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A novel 2-oxoglutarate-dependent dioxygenase involved in vindoline biosynthesis: characterization, purification and kinetic properties

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Abstract

The enzyme, desacetoxyvindoline 4-hydroxylase, was purified to apparent homogeneity from *Catharanthus roseus* by ammonium sulfate precipitation and successive chromatography on Sephadex G-100, green 19-agarose, hydroxylapatite, α-kg sepharose and Mono Q. The 4-hydroxylase was characterized by its strict specificity for position 4 of desacetoxyvindoline suggesting it to catalyze the second to last step in vindoline biosynthesis. The molecular mass of the native and denatured 4-hydroxylase was 45 kDa and 44.7 kDa, respectively, suggesting that the native enzyme is a monomer. Two-dimensional isoelectric focusing under denaturing conditions resolved the purified 4-hydroxylase into three charge isoforms of pIs 4.6, 4.7 and 4.8. The purified 4-hydroxylase exhibited no requirement for divalent cations, but inactive enzyme was reactivated in a time-dependent manner by incubation with ferrous ions. The enzyme was not inhibited by EDTA or SH-group reagents at concentrations up to 10 mM. The mechanism of action of desacetoxyvindoline 4-hydroxylase was investigated. The results of substrate interaction kinetics and product inhibition studies suggest an Ordered Ter Ter mechanism where α-kg is the first substrate to bind followed by the binding of O_2 and desacetoxyvindoline. Their K_m values for α-kg, O_2 and desacetoxyvindoline are 45 μM, 45 μM and 0.03 μM, respectively. The first product to be released was deacetylvindoline followed by CO_2 and succinate, respectively.

Abbreviations: α-kg – α-ketoglutarate or 2-oxoglutarate, NMT – N-methyltransferase, SAM – S-adenosyl-L-methionine, TLC – thin layer chromatography, VBL – vinblastine, VCR – vincristine

Introduction

Alkaloids are widely distributed throughout the plant kingdom. It has been estimated that 15–30% (Robinson 1981) of plants produce alkaloids and that over 25% of these are derived from tryptophan. The family Apocynaceae which consists of the genera *Rauwolfia*, *Catharanthus* and *Aspidosperma* are a very rich source of tryptophan-derived indole alkaloids. The indole alkaloids of this family have been extensively investigated mostly because of their physiological effects on man and their use in pharmacy. The *Vinca* alkaloids represent a class of natural drugs derived from the periwinkle plant, *Catharanthus roseus*. Two commercially important bis-indole alkaloids, vinblastine (VBL) and vincristine (VCR), are known to accumulate in the aerial parts of *C. roseus*. These dimeric indole alkaloids have been extensively investigated because of their usefulness in treating certain neoplasms.

In contrast with the extensive studies on the structural elucidation of indole alkaloids in *C. roseus* (Robinson 1981) which led to knowledge of the biosynthetic pathway of VBL and VCR, less is known of the enzymology of this pathway (De Luca 1993). This situation may be explained by the inherent difficulties in the preparation of specifically substituted indole alkaloid substrates and reaction products that are required to conduct enzymatic studies. The recent advances in our understanding of the late stages of vindoline biosynthesis (Balsevich et al. 1986) has facilitated the synthesis of appropriate substrates for the development of enzyme assays.

Since VBL and VCR accumulate in low amounts, a considerable amount of research has been devoted

to study the production of these alkaloids by cell and tissue culture methods (De Luca & Kurz 1988). Unfortunately, this approach has not yet succeeded to produce bis-indole alkaloids since cell cultures are unable to synthesize vindoline, one of the monomeric precursors of VBL and VCR. In order to study enzymes involved in the late stages of vindoline biosynthesis, we decided to use intact *C. roseus* plants which produce and accumulate vindoline.

The biosynthesis of vindoline from tabersonine involves three hydroxylations, one *O*-methylation, one *N*-methylation and an *O*-acetylation (Fig. 1). Recently, a number of these enzymes have been purified and characterized from *C. roseus*. The third last step in vindoline biosynthesis is catalyzed by *N*-1 desmethyldesacetoxyvindoline (16-methoxy-2,3-dihydro-3-hydroxytabersonine) *N*-methyltransferase which has been partially purified (Dethier & De Luca 1993). The last step in vindoline biosynthesis is catalyzed by deacetylvindoline 4-*O*-acetyltransferase (DAT) which has been purified to homogeneity (Power et al. 1990). The enzyme which catalyzes the second last step in vindoline biosynthesis requires indole alkaloid substrate, α-kg, ascorbate, ferrous ions and molecular oxygen for activity and thus is classified as a 2-oxoglutarate-dependent dioxygenase (De Carolis et al. 1990).

The fact that hydroxylation at position 4 is critical for the enzymatic synthesis of vindoline and that desacetoxyvindoline 4-hydroxylase is absent in cell cultures prompted us to develop a protocol for the purification to homogeneity of desacetoxyvindoline 4-hydroxylase. Having developed a purification procedure which yields a highly purified enzyme preparation as well as having a direct enzyme assay which is simple, fast and accurate we attempted to elucidate the kinetic mechanism of desacetoxyvindoline 4-hydroxylase.

Results and discussion

Synthesis of N(1)-[$^{14}CH_3$] desacetoxyvindoline

The assay for desacetoxyvindoline 4-hydroxylase is based on the availability of the substrate, *N*(1)-[$^{14}CH_3$] desacetoxyvindoline (De Carolis et al. 1990). Briefly, crude desalted extracts containing desmethyldesacetoxyvindoline NMT activity from young leaves of *C. roseus* were used to synthesize *N*(1)-[$^{14}CH_3$] desacetoxyvindoline using desmethyldesacetoxyvindoline as the methyl acceptor and *S*-adenosyl-*L*-[$^{14}CH_3$]methionine (SAM) as the methyl donor (Fig. 1). The NMT assay mixture contained 24.5 µM of desmethyldesacetoxyvindoline, 25 nmol labelled SAM (containing 2,750,000 dpm), and 2 mg of protein exhibiting NMT activity (in 100 mM Tris-HCl [pH 8.0]) in a final volume of 320 µl. The enzyme reaction was started by addition of protein and the mixture was incubated for 80 min at 30 °C. The reaction was stopped by addition of 100 µl of 1 N NaOH. Desmethyldesacetoxyvindoline and *N*(1)-[$^{14}CH_3$] desacetoxyvindoline were extracted in 3 × 150 µl of ethyl acetate. The alkaloids were separated by preparative TLC (silica) using 10% methanol in ethyl acetate as the solvent system. The band corresponding to *N*(1)-[$^{14}CH_3$] desacetoxyvindoline was identified, scraped and extracted from the silica powder with methanol. A single product corresponding to *N*(1)-[$^{14}CH_3$]desacetoxyvindoline was obtained with a 36% yield.

Desacetoxyvindoline 4-hydroxylase assay

Desacetoxyvindoline 4-hydroxylase was assayed by measuring the formation of *N*(1)-[$^{14}CH_3$]deacetylvindoline (Fig. 1). The assay mixture contained 0.56 nmol of labelled alkaloid substrate (containing 44,600 DPM), 10 mM α-kg, 7.5 mM ascorbate and 10 µM Fe^{2+} and up to 150 µg of protein in a total volume of 200 µl. The 4-hydroxylase catalyzes the incorporation of one atom of molecular oxygen into position 4 of the alkaloid moiety while the second is incorporated into the keto group of α-kg. The labile intermediate is then decarboxylated leading to the liberation of CO_2 and the formation of succinate. The assay was started by the addition of enzyme and the mixture incubated at 30 °C for 15 min. The reaction was stopped by the addition of 100 µl of 1 M NaOH and the aqueous phase was extracted for indole alkaloids with ethyl acetate. The organic phase was recovered and after evaporation to dryness, the substrate (R_f 0.52) and product (R_f 0.22) were separated by TLC (silica) using 10% methanol in ethyl acetate as the solvent. After chromatography *N*(1)-[$^{14}CH_3$]deacetylvindoline was isolated from the silica support and the radioactivity counted using a scintillation counter.

Figure 2 shows a photograph of an autoradiogram of the chromatographed reaction product of desacetoxyvindoline 4-hydroxylase activity. The lanes represent fractions eluting from a gel filtration column. The autoradiogram shows that hydroxylase activity elutes

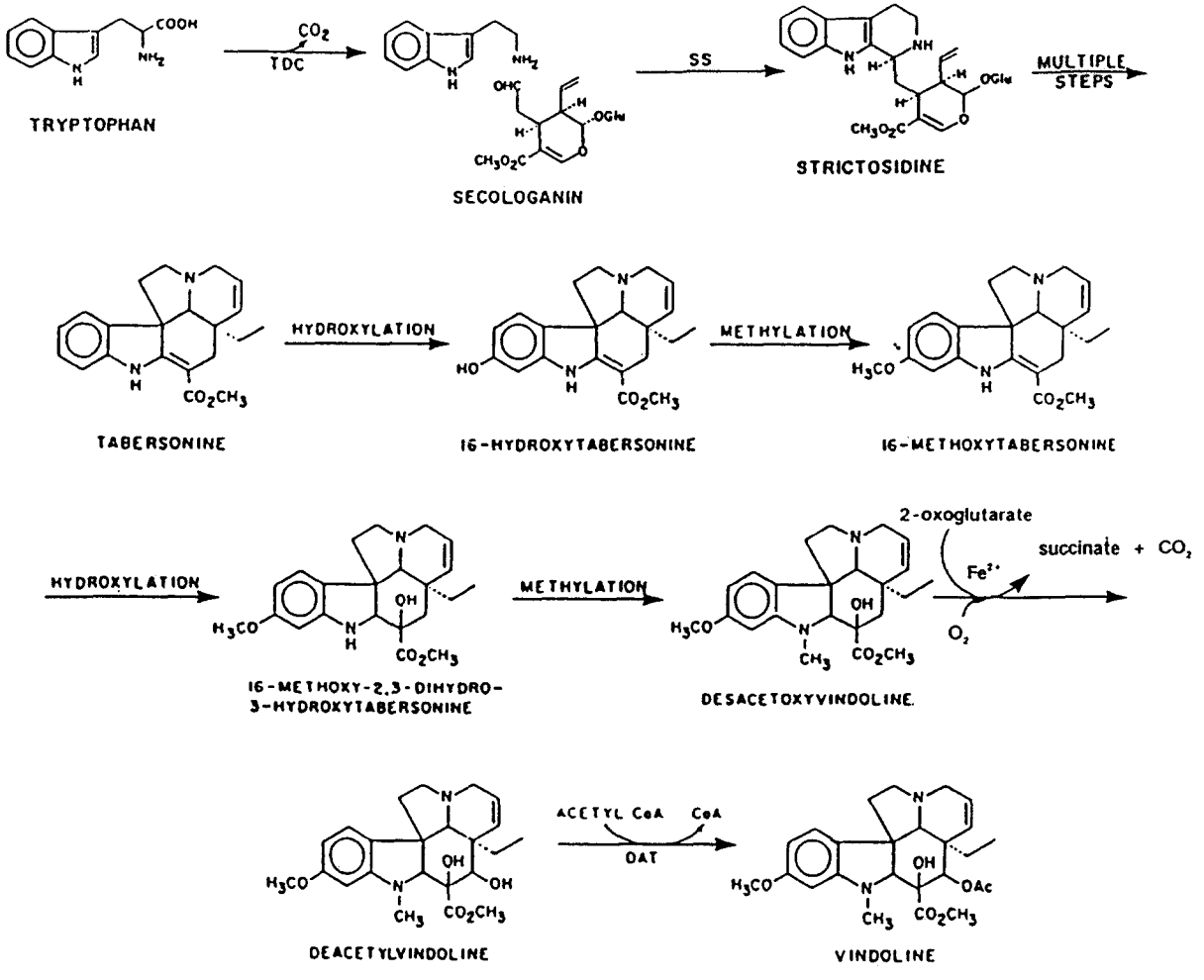


Fig. 1. The biosynthetic pathway from tryptophan to vindoline.

in lanes F to I and that maximal activity occurs in lane H. The efficiency of this assay rendered it suitable for routine use in protein purification on different columns, or in conducting enzyme kinetic studies.

Desacetoxyvindoline 4-hydroxylase is a 2-oxoglutarate dependent dioxygenase

When a protein preparation exhibiting 4-hydroxylase activity was incubated with *N*(1)-[$^{14}CH_3$]desacetoxyvindoline, no reaction product corresponding to *N*(1)-[$^{14}CH_3$]deacetylvindoline was observed. Addition of ascorbic acid or ferrous sulfate or both to the reaction mixture did not result in any product formation. However, the addition of α-kg produced a radiolabeled product of lower R_f value corresponding to *N*(1)-[$^{14}CH_3$]deacetylvindoline. Addition of ascorbic acid and ferrous sulfate enhanced 4-hydroxylase activity. Removal of molecular oxygen from the reaction mixture containing α-kg resulted in complete inhibition of 4-hydroxylase activity. These results establish that the enzyme is a 2-oxoglutarate-dependent dioxygenase (De Carolis et al. 1990).

Purification of desacetoxyvindoline 4-hydroxylase

As shown previously, a cell free extract of *C. roseus* catalyzed the 4-hydroxylation of *N*(1)-[$^{14}CH_3$]desacetoxyvindoline. Based on these results we used the radiolabeled indole alkaloid as substrate

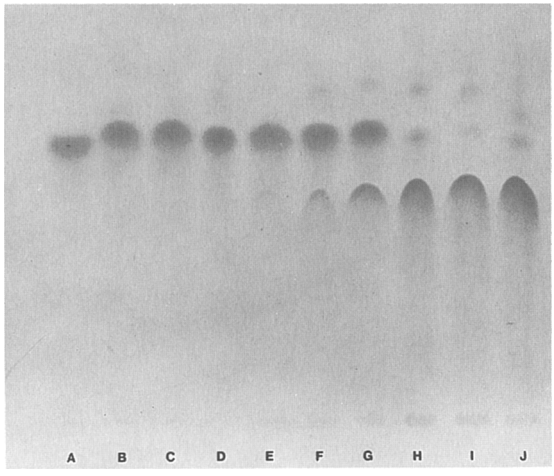


Fig. 2. Photography of an autoradiogram of the chromatographed reaction product of the 4-hydroxylase using the direct assay mixture. Each lane represents fractions eluting from a gel filtration column. In the absence of 4-hydroxylase activity (lanes A–E) only the substrate is present. However, in the presence of increasing 4-hydroxylase activity (lanes F–J), the substrate is converted to product, N(1)-[$^{14}CH_3$]deacetylvindoline. Optimal 4-hydroxylase activity was observed in lane I. Methanol-ethyl acetate was used to develop the silica TLC plate.

to detect 4-hydroxylase activity during enzyme purification (De Carolis & De Luca 1993).

The protein extract of *C. roseus* was fractionated with ammonium sulfate and the protein which precipitated between 35 and 75% saturation was chromatographed by gel filtration on a Sephadex G-100 column. The 4-hydroxylase eluted as a discrete peak with an apparent molecular weight of 45 kDA.

The active fractions were pooled and applied on a green 19-agarose dye-affinity column. The bound protein was eluted with a linear 0.0 to 1.0 M NaCl gradient. The 4-hydroxylase eluted between 0.49 and 0.68 M NaCl and resulted in the elimination of a considerable amount of protein and pigmentation.

The green 19-agarose purified fractions exhibiting 4-hydroxylase activity were chromatographed on a hydroxylapatite column. The bound 4-hydroxylase was eluted with a linear gradient of 10 to 200 mM sodium phosphate. The active 4-hydroxylase eluted at 165 mM phosphate.

Phosphate was removed from the fractions exhibiting 4-hydroxylase activity by chromatography on Sephadex G-25 and the preparation was applied to an α-kg sepharose cosubstrate affinity column. The column was extensively washed and the bound enzyme was selectively eluted with a linear α-kg gradient of 0.0 to 50.0 mM. The 4-hydroxylase eluted at 29.5 mM α-kg. Other bound proteins were nonselectively eluted from the α-kg sepharose column by applying a linear salt gradient of 0.0 to 2.0 M NaCl.

Fractions containing 4-hydroxylase activity which eluted from the α-kg sepharose still contained minor contaminants. As a result, the enzyme was further purified by high performance ion-exchange chromatography on a Mono Q HR 5/5 column. The protein was applied to the column and was eluted with a linear salt gradient of 0.0 to 0.2 M NaCl. The enzyme eluting at 105 mM NaCl was free from other contaminating proteins (De Carolis & De Luca 1993).

This five step purification protocol enriched the enzyme over 2000-fold with a recovery of 1.6% (Table 1) and the specific activity at the Mono Q stage was 86.15 pkat/mg for the 4-hydroxylase. The results obtained with gel filtration chromatography and SDS-PAGE suggested that the native enzyme exists as a monomeric protein of 44.7 kDA (De Carolis & De Luca 1993).

The strategy used to purify the 4-hydroxylase involved the development of a complementary five step protocol which minimized the time required to purify this labile enzyme and diminished the probability for protein denaturation, modification and degradation. However, in several purifications, the typical SDS-PAGE protein profile at the Mono Q step showed a second 40.2 kDa protein which coeluted with the purified 4-hydroxylase (Fig. 3). To examine the similarities between the 4-hydroxylase (44.7 kDa) and the 40.2 kDa both proteins were subjected to protease digestion by the procedure of Cleveland et al. (1977). Proteolysis by trypsin gave identical peptide patterns for the 4-hydroxylase and the 40.2 kDa protein as observed by SDS-PAGE (Fig. 4, lanes A,B). Trypsin, which cleaves preferentially to the carboxy side of basic amino acids gave peptide fragments of 25.8 kDa, 19.2 kDa, 16.6 kDa and 13.6 kDa. Endoproteinase, which cleaves specifically at the carboxyl end of lysine also yielded identical peptide fragments of 25.0 kDa and 20.0 kDa and a minor band of 14.4 kDa for the two proteins (Fig. 4 lanes E,F).

An important factor responsible for the purification of the *C. roseus* 4-hydroxylase was the reactivation and subsequent stabilization of enzyme activity observed when incubating it with ferrous ions. Initially, we found that the enzyme was inactivated after several steps of purification. Studies in our laboratory have shown that Fe^{2+} had little effect on the activity of

Table 1. Purification of desacetoxyvindoline 4-hydroxylase

Step	Total protein mg	Specific activity pkatal/mg	Total activity pkatal	Purification x-old	Recovery %
Crude	1955	0.043	83.5	-	100
Sephadex G-100	100	1.46	146.6	34	176
Green 19-agarose	4.3	15.85	67.4	371	81
Hydroxyapatite	1.4	24.57	34.3	575	41
α-kg Sepharose	0.188	56.83	10.7	1331	12.8
Mono-Q	0.015	86.15	1.3	2018	1.6

One katal of desacetoxyvindoline 4-hydroxylase is defined as the amount of enzyme that catalyzes the conversion of one mole of substrate per second using the direct assay method.

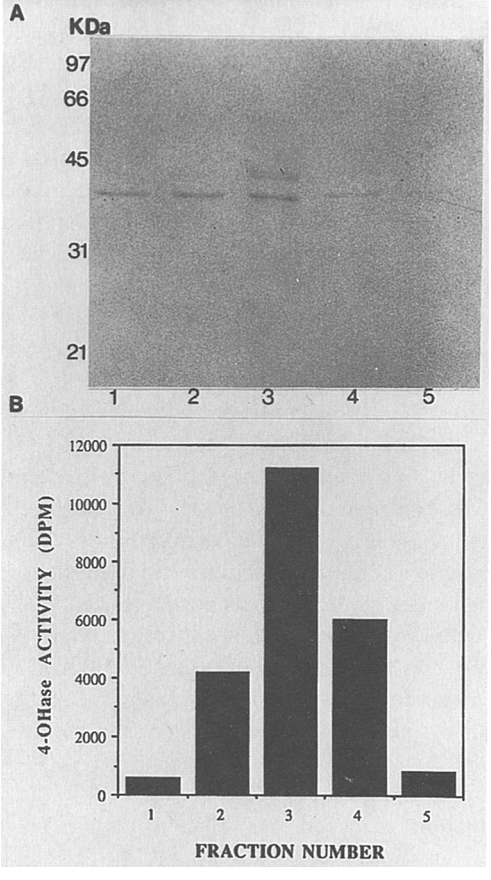


Fig. 3. SDS-PAGE of fractions collected during Mono Q chromatography step. (*A*), protein profile of a Mono Q whereby the 40.2 kDA protein co-eluted with the 4-hydroxylase. (*B*), the 4-hydroxylase activity profile of the Mono Q illustrating the 44.7 kDA band peaked in staining intensity in the fraction containing peak activity. The molecular mass markers are indicated on the left in kDA.

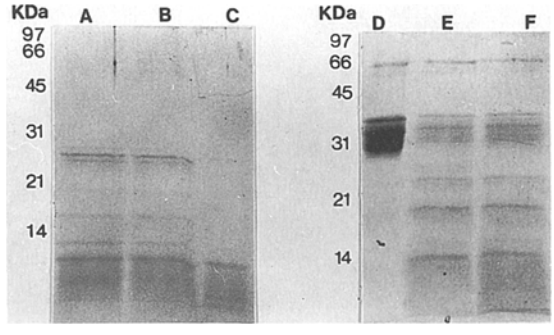


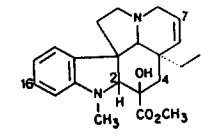
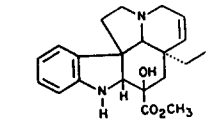
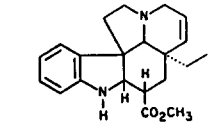
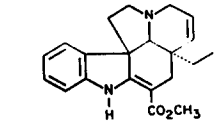
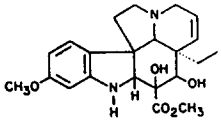
Fig. 4. Peptide maps of the 44.7 kDA and 40.2 kDA proteins. The 44.7 kDA and 40.2 kDA, each at *ca.* 5 μg in sample buffer were incubated at 37 °C for 45 min with 5 μg of protease. *Lanes A and B*, digestion of 44.7 kDA and 40.2 kDA, respectively with trypsin. *Lane C*, trypsin incubated at 37 °C for 45 min. *Lane D*, endoproteinase incubated at 37 °C for 45 min. *Lanes E and F*, digestion of 44.7 kDA and 40.2 kDA, respectively with endoproteinase. The molecular mass markers are indicated on the left in kDA.

partially purified enzyme (De Carolis et al. 1990), but experiments with the highly purified preparation have shown an absolute requirement for Fe^{2+} (De Carolis & De Luca 1993). Addition of ascorbate to the purified 4-hydroxylase did not reactivate the enzyme, whereas addition of Fe^{2+} increased the enzyme activity by 3.8-fold. Furthermore, reactivation of the enzyme by Fe^{2+} was time-dependent. This suggests that the enzyme loses Fe^{2+} during purification.

Substrate specificity

The desacetoxyvindoline 4-hydroxylase exhibited a strict specificity for position 4 of various indole alkaloid substrates. The most efficient substrates were in the following descending order: [1] > [3] > [2] > [4, 5] (Table 2). Substrates [2] and [3] which lacked the *N*-methyl and both the *N*-methyl and 3-hydroxyl group, respectively were poor substrates. Substrate [4] and

Table 2. Substrate specificity of *C. roseus* 4-hydroxylase.

Substrate[a]	Relative activity
[1] 	100
[2] 	0.6
[3] 	20
[4] 	0
[5] 	0

[a] Substrate specificity studies of the 4-hydroxylase were conducted using the standard assay for dioxygenases (Rhoads & Udenfriend 1968). The assay contained 9.2 μM [1-^{14}C]α-kg, 5 μM of unlabelled alkaloid, 7.5 μM ascorbate, 0.5 mg catalase, and up to 0.3 mg of protein in a final volume of 1 ml. The release of $^{14}CO_2$ from [1-^{14}C]α-kg is characteristic of 2-oxoglutarate-dependent dioxygenase activity.

[1] 2,3-dihydro-3-hydroxy-*N*(1)-methyltabersonine
[2] 2,3-dihydro-3-hydroxytabersonine
[3] 2,3-dihydrotabersonine
[4] tabersonine
[5] 16-*O*-methyl-2,3-dihydro-3,4-dihydroxytabersonine

[5] exhibited no 4-hydroxylase activity. These results are consistent with the natural occurrence of vindoline in young leaves of *C. roseus* and suggest that desacetoxyvindoline 4-hydroxylase is involved in the second last step in vindoline biosynthesis.

Kinetic analysis

The reaction mechanism and kinetic parameters for desacetoxyvindoline 4-hydroxylase have been elucidated. The intersecting initial velocity patterns observed for all possible substrates (α-kg, O_2 and desacetoxyvindoline) (De Carolis & De Luca 1993) suggest that the binding of these substrates to the 4-hydroxylase occurs by a sequential mechanism (Cleland 1967). The order of binding of O_2 and desacetoxyvindoline was determined by studying the effect on the initial velocity plots at limiting and saturating O_2 concentrations. When O_2 was limiting and desacetoxyvindoline was the fixed substrate the initial velocity pattern gave intersecting lines. However, at saturating O_2 concentrations a plot of α-kg as the variable substrate at changing fixed concentrations of desacetoxyvindoline yielded parallel lines. These differences in initial velocity patterns suggest that O_2 is the second substrate to bind followed by desacetoxyvindoline (Cleland 1967). The first product to be released is deacetylvindoline since noncompetitive inhibition was observed with all three substrates (De Carolis & De Luca 1993). The third product to be released should give competitive inhibition with respect to α-kg and noncompetitive inhibition with O_2 and desacetoxyvindoline. Succinate was the only product which gave this set of patterns and consequently should be the third product to be released. The present results would suggest that α-kg is the first substrate to bind followed by O_2 and desacetoxyvindoline while deacetylvindoline is the first product released followed by CO_2 and succinate. The data is consistent with an Ordered Ter Ter mechanism.

The K_m values for α-kg and O_2 were identical (45 μM). The high affinity of the 4-hydroxylase for desacetoxyvindoline (0.03 μM) may reflect the low concentration of these metabolites inside the cell.

The kinetic mechanism proposed here is in agreement with those of mammalian (Myllyla et al. 1977, Puistola et al. 1980) as well as bacterial (Holme 1975) 2-oxoglutarate-dependent dioxygenases and suggests that this is a general feature of this class of enzyme.

Conclusions

The development of the purification protocol of desacetoxyvindoline 4-hydroxylase has supplied the necessary tools for the molecular cloning of the 4-hydroxylase gene. This clone will be used to study the regulation of the 4-hydroxylase activity in *C. roseus* in response development-specific cues and to environmental stimuli such as light. In addition, the availability of this clone will permit overexpression of this gene in the intact plant and in tissue cultures.

Acknowledgements

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