Selective Inhibition of Proline Hydroxylation by 3,4-Dehydroproline

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ABSTRACT
The effect of proline analogs on peptidyl proline hydroxylation has been studied in vivo using aerated root slices of Daucus carota. One analog, 3,4-dehydroproline, acted at micromolar concentrations to rapidly and selectively inhibit peptidyl proline hydroxylation. A structurally altered hydroxyproline-rich cell wall glycoprotein was synthesized and secreted by dehydroproline-treated tissue. The capacity to hydroxylate proline recovered slowly following a short pulse treatment with the analog, with a halftime for recovery of about 24 hours. Recovery was not altered by supplying exogenous proline. Dehydroproline had little effect on the induction of nitrate reductase by nitrate, nor on wound-induced increases in amino acid uptake and protein synthesis. In contrast, other proline analogs inhibit proline hydroxylation only at millimolar concentrations. It is hypothesized that dehydroproline acts as an enzyme-activated suicide inhibitor of prolyl hydroxylase. This analog should become a useful tool for elucidating the functional significance of hydroxyproline-rich glycoproteins.

HRGPs are ubiquitous components of plant extracellular matrices, yet their physiological and developmental significance is poorly understood. The use of inhibitors of prolyl hydroxylation to produce structurally altered glycoproteins is one experimental approach to a better understanding of the possible roles of HRGPs. Specific inhibition is phenotypically analogous to site-specific mutagenesis of the prolyl hydroxylase gene. Inhibitor studies have indicated roles for the HRGPs in growth control (1, 6, 23, 28), disease resistance (8), and morphogenesis (2). Unfortunately, these experiments have relied on the use of inhibitors (α,α'-dipyridyl; cis- and trans-hydroxy-l-proline) which are not specific for prolyl hydroxylase. Thus, interpretation of the observations is difficult.

Aerated slices of carrot root synthesize large amounts of HRGPs (4). The salt-extractable wall HRGP has been purified and characterized (25, G. J. van Holst and J. E. Varner, unpublished). Its chemical composition closely resembles that of (a) bacterial agglutinins isolated from tobacco callus and potato tubers (18, 19), (b) cell wall proteins deposited during growth cessation in pea epicotyl and bean hypocotyl (16, 26), and (c) the nonextractable wall glycopeptides from cultured tomato cells (17). The soluble carrot glycoprotein is slowly insolubilized in the cell wall, probably through the oxidative formation of isodityrosine (7). Because as much as 30% of exogenously supplied proline is hydroxylated after incorporation into peptide linkage (5), the carrot system is ideal for studies on the biosynthesis of hydroxyproline. We have used this system to investigate the effects of several proline analogs on peptidyl proline hydroxylation, and to characterize the effects of the most potent of these analog inhibitors, 3,4-dehydroproline.

MATERIALS AND METHODS
Biochemicals. All reagents were from Sigma except: CsCl from Boehringer-Mannheim; sulfaniamide and N-(1-naphthyl)-ethylenediamine dihydrochloride from Eastman; diphenylazole (PPO), Triton X-100, and toluene from Research Products International (Mount Prospect, IL); and chromatography solvents from Fisher. Scintillation cocktail was made by mixing 2 L toluene, 1 L Triton X-100, 8 g PPO, and 0.2 g (1,4-bis[2-methyl-5-phenoxazolyl])benzene. Radiochemicals were obtained from New England Nuclear with the following specific activities: l-[2,3-3H]proline, 29.0 Ci/mmol; l-[5-3H]proline, 22.0 Ci/mmol; l-[4-3H]proline, 28.6 Ci/mmol; l-[3,4,5-3H]leucine, 110 Ci/mmol; and l-[ring-3H]tyrosine, 74.6 Ci/mmol.

Tissue Preparation. Large tap roots of Daucus carota were obtained from a local merchant and stored at 4°C until use. Sterile discs of phloem parenchyma tissue (7 mm diameter by 1.5 mm thick) were washed extensively with sterile water and aerated at 25°C in shaking Erlenmeyer flasks for at least 30 h to induce synthesis of HRGPs (5) (less than 5 g tissue in 50 ml water in each 500 ml flask, shaking at 120 cycles/min).

Hydroxylation Assays. Peptidyl proline hydroxylation was measured by the in vivo tritium-loss method of Varner and Burton (27). This method is based on the specific labilization of the 4-trans-hydrogen during proline hydroxylation. Using [4-3H]proline as a tracer, the labile tritium exchanges with H2O and the resulting [3H]OH can be collected by distillation. Specifically tritiated prolines were purified before use by TLC on silica gel plates (Analytech; Newark, DE) using CHCl3/methanol/NH3 (2/2/1). Two discs were incubated with [3H]proline at a final concentration of 1 μM in 1.0 ml of 50 mM K-phosphate (pH 6.0) in a 20-ml scintillation vial. Tritium loss data are expressed as follows: 'uptake' is the percentage of [3H]proline disappearing from the incubation medium during the 90-min experiment; 'incorporation' is the percentage of [3H]proline in the discs which remained insoluble in 80% ethanol and 'hydroxylation' is the percentage of the [3H]proline incorporated which was hydroxylated at the 4-trans position (measured by tritium loss from [4-3H]proline, assuming that 75% of the label is at the 4-trans position as reported by the manufacturer, and corrected for nonspecific tritium loss from [2,3-3H]proline or [5-3H]proline). Control experiments showed that about 85% of the [3H]proline was taken up by the discs and incorporated into protein in a 90-min period. Less than 1% of the tritium was lost from either [2,3-3H]proline or [5-3H]proline, while 15 to 20% of the tritium was lost from [4-trans-3H]proline. This specific tritium loss is a
good measure of peptidyl proline hydroxylation (25). Prolyl hydroxylation, as measured by this method, was completely blocked by 0.1 mM α,α'-dipyridyl (not shown), as has been shown by others (4).

**Cell Wall HRGP Isolation and Analysis.** The salt-extractable cell wall HRGP was prepared by the method of Stuart and Varner (25). Labeled root discs were homogenized with a mortar and pestle, and a cell wall fraction was isolated by centrifugation at 1000g for 3 min. This cell pellet was washed extensively with water by differential centrifugation (4°C, about 1 L/g fresh weight) before extraction with 0.5 M CaCl2 at 4°C. The labeled salt extracts were analyzed on isopycnic density gradients of CsCl. A CsCl solution (density 1.45 g/ml) containing labeled HRGP was centrifuged at 50,000 rpm for 48 h in a Beckman SW 65 rotor. Gradients were fractionated by dripping through a hollow needle puncture. The refractive index of an aliquot of every fifth fraction was measured with a Bausch and Lomb refractometer to calculate the density, and the fractions were counted.

**Nitrate Reductase Induction and Assay.** Two aged discs were incubated in 1.0 ml of 0.2 mM KNO3 for 6 h to induce synthesis of nitrate reductase. Activity of the enzyme was measured in situ by modifying the method of Ferrari and Varner (9). Two discs were vacuum infiltrated in 1.0 ml of 50 mM K-phosphate (pH 7.5), 0.3 mM KNO3, 1% ethanol, 0.1% Triton X-100, 0.1 mM KCl. 

**Fig. 1.** Prolyl hydroxylation inhibition by dehydropropoline. Loss of tritium from [4-3H]proline was measured by distillation in unextracted root discs (X) and in discs treated with 100 μM dehydro-D,L-proline at 0 min (O) or at 30 min (C). Uptake and incorporation of proline were unaffected by dehydropropoline treatment in these experiments. These data are averages of six experiments.

**Fig. 2.** Recovery following dehydropropoline treatment. Carrot discs were treated with 100 μM dehydro-D,L-proline for 40 min. The dehydropropoline was removed and proline uptake (O), incorporation into proteins (C), and hydroxylation (X) were measured at each time point with tritium-loss experiments. These data are averages of two experiments.

**Table I.** Effects of 3,4-Dehydro-D,L-Proline on Proline Metabolism

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Uptake</th>
<th>Incorporation</th>
<th>Hydroxylation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>86</td>
<td>86</td>
<td>16.9</td>
</tr>
<tr>
<td>0.5</td>
<td>87</td>
<td>85</td>
<td>13.1</td>
</tr>
<tr>
<td>5.0</td>
<td>75</td>
<td>86</td>
<td>5.2</td>
</tr>
<tr>
<td>50.0</td>
<td>66</td>
<td>65</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Antimycin A, and incubated for 60 min at room temperature. Nitrite produced during this period was assayed by mixing 1.0 ml of the incubation medium with 0.25 ml of 1% sulfanilamide in 1.5 N HCl and 0.25 ml of 0.1% of N-(1-naphthyl)ethylenediamine dihydrochloride and measuring the A at 540 nm.

**RESULTS AND DISCUSSION**

**Effect of 3,4-Dehydropropoline on Proline Hydroxylation.** The proline analog 3,4-dehydro-D,L-proline was an effective inhibitor of peptidyl proline hydroxylation at μM concentrations (Table I). A concentration of 5 μM inhibited hydroxylation by nearly 70% while only slightly affecting proline uptake and incorporation. Hydroxylation was inhibited 90% using 50 μM dehydropropoline although both uptake and incorporation were inhibited by about 24%. Similar results (not shown) were obtained in two experiments using one-half these concentrations of 3,4-dehydro-D,L-proline.

Dehydropropoline has been reported to be a potent in vivo inhibitor of prolyl hydroxylase in animal cells (13, 22) where only the i-isomer was recognized by the prolyl-α<sub>I</sub>-RNA synthetase and was active as an inhibitor of prolyl hydroxylation (14, 15). A decrease in specific activity of the purified enzyme was measured, with no change in the levels of hydroxylase antigen (21). Free dehydropropoline showed no inhibition of the enzyme activity in vitro, but dehydropropoline-containing precollagen peptides inac-
tivated the isolated enzyme by forming a stable peptide-enzyme complex (21, 22).

The inhibition observed in carrot discs was rapid; within 15 min peptideyl proline hydroxylation, as measured by differential tritium loss, was blocked by 100 μM dehydro-D,L-proline (Fig. 1). Dehydroproline was rapidly taken up and incorporated into proteins by mung bean and carrot cells (10; J. B. Cooper, unpublished). The inhibition kinetics observed in this study are similar to those observed inhibiting the purified animal hydroxylase with synthetic dehydroproline-containing peptides (21), and were much faster than those observed with animal cell systems (14).

The capacity to hydroxylate peptideyl proline recovered slowly following a pulse of 100 μM dehydroproline (Fig. 2). Twenty-four h following a 40-min pulse, proline hydroxylation had recovered by only about 50%. Proline uptake and incorporation recovered rapidly, indicating that the analog was not generally toxic. Recovery was not affected by a proline chase (Table II). Inasmuch as proline and proline analogs are rapidly incorporated into proteins by aerated carrot slices, our data support the hypothesis that dehydroproline permanently inactivates prolyl hydroxylase. Covalent binding could occur when the activated O2 radical at the active site (20) attacks the C-4 position of a dehydropropyl ring, generating a peptideyl-dehydroproline radical which could covalently attack the enzyme, binding the substrate analog to the active site. Experimental tests of this hypothesis using a purified prolyl hydroxylase are now under way. Assuming the enzyme remained inactivated until degradation, the half time for prolyl hydroxylase turnover can be estimated to be about 24 h in this tissue.

HRGP Synthesis following Dehydroproline Treatment. Dehydroproline-treated carrot tissue synthesized and secreted underhydroxylated, and thus underglycosylated, cell wall HRGP (Fig. 3). Salt-soluble wall proteins prepared from tyrosine-labeled root discs were fractionated on isopycnic CsCl density gradients. HRGP extracted from untreated discs banded with a buoyant density of about 1.44 g/ml (24, 25). At least 80% of the salt-extractable label was contained in this peak when discs were labeled with proline, tyrosine, lysine, or histidine (data not shown). Soluble wall proteins extracted from root discs labeled 6 h after hydroxylation inactivation (100 μM dehydro-D,L-proline for 40 min) had a decreased buoyant density (to about 1.34 g/ml) consistent with a decreased carbohydrate content. A similar shift in density of the cell wall HRGP was observed using α,α'-dipyridyl to inhibit prolyl hydroxylase (24), although dipyridyl was toxic to carrot cells in long term experiments.

The identity of the peak with decreased density was confirmed by a double labeling experiment using [3H]tyrosine and [14C]leucine. Due to its biased amino acid composition (25), the 3H/14C ratio of the carrot HRGP was enriched 10-fold in 3H over other proteins. The lower density peak labeled in carrot slices treated 6 h earlier with dehydroproline (Fig. 3) also had a 3H/14C ratio enriched 10-fold in 3H. The results of these experiments confirm the data presented in Table I and Figure 2, demonstrating that dehydroproline-treated carrot cells continue to synthesize and secrete structurally altered HRGP.

Specificity of Hydroxylase Inhibition by Dehydroproline. The rapid recovery of both proline uptake and incorporation following a pulse of dehydroproline (Fig. 2) indicated that this analog was a relatively specific inhibitor of peptideyl proline hydroxylation. Because the interpretation of all inhibitor experiments depends upon specificity, the effects of dehydroproline treatment on several activities not related to hydroxyproline biosynthesis have been examined.

Table IV. Effect of Dehydroproline on Nitrate-Induced Nitrate Reductase Activity

<table>
<thead>
<tr>
<th>Incubation Medium</th>
<th>Nitrate Reductase Activity</th>
<th>μmol/g fresh wt h</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>NO3−</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>NO3− + DHP</td>
<td>0.89</td>
<td></td>
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</tbody>
</table>

Aerated root discs were incubated with H2O, 0.2 mM KNO3, or 0.2 mM KNO3 + 100 μM 3,4-dehydro-D,L-proline. After 6 h, nitrate reductase activity was measured against nitrite standards. These results are the averages of four experiments.
INHIBITION OF PROLYL HYDROXYLASE

Table V. Effects of Other Proline Analogs on Proline Metabolism

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Uptake</th>
<th>Incorporation</th>
<th>Hydroxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>mm</td>
<td></td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>A. Hydroxyproline (isomers)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>0.0</td>
<td>65</td>
<td>85</td>
</tr>
<tr>
<td>trans</td>
<td>2.0</td>
<td>32</td>
<td>82</td>
</tr>
<tr>
<td>cis</td>
<td>0.5</td>
<td>48</td>
<td>80</td>
</tr>
<tr>
<td>B. Thioproline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>74</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>59</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>28</td>
<td>88</td>
</tr>
</tbody>
</table>

Slicing and aeration of plant storage tissue induces a variety of metabolic changes in addition to HRGP accumulation (12). Among these are large increases in membrane transport and protein synthesis. Carrot tissues aerated for 4 h had a limited capacity for amino acid uptake and incorporation into protein (Table III). Discs aerated for 24 h in the presence of dehydroproline developed nearly the same capacity for leucine and proline uptake as discs aerated in H₂O alone. Likewise, leucine and proline incorporation into proteins was affected only slightly by aeration in the presence of this analog.

De novo synthesis of nitrate reductase can be induced in many cells by exposure to nitrate (9, 29). The induced activity of this enzyme has been shown to be sensitive to the incorporation of amino acid analogs (3). While aerated carrot tissue contained some nitrate reductase activity, this activity was increased about 4-fold following a 6-h exposure to 0.2 m KNO₃ (Table IV). Dehydroproline, supplied to the tissue during this induction period, inhibited the increase in enzyme activity by only about 15%.

Effects of Other Proline Analogs on Proline Metabolism.

Proline uptake was inhibited by about 75% in root discs incubated with mM concentrations of either cis- or trans-hydroxyproline (Table V, A). These analogs had little effect on proline incorporation, while hydroxylation was inhibited 35% using the trans-isomer at 2 mM and about 50% using the same concentration of the cis-isomer. Both isomers when present at mM concentrations are incorporated into proteins directly (6, 11). Our results with root discs are in agreement with those of others who demonstrated that the cis-isomer was more active than the trans-inhibiting the conversion of [¹⁴C]proline to [¹⁴C]hydroxyproline in oat coleoptiles and pea roots (6, 28). The poor inhibition of hydroxylation observed in the present study might be due to a high endogenous proline concentration, since hydroxyproline is poorly recognized by the prolyl-tRNA synthetase (15) and cytoplasmic proline concentrations increase in many plant tissues following a variety of stresses, including wounding.

Millimolar concentrations of thioproline inhibited proline uptake to about the same extent as did hydroxyproline (Table V, B). Proline incorporation was unaffected, while hydroxylation was preferentially inhibited. About 50% of the peptide proline hydroxylation was blocked by 0.5 mM, and about 85% was blocked by 2 mM thioproline. These results differ from those with animal cells where thioproline was recognized by the prolyl-tRNA synthetase and incorporated into proteins, but did not inhibit hydroxylation (13, 15). Thus, thioproline may be useful for understanding the differences between the plant and animal prolyl hydroxylases.

Conclusions. The proline analog 3,4-dehydroproline is an active inhibitor of prolyl hydroxylase in vivo. The low concentration and brief treatment required for specific inhibition of hydroxylase activity offer many advantages over other hydroxylase inhibitors. Thus, dehydroproline should become a useful tool for understanding the physiological and developmental functions of HRGPs.

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