Tetrahedron Letters, Vol.31, No.34, pp 4855-4858, 1990
Printed in Great Britain

0040-4039/90 $3.00 + .00
Pergamon Press plc

ENZYMIC FORMATION OF (R)-RETICULINE FROM 1,2-DEHYDRORETICULINE IN THE OPIUM POPPY PLANT

W. De-Eknamkul and M.H. Zenk

Lehrstuhl für Pharmazeutische Biologie, Universität München,
Karlstrasse 29, D-8000 München 2, West Germany

Abstract: A cytosolic $NADPH_2$ dependent enzyme which reduces stereospecifically 1,2-dehydroreticuline to yield (R)-reticuline, the biosynthetic precursor of the morphinan alkaloids, has been discovered, purified to homogeneity, and characterized from Papaver somniferum seedlings.

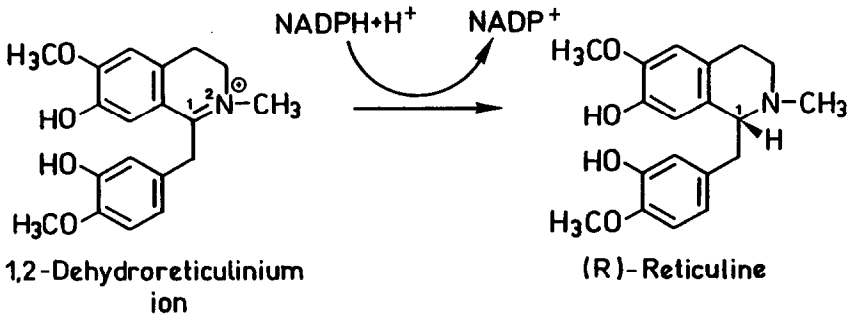
(R)-Reticuline is firmly established in Papaver somniferum as the precursor of morphinan type alkaloids possessing also the (R)-configuration.[1,2] (S)-Reticuline, however, is the central intermediate in isoquinoline alkaloid biosynthesis[3] which is formed from (S)-norcoclaurine.[4] Therefore, isomerization of (S)-reticuline to its (R)-antipode was postulated.[1,5] This inversion of configuration was most plausibly explained by the intermediate formation of the 1,2-dehydroreticulinium ion originating from (S)-reticuline followed by stereospecific reduction to yield the desired (R)-counterpart.[1] To support this proposal, 1,2-dehydroreticuline was synthesized and shown to be efficiently incorporated into opium alkaloids.[1] The nature of the intermediate claimed to be 1,2-dehydroreticulinium ion remained uncertain and it was only 13 years later that the authentic dehydro compound was synthesized, fully characterized, and its metabolic activity as a precursor for the morphinan type alkaloids unequivocally established[5]. Furthermore, the natural occurrence of the 1,2-dehydroreticulinium ion was demonstrated and its pool size in the poppy plant determined.[5] These experiments proved unequivocally the intermediacy of this quaternary base in the isomerization of (S)- to (R)-reticuline.

A novel oxidase ((S)-tetrahydroprotoberberine oxidase) has previously been discovered in our laboratory in Berberidaceae that catalyzes in the presence of oxygen the dehydrogenation of (S)-tetrahydroprotoberberines.[6] This flavoprotein is compartmentalized in a specific vesicle[7] and can stereospecifically oxidize (S)-benzylisoquinolines to their corresponding 1,2-dehydro analogues.[8] The rate of oxidation of (S)-reticuline is approximately 1% of that of (S)-norreticuline.[6] This enzyme activity directed towards tetrahydroprotoberberine alkaloids has been shown also to occur in Papaver somniferum roots and leaves.[8] While previously authentic 1,2-dehydroreticulinium ion had to be

prepared (with low specific activity) in a 7-stage chemical synthesis, we now could prepare the desired dehydro-derivative of norreticuline and reticuline as well as other benzylisoquinoline alkaloids with high specific activity and a maximum of yield by employing this very stable enzyme as a catalyst.

[N-$^{14}CH_3$]-1,2-Dehydroreticuline (spec. act. 56 µCi/µmol) was synthesized[8] and fed to 5-day-old seedlings of Papaver somniferum through the root system and allowed to metabolize for 72 hrs. Thebaine was isolated[5] and a 3.8% incorporation observed, that is in the same range as previously reported (2.7%).[5] This experiment proved that the enzymatically generated 1,2-dehydroreticulinium ion was metabolically active. Subsequently a crude cell-free extract of 5-day-old poppy seedlings was prepared and incubated with [N-$^{14}CH_3$]-1,2-dehydroreticuline in the presence of NADPH and at a pH 8.5. Work up of the incubation mixture yielded a compound indistinguishable from authentic reticuline. The product obtained by large scale (500-fold) incubation was unequivocally identified as reticuline by mass spectral analysis (EI; 70eV): m/e (% relative intensity) = 192 (base peak) (100), 177 (21), 149 (5), 137 (benzylic fragment) (3). The absolute configuration of reticuline was further examined by using three different methods. Firstly, the product was analyzed by a (R)- and (S)-specific radioimmunoassay[9] and was clearly shown to have (R)-configuration. Absolutely no evidence for the formation of the (S)-enantiomer was found even if the time course for the formation of reticuline was followed for up to 8 hrs. Secondly, the CD-spectrum of the enzymic product corresponded to (R)-reticuline and thirdly, the highly radio-labelled metabolite generated from 1,2-dehydroreticuline was not biotransformed to scoulerine by the highly (S)-specific berberine bridge enzyme,[10] which represents one of the most sensitive assays for (S)-reticuline. In the absence of NADPH no transformation of the iminium salt occurred. The enzyme was subsequently purified and catalytic activity determined using standard incubation mixture which consisted in a total volume of 150 µl of: 330 mM glycine-NaOH buffer pH 8.5, 0.67 mM NADPH, 13.3 µM [N-$^{14}CH_3$]-1,2-dehydroreticuline (15 000 dpm) and protein up to 0.1 mg. The reaction was terminated after 30 min incubation at 30°C by addition of toluene and the radioactivity of the organic phase determined. Under these conditions the substrate remains in the aqueous phase whereas reticuline is extracted into the organic layer. Using a 6-step purification procedure, beginning with ammonium sulphate precipitation (60 - 85%) followed by column chromatography with a) Phenyl-Sepharose, b) DEAE-Sephacel, c) Ultrogel AcA 34, d) Hydroxyapatite, and a final FPLC step employing Superose 12, the enzyme was purified about 1400-fold to homogeneity with 20% recovery. The pure enzyme shows an apparent molecular weight of 30 $\pm$ 2 kD (SDS-PAGE and gel filtration), a pH-optimum at 8.5 and a temperature optimum at 30°C. The dependence of the rate of (R)-reticuline formation on 1,2-dehydroreticuline afforded a K_M value of 10 µM. NADPH yielded a K_M value of 7 µM (no activity was observed with NADH, NAD^+ or $NADP^+$). The enzyme transfers the pro-S-hydride from NADPH to C-1 of the 1,2-dehydroreticulinium ion and is highly substrate-specific. No reduction was detected with 1,2-dehydronorreticuline, 1,2-dehydrococlaurine, salutaridine, codeinone or acetaldehyde. Careful kinetic analysis revealed that for each mole of substrate consumed, one mole of (R)-reticuline is formed. We propose the designation 1,2-dehydroreticuline reductase for

this highly specific enzyme which catalyzes the reaction stereo-specifically as depicted in Scheme 1.



Scheme 1. Reaction sequence catalyzed by 1,2-dehydroreticuline reductase [(R)-specific] from Papaver somniferum

Up to now no evidence could be obtained for the reversibility of the reaction under varying pH conditions and cosubstrate requirements. The purified enzyme is stabilized by addition of NADPH (1 mM), showing half-maximal activity after 20 days or 30 days when kept at 4°C or -20°C, respectively. Omission of NADPH during the storage process effects rapid loss of activity with a half-life of 2 days or 1 day when stored at 4°C or -20°C, respectively. This novel enzyme is evidently a cytosolic protein and no indication was found for subcellular compartmentation using sucrose density (15 - 45%) gradient centrifugation. A time course study of the reductase activity in P. somniferum seedlings showed that the measurable onset of enzyme activity was at day 2 after germination. The enzyme activity then increased rapidly to reach a maximum value (22 pkat/g dwt) at day 4. Thereafter (day 10), turnover declined gradually to 14 pkat/g dwt. In addition to P. somniferum, the reductase was found in differentiated plants of P. bracteatum (6.8 pkat/g dwt). No enzyme activity was detected, however, in differentiated plants of P. persicum, P. oreophilum, Argemone hunnemannii, Dicentra oregana, Fumaria macrosepta. Also, no activity was found in cell cultures of members of the genus Papaver such as P. somniferum, P. rhoeas, P. bracteatum, P. feddei, P. dubium.

There can be no doubt that this highly species- and substrate- as well as stereospecific enzyme catalyzes the provision of (R)-reticuline for the formation of morphinandienone alkaloids also possessing the (R)-configuration at the chiral center. The naturally occurring[5] branch point intermediate 1,2-dehydroreticuline is specifically reduced as shown by in vivo[5] and in vitro experiments to (R)-reticuline, thus opening the pathway leading to the opium alkaloids. The question now remaining is how the substrate molecule 1,2-dehydroreticuline is formed in the poppy plant. Is (S)-tetrahydroprotoberberine oxidase,[6-8] which

shows low substrate specificity but was efficiently used to generate the quaternary base *in vitro*, involved in the generation of the iminium intermediate? Does the oxidase, detected in *Papaver somniferum* by using tetrahydroprotoberberines as substrates, show activity towards (S)-reticuline *in vivo* and are the enzymatic steps involved in the conversion of (S)- to (R)-reticuline reversible?

Acknowledgement:
Our thanks are due to Dr. R. Stadler for linguistic help in preparation of the manuscript, Prof. G. Snatzke, Bochum, for measuring and interpretation of the CD-spectra of (R)-reticuline. W. De-Eknamkul thanks the Alexander von Humboldt-Stiftung for a fellowship. Supported by SFB 145 of Deutsche Forschungsgemeinschaft, Bonn, and Fonds der Chemischen Industrie.

References:

1. A.R. Battersby, D.M. Foulkes and R. Binks, J. Chem. Soc. 3323 (1965).
2. S. Loeffler, R. Stadler and M.H. Zenk, see foregoing publication.
3. Summarized and referenced in G.A. Cordell, Introduction to Alkaloids. John Wiley & Sons, New York 1981, and literature cited therein.
4. R. Stadler, T.M. Kutchan, S. Loeffler, N. Nagakura, B. Cassels, and M.H. Zenk, Tetrahedron Letters 28, 1251 (1987).
5. P.R. Borkowski, J.S. Horn and H. Rapoport, J. Am. Chem. Soc. 100, 276 (1978).
6. M. Amann, N. Nagakura and M.H. Zenk, Eur. J. Biochem. 175, 17 (1988).
7. M. Amann, G. Wanner and M.H. Zenk, Planta 167, 310 (1986).
8. M. Amann and M.H. Zenk, Phytochemistry 26, 3235 (1987).
9. U. Wieczorek, N. Nagakura, C. Sund, S. Jendrzejewski, and M.H. Zenk, Phytochemistry 25, 2639 (1986).
10. P. Steffens, N. Nagakura and M.H. Zenk, Phytochemistry 24, 2577 (1985).

(Received in Germany 27 June 1990)