Purification, Characterization, and Kinetic Analysis of a 2-Oxoglutarate-dependent Dioxygenase Involved in Vindoline Biosynthesis from Catharanthus roseus*

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A 2-oxoglutarate-dependent dioxygenase (EC 1.14.11.11) which catalyzes the hydroxylation at position 4 of the indole alkaloid, desacetoxyvindoline has been purified to near homogeneity from Catharanthus roseus. The purification procedure combined conventional chromatographic methods and cosubstrate affinity chromatography on α-ketoglutarate-Sepharose. The specific activity of the 4-hydroxylase was enriched over 2000-fold compared to the crude homogenate with a recovery of 1.6%. The molecular mass of the native and denatured 4-hydroxylase was found to be 45 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 pl values 4.6, 4.7, and 4.8. The enzyme did not require most divalent cations, but inactive enzyme was reactivated in a time-dependent manner by incubation with ferrous ions.

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 2-oxoglutarate is the first substrate to bind followed by the binding of O2 and desacetoxyvindoline. The first product to be released was deacetyl vindoline followed by CO2 and succinate, respectively.

Alkaloids, in general, may be defined as structurally complex organic bases which contain nitrogen and are formed in plants and fungi. In addition, alkaloids comprise a variety of important classes of drugs that have widespread experimental and therapeutic application. The Vinca alkaloids represent a class of natural drugs derived from the periwinkle plant, Catharanthus roseus. Two commercially important bis-indole alkaloids, vinblastine (VBL)1 and vincristine, are known to accumulate in the aerial parts of C. roseus (1). Vinblastine is typically used to treat Hodgkin's disease while vincristine is used in cases of acute leukemia (2). Since VBL and vincristine 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 (3). Unfortunately, this approach has not yet succeeded in producing bis-indole alkaloids since cell cultures are unable to synthesize vindoline, one of the monomeric precursors of VBL and vincristine (4).

To gain an understanding of why vindoline biosynthesis is repressed in cell cultures, studies have been performed on intact C. roseus plants with particular interest in the isolation of intermediates and enzymes involved in this pathway. Recent studies have indicated that key enzymes involved in vindoline biosynthesis are under strict developmental regulation (3). 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 to last step in vindoline biosynthesis is catalyzed by N-1-desmethyldesacetoxyvindoline (16-methoxy-2,3-dihydro-3-hydroxytabersonine)2 N-methyltransferase which has been partially purified (5) and is localized in the chloroplast (6). The last step in vindoline biosynthesis is catalyzed by deacetylvinindoline 4-O-acetyltransferase which recently has been purified to homogeneity (7). The enzyme which catalyzes the second to last step in vindoline biosynthesis requires indole alkaloid substrate, 2-oxoglutarate, ascorbate, ferrous ions, and molecular oxygen for activity and thus is classified as a 2-oxoglutarate-dependent dioxygenase (8). 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 highly purified enzyme preparation as well as having a direct enzyme assay (8) which is simple, fast, and accurate we attempted to elucidate the kinetic mechanism of desacetoxyvindoline 4-hydroxylase.

This report describes the purification to near homogeneity and a detailed kinetic analysis of a 2-oxoglutarate-dependent dioxygenase involved in vindoline biosynthesis from C. roseus.

EXPERIMENTAL PROCEDURES

Plant Material

The C. roseus plants used in this report were grown under greenhouse conditions. Terminal buds and the first two pairs of fully expanded leaves were used for enzyme extraction.

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Desacetoxyvindoline 4-Hydroxylase from C. roseus

**Chemicals**

S-Adenosyl-L-[methyl-14C]methionine and [1-14C]2-oxoglutaric acid were purchased from Amersham (Mississauga, ON). Sephadex G-25, Sephadex G-100, AH-Sepharose 4B, Mono Q HR 5/5, Sephacryl S-75 HR 10/30, and the FPLC System were all purchased from Pharmacia (Montreal, PQ). Green 19-agarose, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide, ferrous sulfate, and ascorbate were from Sigma. The Mini-Protean II dual slab cell, 2-D cell, SDS-PAGE, 2-D SDS-PAGE standards, hydroxylapatite, and the protein dye reagent were purchased from Bio-Rad (Mississauga, ON). Unlabeled 2-oxoglutarate and a-ketoglutaric acid were from Boehringer Mannheim (Montreal, PQ). All indole alkaloids used in this study were from our laboratory collection. These compounds were synthesized by previously described methods and their identity confirmed by spectroscopic techniques (9). All other chemicals were of analytical grade.

**Buffers**

The following buffers were used: 200 mM Tris-HCl, pH 7.5, containing 10 mM dithiothreitol and 5 mM EDTA (A); 50 mM Tris-HCl, pH 7.5, containing 28 mM 2-mercaptoethanol (B); 10 mM sodium phosphate, pH 6.8, containing 28 mM 2-mercaptoethanol (C).

**Desacetoxyvindoline 4-Hydroxylase Assay**—The direct enzyme assay developed for the 2-oxoglutarate-dependent dioxygenase in C. roseus (8) contained 0.56 nmol of labeled indole alkaloid substrate (containing 44,600 disintegration/minute), 10 mM 2-oxoglutarate, 7.5 mM ascorbate, and the protein dye reagent were purchased from Sigma. The Mini-Protean II dual slab cell, 2-D cell, SDS-PAGE, 2-D SDS-PAGE standards, hydroxylapatite, and the protein dye reagent were purchased from Bio-Rad (Mississauga, ON). Unlabeled 2-oxoglutarate and a-ketoglutaric acid were from Boehringer Mannheim (Montreal, PQ). All indole alkaloids used in this study were from our laboratory collection. These compounds were synthesized by previously described methods and their identity confirmed by spectroscopic techniques (9). All other chemicals were of analytical grade.

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**Desacetoxyvindoline 4-Hydroxylase Assay**—The direct enzyme assay developed for the 2-oxoglutarate-dependent dioxygenase in C. roseus (8) contained 0.56 nmol of labeled indole alkaloid substrate (containing 44,600 disintegration/minute), 10 mM 2-oxoglutarate, 7.5 mM ascorbate, and up to 150 μg of protein in a final volume of 200 μl. The reaction was started by addition of the enzyme protein and was 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 500 μl of ethyl acetate. The two phases were separated by centrifugation, and the organic layer was recovered. The organic layer which contained the 4-hydroxylated product as well as the unreacted substrate was evaporated to dryness. The residue was dissolved in 10 μl of methanol and was separated by thin layer chromatography (silica) using 10% methanol in ethyl acetate as the solvent, in order to separate the product (RF 0.22) and substrate (RF 0.52). After chromatography, the labeled product was isolated on the silica plate and removed. The amount of product formed was based on the radioactivity recovered from the silica plate. The radioactivity was determined by liquid scintillation counting in Optiphase Hisafe II (LKB) scintillation fluid.

The 4-hydroxylase was also assayed according to a previously described method (10). Briefly, the assay contained 9.2 μM [1-14C]2-oxoglutarate with a specific activity of 50 mCi/mmol, 5 μM unlabeled indole alkaloid substrate, 7.5 mM ascorbic acid, 0.5 mg of catalase, and up to 0.3 mg of enzyme protein (50 mM Tris-HCl, pH 7.5, containing 28 mM 2-mercaptoethanol) in a final volume of 1 ml. The enzyme reaction was started by the addition of enzyme protein, and the mixture was incubated for 60 min at 30 °C. The reaction was stopped with the addition of 100 μl of 6 M HCl, and the formation of 2,3-dihydro-3,4-dihydroxy-N(1)-methyltabersonine was based on the decarboxylation of [1-14C]2-oxoglutarate leading to the formation of succinate and the liberation of 14CO₂.

**Variation in Oxygen Concentration**

The Reacti-Vials containing active 4-hydroxylase were sealed with Teflon/silicone discs (22 mm) and placed on ice. The vials were then flushed with N₂ for 30 min prior to the addition of known concentrations of the substrates which were further flushed with N₂ for another 15 min. The appropriate O₂ concentration introduced into each vial was based on the percent by volume in the incubation atmosphere. Once O₂ was added to the reaction mixture, the vials were incubated in a water bath for 30 min at 30 °C. The reaction was stopped with the addition of 100 μl of 1 M NaOH into the vials, and the 4-
hydroxylated derivative was recovered as mentioned in the direct assay method. The $K_r$ for $O_2$ is expressed as micromolar concentration on the basis of the known solubility of $O_2$ at 30 °C. The accuracy of $O_2$ concentration introduction into the incubation atmosphere was verified by taking a sample of the incubation atmosphere from controls and monitoring 20% using a Thermal Conductivity Detector on a gas chromatograph (Hewlett-Packard 5890 II Chromatograph).

Preparation of $\alpha$-Ketoglutarate-Sepharose

The $N,N'$-disubstituted carbodiimides promote the condensation between a free amino and a free carboxyl group to form a peptide link. Thus, AH-Sepharose 4B, which contains free amino groups was cupped randomly with the carboxyl groups of the ligand, $\alpha$-ketoglutaric acid (11). Briefly, all operations in the carbodiimide coupling of $\alpha$-ketoglutaric acid were performed at room temperature. Freeze-dried AH-Sepharose 4B (2 g) was allowed to swell in an excess of 0.5 M NaCl (200 ml/g) for a minimum of 15 min. The gel was washed with 0.5 M NaCl (400 ml) to remove additives originally present with the AH-Sepharose 4B powder. The NaCl was then removed by washing the gel with distilled water (adjusted to pH 4.5). The ligand, $\alpha$-ketoglutaric acid ($\alpha$-Kg), (170 mg) was dissolved in 5 ml of distilled water (adjusted to pH 4.6) and added to the swollen gel. The suspension was gently stirred, and the pH was maintained at 4.5. Carbodiimide hydrochloride (1.4 g) was dissolved in 5 ml of distilled water (adjusted to pH 4.5) and added to the suspension over a 1-h period with continuous stirring at pH 4.5. The reaction was allowed to proceed for 24 h. The gel was washed with 400 ml of distilled water, pH 4.5, followed by equilibration with the appropriate buffer and packed into a column. The affinity chromatography on $\alpha$-Kg-Sepharose and on a $\alpha$-Kg-Sepharose column by applying a linear salt gradient (500 ml) of 0.0–1.0 M NaCl in buffer B. Fractions of 4.0 ml were collected and assayed for 4-hydroxylase activity using the direct assay method.

The 4-hydroxylase peak activity eluted between 0.49 and 0.56 M NaCl in buffer B. Fractions of 2.5 ml were collected and assayed for 4-hydroxylase activity using the direct assay method. The active 4-hydroxylase eluted at 165 mM phosphate.

Chromatography on Hydroxylapatite—The green 19-agarose-purified fractions exhibiting 4-hydroxylase activity were chromatographed on a hydroxylapatite column (1.6 × 10.5 cm) which had been previously equilibrated in buffer C. The column was washed with buffer C, and the bound 4-hydroxylase was eluted with a linear gradient (50 ml) of 0.0–1.0 M sodium phosphate (pH 9.0). Fractions of 2.5 ml were collected and assayed for 4-hydroxylase activity using the direct assay method. The 4-hydroxylase eluted at 165 mM phosphate.

Chromatography on Mono Q—Fractions containing 4-hydroxylase activity which eluted from the $\alpha$-Kg-Sepharose were pooled and chromatographed on a Mono Q column previously equilibrated in buffer C. This preparation still contained minor contaminants, and the 4-hydroxylase was further purified by subjecting it to high performance ion-exchange chromatography on Mono Q HR 5/5 column. The protein was applied to the column which had been previously equilibrated in buffer C and the first two pairs of fully expanded leaves (~225 g) were mixed with polyvinylpyrrolidone (10% w/w) and homogenized in a Waring blender for 1 min at maximum speed containing ice-cold buffer A (1.3 w/v). The homogenate was filtered through cheesecloth, and the filtrate was centrifuged at 20,000 × g for 15 min. The filtrate was fractionated with solid ammonium sulfate, and the protein was precipitated between 35 and 75% salt saturation was collected by centrifugation.

RESULTS

Purification of Desacetoxypodophyline 4-Hydroxylase—The desacetoxypodophylne 4-hydroxylase was purified from C. roseus by ammonium sulfate precipitation and successive chromatography on Sephadex G-100, green 19-agarose, hydroxylapatite, affinity chromatography on $\alpha$-Kg-Sepharose and on a Mono Q column using the FPLC system. A summary of the 4-hydroxylase purification protocol is shown in Table I. An average of 2,000-fold increase in specific activity could be obtained with a recovery of 1.6%. The final preparation had a specific activity of 86.15 picokatalas/mg protein. The typical
**TABLE I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Specific activity Picokatal/mg</th>
<th>Total activity (fold)</th>
<th>Purification Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
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<td>0.043</td>
<td>83.5</td>
<td>100</td>
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<tr>
<td>Sephadex G-100</td>
<td>100</td>
<td>1.46</td>
<td>146.6</td>
<td>34</td>
</tr>
<tr>
<td>Green 19-agarose</td>
<td>4.3</td>
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<td>67.4</td>
<td>371</td>
</tr>
<tr>
<td>Hydropyrolatite</td>
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<td>24.57</td>
<td>34.3</td>
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</tr>
<tr>
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<td>56.83</td>
<td>10.7</td>
<td>1331</td>
</tr>
<tr>
<td>Mono-Q</td>
<td>0.015</td>
<td>86.15</td>
<td>1.3</td>
<td>2018</td>
</tr>
</tbody>
</table>

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

Coomassie Blue-staining pattern obtained at different stages of purification is illustrated in Fig. 2. The Mono Q-purified 4-hydroxylase migrated as a single band with an apparent molecular weight of 44,700 as observed on SDS-PAGE. To further demonstrate that the protein band observed on SDS-PAGE corresponded to the 4-hydroxylase, the Mono Q-purified enzyme was submitted to gel filtration on a Superdex-75 column. The fraction containing maximum 4-hydroxylase activity corresponded to a protein with a molecular weight of 45,000 suggesting that the native enzyme exists as a monomer.

**High Resolution Two-dimensional Polyacrylamide Gel Electrophoresis**—The Mono Q-purified 4-hydroxylase preparation was subjected to two-dimensional polyacrylamide electrophoresis according to the method of O’Farrell (14) using isoelectric focusing tube gels with a pH gradient of 3–10. Three protein charge isoforms were visualized after SDS-PAGE and their corresponding isoelectric points were determined from a standard curve. Two major protein charge isoforms were observed with pI values of 4.7 and 4.8 followed by a minor one with a pI of 4.6 (Fig. 3).

**Requirements for Fe$^{3+}$**—During the purification of desacetoxyvindoline 4-hydroxylase, we found that its activity was rapidly lost after the dye-ligand affinity chromatography step. The addition of glycerol (up to 10%), a reagent known to stabilize enzymatic activity (15), did not maintain 4-hydroxylase activity. However, the enzyme was completely reactivated by preincubating the enzyme with all its cofactors (Table II). The results also suggest that Fe$^{3+}$ is primarily responsible for the reactivation of the 4-hydroxylase. The reactivation observed by the addition of Fe$^{3+}$ (8.5 μM) alone (Table II) is time-dependent, and maximal activity is obtained after incubation for 3 h at 4 °C (data not shown).

The 4-hydroxylase did not require other divalent cations (Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$) for activity nor could they replace Fe$^{3+}$. In addition, the enzyme was not inhibited by the sulfhydryl group reagents iodoacetamide and N-ethylmaleimide when present at concentrations of 0.1–10 mM.

**Kinetic Analysis**—The kinetic analysis was performed using purified 4-hydroxylase in Tris·HCl containing 28 mM 2-mercaptoethanol, pH 7.5. The enzyme assays used in the kinetic studies were linear with time and enzyme concentration under all conditions tested. Furthermore, the direct enzyme assay established for desacetoxyvindoline 4-hydroxylase (8) was used to monitor the formation of the 4-hydroxylated derivative. In experiments where there was any doubt about the pattern in the reciprocal plot, it was repeated several times until a consistent pattern was established.

**Initial Velocity Patterns**—Detailed kinetic studies were carried out by varying the concentration of one substrate in the presence of different fixed concentrations of the second substrate, while the concentration of the third was held constant. Double-reciprocal plots with desacetoxyvindoline as the vari-

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**FIG. 2.** SDS-PAGE of fractions exhibiting 4-hydroxylase activity at different stages of purification. Lane A, green 19-agarose (~10 μg); lane B, hydroxylatite (~10 μg); lane C, α-ketoglu-tarate-Sepharose (~3 μg); lane D, Mono Q (~2 μg). The molecular mass markers are indicated on the left in kDa.

**FIG. 3.** Two-dimensional electrophoresis of desacetoxyvindoline 4-hydroxylase. The 2-D electrophoretic pattern of the purified 4-hydroxylase (~2 μg) was carried out in the first dimension (3–10 pH range ampholites) and SDS-PAGE in the second dimension (15% acrylamide). The molecular mass markers are indicated on the left in kDa and the apparent isoelectric points on the top. The pI values of the respective 4-hydroxylase protein spots are referred to under “Results.”

**TABLE II**

<table>
<thead>
<tr>
<th>Incubation mixture$^a$</th>
<th>Relative activity</th>
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<td>Control$^d$</td>
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<tr>
<td>+α-KG</td>
<td>1.5</td>
</tr>
<tr>
<td>+α-KG and ascorbate</td>
<td>1.3</td>
</tr>
<tr>
<td>+α-KG and Fe$^{3+}$</td>
<td>3.6</td>
</tr>
<tr>
<td>+α-KG, ascorbate, and Fe$^{3+}$</td>
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</tr>
<tr>
<td>+Ascorbate</td>
<td>1.3</td>
</tr>
<tr>
<td>+Ascorbate and Fe$^{3+}$</td>
<td>3.2</td>
</tr>
<tr>
<td>+Fe$^{3+}$</td>
<td>3.8</td>
</tr>
</tbody>
</table>

$^a$ The control consists of green 19-agarose-purified 4-hydroxylase in buffer B which had a specific activity of 10.2 pkat/mg (relative activity = 1).

$^b$ The concentration of α-KG, ascorbate, and Fe$^{3+}$ were 100, 250, and 8.5 μM, respectively.

$^c$ Incubation mixture consisted of adding the reagents and incubating for 3 h at 4 °C after which the mixture was assayed for 4-hydroxylase activity.

$^d$ The direct enzyme assay was used.
able substrate at several fixed concentrations of α-ketoglutarate saturated with a constant $O_2$ concentration (in air) gave an intersecting pattern (Fig. 4A). When α-ketoglutarate was plotted as the variable substrate with $O_2$ as the changing fixed substrate the same type of pattern was obtained (Fig. 4B). Similar intersecting lines were also obtained when $O_2$ concentration (11, 40, and 55 μM) was limiting at different fixed concentrations of desacetoxyvindoline (Fig. 4C). However, parallel lines were obtained when α-ketoglutarate was the variable substrate at fixed concentrations of desacetoxyvindoline with saturating $O_2$ (960 μM) concentration (Fig. 4D).

As calculated from the replots, the $V_{max}$ for the conversion of desacetoxyvindoline to deacetylvindoline was 3.85 picokatal/mg protein. The $K_m$ values for 2-oxoglutarate, $O_2$, and desacetoxyvindoline were found to be 45.0, 45.0, and 0.03 μM, respectively (Table III). In addition, the $K_m$ values for ascorbate and Fe$^{2+}$ were found to be 0.2 mM and 8.5 μM, respectively.

**Product Inhibition Patterns**—The order of substrate binding and product release was further determined from the product inhibition studies. Succinate was found to be a competitive inhibitor with respect to 2-oxoglutarate (Fig. 5A) but noncompetitive with respect to desacetoxyvindoline (data not shown). Deacetylvindoline was a noncompetitive inhibitor with respect to 2-oxoglutarate (Fig. 5B), $O_2$ (Fig. 5C), and desacetoxyvindoline (Fig. 5D). The $K_i$ values for deacetylvindoline and succinate were 115 μM and 9 mM, respectively (Table IV) whereas the inhibitor concentration of $CO_2$ required for 50% inhibition [$CO_2]_{50}$ was found to be 7.5 mM. The high levels of $CO_2$ required to inhibit activity made it impossible to perform further kinetic studies.

**DISCUSSION**

The desacetoxyvindoline 4-hydroxylase of *C. roseus* has been purified to near homogeneity using a five-step purification procedure. The protocol described here yielded an overall purification of 2000-fold with a recovery of 1.6%. The pure enzyme had a specific activity of 86.15 picokatal/mg protein. The key features of this purification protocol involved the utilization of green Wagarose and 2-oxoglutarate cosubstrate affinity chromatography on α-kg-Sepharose. The 4-hydroxylase bound tightly to green 19-agarose since salt concentrations below 0.45 M NaCl, a sufficient concentration to elute most proteins from the dye-ligand, did not elute the enzyme.
The separation on green 19-agarose resulted in a 371-fold purification, a 10-fold increase over the previous step with a typical recovery of 80–85%. This was followed by the immobilization of the 4-hydroxylase to an α-kg-Sepharose affinity column and selective elution with a 2-oxoglutarate gradient.

With this affinity step, a 2–1330-fold purification was achieved and the 4-hydroxylase preparation contained several minor contaminants, as observed by SDS-PAGE. Based on the initial 4-hydroxylase activity of the crude preparation, the enzyme constitutes ~0.05% of the soluble protein. Due to the low abundance of the enzyme in  C. roseus, the establishment of an affinity chromatography step specific for the 4-hydroxylase was essential. A similar affinity chromatography strategy using coenzyme A as the affinity ligand has been used to purify to homogeneity the equally low abundant (~0.03%) 2-oxoglutarate-dependent dioxygenase involved in vindoline biosynthesis from C. roseus (7, 16).

Other 2-oxoglutarate-dependent dioxygenases have been purified. The prolyl 4-hydroxylase from chick embryos has been purified by linking a peptide substrate with a high affinity for the enzyme to agarose and specifically eluting the enzyme with a second peptide substrate (17). The dioxygenases involved in scopolamine (18) and cephalosporin (19) synthesis are involved in vindoline biosynthesis from  C. roseus (7, 16).

TABLE III

<table>
<thead>
<tr>
<th>Varied ligand</th>
<th>Changing fixed ligand</th>
<th>Constant ligand</th>
<th>Kinetic pattern</th>
<th>Kinetic parameter µM</th>
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<td>K₄ 45.0</td>
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<tr>
<td>α-kg</td>
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<td>α-kg</td>
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<tr>
<td>O₂(³⁻)³⁻</td>
<td>DAV</td>
<td>α-kg</td>
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<tr>
<td>α-kg</td>
<td>DAV</td>
<td>O₂(³⁻)³⁻</td>
<td>Parallel</td>
<td></td>
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</table>

*a The replots from the substrate interaction kinetics were used to determine the numerical value of kinetic parameters.

*b The varying concentrations for O₂(³⁻)³⁻ were 11, 40, and 55 µM while the O₂(³⁻)³⁻ concentration was 960 µM.

The third product to be released should give an intersecting initial velocity pattern as observed (Fig. 4C). If the second substrate to bind is truly O₂, then the initial velocity plot of  α-ketoglutарат should yield a parallel pattern as observed (Fig. 4D). This suggests that the normally reversible reaction sequence from E to (EABC) becomes irreversible when saturating with O₂ and thus the  α-ketoglutарат (A)-desacetoxyvindoline (C) initial velocity pattern becomes parallel (28). As a result, the order of binding of O₂ and desacetoxyvindoline was determined by studying the effect on the initial velocity plots when limiting and saturating with O₂ concentrations. When O₂ concentration was limiting and desacetoxyvindoline was the fixed substrate the initial velocity pattern gave intersecting lines (Fig. 4C). If the second substrate to bind is truly O₂, then the initial velocity plot of  α-ketoglutарат as the variable substrate at changing fixed concentrations of desacetoxyvindoline and at saturating O₂ should yield a parallel pattern as observed (Fig. 4D). This suggests that the normally reversible reaction sequence from E to (EABC) becomes irreversible when saturating with O₂ and thus the  α-ketoglutарат (A)-desacetoxyvindoline (C) initial velocity pattern becomes parallel (28). As a result, the order of binding of substrates would have  α-ketoglutarat binding first, due to its competitive inhibition with succinate followed by the binding of O₂ and desacetoxyvindoline. The first product released was desacetylvinodine due to the non-competitive inhibition observed with  α-ketoglutarat, O₂, and desacetoxyvindoline. This suggestion would agree with a common feature of ordered mechanisms whereby the product of the last substrate to combine to the enzyme is the first to be released (29). The third product to be released should give competitive inhibition with respect to  α-ketoglutarat and noncompetitive with O₂ 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 desacetylvinodine, CO₂, and succinate are released in this order. The data discussed above are consistent with an Ordered Ter Ter mechanism (28) and are in agreement with the kinetic mechanism of prolyl 4-hydroxylase (25), lysyl hydroxylase (26), and thymine 7-hydroxylase (27) indicating that this is a general feature of 2-oxoglutarate-dependent dioxygenases. However, more complete studies on product inhibition will be necessary to verify the place in the reaction mechanism for Fe³⁺ and ascorbate,
FIG. 5. Product inhibition of desacetoxyvindoline 4-hydroxylase catalyzing the conversion of desacetoxyvindoline to deacetylvindoline. Inhibition of the 4-hydroxylase reaction by succinic acid with respect to 2-oxoglutarate (A). The inhibition of the 4-hydroxylase reaction by deacetylvindoline with respect to 2-oxoglutarate (B), O₂ (C), and desacetoxyvindoline (D). The deacetylvindoline concentration (A–C) was fixed at 0.25 μM. The α-kg concentration (C and D) was fixed at 100 μM. The concentrations of Fe²⁺ and ascorbate were 10 μM and 0.25 mM, respectively, while the O₂ concentration in air-saturated reaction mixtures was taken as 240 μM pkat. picokatals.

both considered not to be true substrates of 2-oxoglutarate-dependent dioxygenase reactions. Prolyl 4-hydroxylase studies have suggested that Fe²⁺ is the first substrate to bind in thermodynamic equilibrium while ascorbate appears to bind before the other substrates or after the release of at least one product (24). We have observed that desacetoxyvindoline 4-hydroxylase becomes bound to the α-kg-Sepharose affinity column in the presence or absence of Fe²⁺ and ascorbate suggesting that 2-oxoglutarate is the first real substrate to bind to the enzyme.

The high affinity of the 4-hydroxylase for desacetoxyvindoline suggests that the metabolite is present in low concentration within the cell. A similar high affinity for plant metabolites has been observed by other 2-oxoglutarate-dependent dioxygenases involved in scopalamine (22), gibberellin (30), and flavanone (31) biosynthesis. Since the $K_p$ for deacetylvindoline is over 3800-fold greater than the $K_m$ for desacetoxyvindoline, this indicates that the hydroxylation step is not inhibited by the high concentration of product formed. Similar lack of product inhibition has been observed for the enzyme catalyzing the last step in vindoline biosynthesis (7).

The availability of a purified desacetoxyvindoline 4-hydrox-
The purified enzyme was submitted to tryptic digestion in order to obtain internal sequence information. Studies at the molecular level will be useful to study the developmental, environmental, and tissue-specific regulation of 4-hydroxylase gene expression in *C. roseus* seedlings (33) will be screened with these probes.

The production of vindoline from *C. roseus* tissue cultures remains a difficult task. The lack of vindoline biosynthesis in cell suspension cultures has been correlated with the lack of expression of the enzymes which catalyze the last three steps of this pathway. Studies at the molecular level will be useful to study the developmental, environmental, and tissue-specific regulation of 4-hydroxylase gene expression in *C. roseus*. Our ultimate goal is to understand the factors which regulate vindoline biosynthesis in *C. roseus* and consequently understand the factors which limit the production of the commercially important alkaloids, vinblastine, and vincristine.

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**REFERENCES**


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