* as biosynthetic precursors of morphinan alkaloids (Battersby et al., 1965b; Wieczorek et al., 1986; Loeffler et al., 1990) (Fig. 5). However, only (*R*)-reticuline has the configuration found in the morphine family of alkaloids such as salutaridine, thebaine, codeine and morphine as well as other intermediates of the morphinan biosynthetic pathway. Therefore, isomerization of (*S*)-reticuline to its (*R*)-counterpart was postulated (Battersby et al., 1965b; Borkowski et al., 1978). This necessary inversion of configuration was most plausibly explained by the intermediate formation of the 1,2-dehydroreticulinium ion originating from (*S*)-reticuline followed by a stereospecific reduction to yield the desired (*R*)-enantiomer for further oxidative coupling. Battersby et al., (1965b) and Borkowski et al. (1978) showed that dehydroreticuline is a natural occurring compound in *P. somniferum* and were able to demonstrate that the dehydro compound was incorporated with high yield into poppy/*Papaver* alkaloids.

In 1992, it was reported that synthetic 1,2-dehydroreticuline had been unequivocally reduced to (*R*)-reticuline by an enzyme isolated from poppy seedlings. This was proven by an (*R*)- and (*S*)-reticuline specific radioimmunoassay and by circular dichroism of the reduction product. Moreover it could be demonstrated that the reticuline formed by the reduction process was not transformed by the (*S*)-reticuline specific berberine bridge enzyme. These results provided clear evidence for the existence of the highly stereospecific 1,2-dehydroreticuline reductase leading to (*R*)-reticuline, which was subsequently also purified to homogeneity and characterized (De-Eknamkul and Zenk, 1992).

However, the question still remained, how 1,2-dehydroreticuline is formed from (*S*)-reticuline in the poppy plant. In this report, we have elucidated one further enzymatic reaction in the supposed pathway from (*S*)-norcoclaurine to (*R*)-reticuline, namely the oxidation of (*S*)-reticuline to 1,2-dehydroreticuline, the known substrate for the above mentioned reductase. A crucial point in this work was the demonstration of the formation of the dehydro compound by a crude enzyme preparation of poppy seedlings, using [1-^{2}H, ^{13}C]-(*S*)-reticuline. This substrate was transformed to [1-^{13}C]-dehydroreticuline by elimination of the deuterium label in position C-1 (Fig. 4.). No soluble cofactors such as NADP, NAD, FMN, FAD were necessary to support this reaction. Unfortunately, the enzyme was instable and sensitive to freezing and storage allowing only 5-fold purification by DEAE column chromatography. Whereas the 1,2-dehydroreticuline reductase (De-Eknamkul and Zenk, 1992) was found to be a cytosolic protein, the reticuline oxidation (1,2-dehydroreticuline synthase; the name reticuline oxidase has been given already to the socalled berberine bridge enzyme: EC 1.21.3.3) was shown to be particle bound. Using sucrose density gradient centrifugation (25–60%), particles containing oxidative enzyme activity were found at sucrose concentrations varying from 33 to

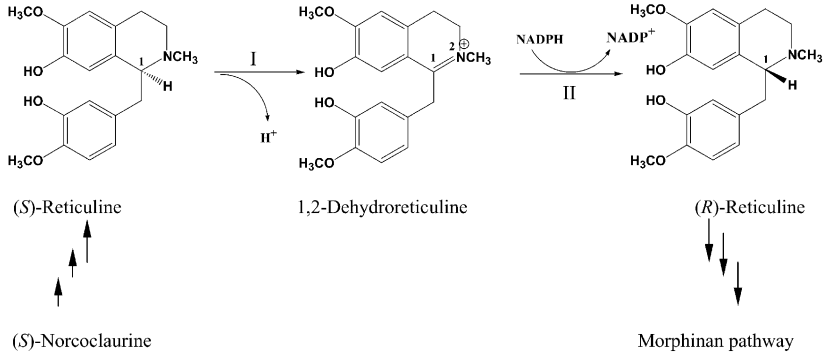

Fig. 5. Isomerisation of (*S*)-reticuline to (*R*)-reticuline via oxidation to the 1,2-dehydroreticulinium ion by 1,2-dehydroreticuline synthase (enzyme I) and subsequent stereoselective reduction to (*R*)-reticuline by 1,2-dehydroreticuline reductase (enzyme II) in *Papaver somniferum* and their involvement in *Papaver* alkaloid biosynthesis.

60%, indicating that the enzyme is located in particles of different size and weight. This demonstrates again, that the morphinan and other isoquinoline alkaloid pathways are highly compartmentalized between specific alkaloid vesicles, the endoplasmatic reticulum, cytosol and latex.

This present study completes the reticuline biosynthetic pathway in *Papaver*, starting from two molecules of L-tyrosine which is converted into one molecule each of dopamine and *p*-hydroxyphenylacetaldehyde. Condensation of these two molecules affords (*S*)-norcloclaurine, which in turn is transformed to (*R*)-reticuline representing the starting point of the morphinan biosynthetic pathway. The 1,2-dehydroreticuline synthase forms an important branching point. While (*S*)-reticuline leads to a multitude of benzophenanthridine and protoberberine alkaloids as well as other metabolites, found in the poppy plant and its latex, the action of the particle housed oxidase on (*S*)-reticuline creates the dehydroreticulinium ion, which is stereoselectively transformed to (*R*)-reticuline possessing the (*R*)-configuartion in position C-1 necessary for entering the morphinan biosynthetic pathway. The oxidase may play a decisive role in funneling the reticuline precursor into the morphinan biosynthetic pathway thus determining also the yield of commercially desired opium alkaloids in the *Papaver* plant.

3. Experimental

3.1. Plant material

The seeds of an inbred high alkaloid producing *Papaver somniferum* line (variety Munich) were used. The seeds (3.5 g) were germinated in transluscent plastic boxes (20×20 cm) containing 30 g of vermiculite, two layers of tissue paper and 150 ml of water. Growth occurred at 20 °C under continuous light (1500 lux cool white fluorescent lamps).

3.2. Chemicals

3.2.1. Synthesis of [1-^{3}H]-(R,S)-reticuline

1,2-Dehydroreticulinium ion (Borkowski et al., 1978) (3 μmol) was reduced in a methanolic solution of Na[B^{3}H$_4$] (10 mCi, spec. act. 12.0 Ci/mmol). The reaction was allowed to proceed on ice for 30 min followed by incubation at room temperature for 2 h. The reaction was terminated by the addition of 80 μl of a mixture of methanol and glacial acetic acid (1:1, v/v). The mixture was purified by thin-layer chromatography on silica gel plates developed with $CHCl_3$:methanol (5:1, v/v) and $CHCl_3$: ethanol: ethylacetate: acetone (6:2:1:1, v/v/v/v). Radioactivity was monitored with TLC-linear analyzer (Tracemaster 20, Berthold, Germany). The R_f-values were 0.23 and 0.13, respectively. The purity of the product was checked by radio-HPLC [Eurosphere-100 C_{18} 125×3.0 mm, 5 μm; eluent: 15% acetonitrile in 1 mM sodium phosphate buffer containing 0.1% (v/v) TEA, pH 2.0; flow rate 1 ml/min; radiodetector Ramona Star (Raytest) equipped with Software D6500 (Merck-Hitachi)]. The retention time of [1-^{3}H]-reticuline was 4.25 min.

3.2.2. Synthesis of [1-^{3}H]-(R,S)-norreticuline

[1-^{3}H]-(*R,S*)-Norreticuline was synthesized analogously to [1-^{3}H]-(*R,S*)-reticuline with practically identical yield and specific activity.

3.3. Enzyme preparation and purification

Deep-frozen 8-day-old seedlings (100 g) were ground to a fine powder in a precooled mortar. The frozen powder was thawed and stirred for 20 min in 100 ml of 100 mM Tricine–NaOH buffer (pH 7.5) containing 1 mM EDTA and 10 mM 2-mercaptoethanol. The mixture was filtered through four layers of cheesecloth and clarified by centrifugation at 10,000 *g* for 10 min. $(NH_4)_2SO_4$ was added to the supernatant to a saturation of 70% followed by centrifugation at 10,000 *g* for 10 min. The obtained protein pellet was resuspended in 30 ml of 50 mM Tricine–NaOH buffer (pH 7.5) containing 1 mM EDTA and 10 mM 2-mercaptoethanol and desalted by dialysis overnight against the same buffer. The crude enzyme extract was applied to an anion exchange column (DEAE-Sephacel, 2.5×12 cm). The column was washed with 50 mM Tricine-NaOH buffer (pH 7.5) and was subsequently developed with a linear gradient of 0–600 mM KCl in the same buffer at a flow rate of 20 ml h^{-1}. The enzyme in question eluted at 0.3 M KCl.

3.4. A sucrose density gradient centrifugation

A sucrose density gradient centrifugation was performed according to the standard procedure as described by Lenz (1994).

3.5. Determination of protein concentration

Protein concentration was determined according to the method of Bradford (1976) with bovine serum albumin as standard.

3.6. Enzyme assay

[1-^{3}H]-(*R,S*)-Norreticuline or [1-^{3}H]-(*R,S*)-reticuline (0.2 nmol, 30,000 cpm) and 1.8 nmol unlabeled substrate were incubated with an enzyme preparation (0.05–1 mg protein) in a final volume of 200 μl of 100 mM sodium borate buffer (pH 8.7) for 2 h at 35 °C. The

incubation was stopped by the addition of 200 μl of dextran coated activated charcoal (10 g of activated charcoal in 100 ml of an isotonic solution containing 250 mg dextran). The mixture was vigorously vortexed for 1 min and centrifuged at 10,000 *g*. 150 μl of the clear supernatant were used for scintillation counting. Controls were performed with boiled enzyme preparations or without the addition of enzyme.

3.7. Product identification

30.4 nmol of [1-^{2}H, 1-^{13}C]-(*S*)-reticuline were incubated with the enzyme preparation obtained after DEAE-column purification (15 mg protein) in 100 mM sodium borate buffer (pH 8.7) at 35 °C in a volume of 4 ml. After overnight incubation, the reaction mixture was frozen in liquid nitrogen and lyophilized to complete dryness. The reaction product was dissolved in methanol and purified by TLC ($CHCl_3$: methanol, 5:1, v/v), respectively. R_f value was 0.1. The purified product was identified by LC-MS-MS and high resolution FT-ICR-MS.

3.8. Mass spectrometry

The positive ion electrospray (ES) mass spectra were obtained from a Finnigan MAT TSQ 7000 instrument (electrospray voltage 4.5 kV; heated capillary temperature 220 °C; sheath gas: nitrogen) coupled with a Surveyor MicroLC system equipped with a RP18-column (1×100 mm, 5 μm, Ultrasep). The solvent system consisted of solvent A: H_2O and B: CH_3CN, each containing 0.2% acetic acid. A linear gradient from 15 to 90% B in A was applied for 15 min, followed by an isocratic step at 90% of B in A for another 15 min. The flow rate was 25 μl min^{-1}. The CID mass spectrum of dehydroreticuline and the selected reaction monitoring (SRM) measurements were performed during the HPLC run using a collision energy of 35 eV; collision gas: argon, collision pressure: 1.8×10^{-3} torr. The CID mass spectra (25 eV) of the standard samples reticuline and [1-^{2}H, ^{13}C]-(*S*)-reticuline were recorded via a syringe pump.

25 eV CID mass spectrum of reticuline, *m*/*z* (rel. int.,%): 330 ($[M+H]^+$, 23), 299 (9), 267 (3), 192 (100), 175 (13), 151 (2), 143 (4), 137 (10).

25 eV CID mass spectrum of [1-^{2}H, ^{13}C]-(*S*)-reticuline, *m*/*z* (rel. int.,%): 332 ($[M+H]^+$, 23), 301 (5), 300 (4), 269 (2), 194 (100), 193 (6), 177 (5), 176 (6), 145 (2), 144 (2), 137 (6).

35 eV CID mass spectrum of dehydroreticuline (RT = 10.50 min), *m*/*z* (rel. int.,%): 328 ($[M]^+$, 43), 313 (39), 312 ($[M–CH_3–H]^+$, 100), 296 (5), 284 (42), 267 (7), 249 (4), 204 (4), 190 (6), 176 (4).

The used reactions for the quantification are based on the most prominent ion in the corresponding electrospray CID MS ($[M–CH_3–H]^+$). Dehydroreticuline: *m*/*z* 328 $[M]^+ \rightarrow m/z$ 312; $^{13}C_1$-dehydroreticuline: *m*/*z* 329 $[M]^+ \rightarrow m/z$ 313.

The high resolution electrospray mass spectra were obtained from a Bruker Apex III 70e Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Billerica, USA) equipped with an Infinity™ cell, a 7.0 Tesla superconducting magnet (Bruker, Karlsruhe, Germany), an RF-only hexapole ion guide and an external electrospray ion source (Agilent, off axis spray). Nitrogen was used as drying gas at 150 °C. The sample solutions were introduced continously via a syringe pump with a flow rate of 120 μl h^{-1}. All data were aquired with 512 k data points and zero filled to 2048 k by averaging 32 scans. The resolution at *m*/*z* 328 was about 90,000.

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