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HYDROXYPROLINE BIOSYNTHESIS IN PLANT CELLS

PEPTIDYL PROLINE HYDROXYLASE FROM CARROT DISKS*

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SUMMARY

Hydroxyproline occurs in many plant proteins and is especially abundant in the proteins associated with the primary cell wall. *In vivo* evidence indicates that the synthesis of peptidyl hydroxyproline occurs by the hydroxylation of peptidyl proline. We have shown that plant cells contain an enzyme which catalyzes this hydroxylation reaction. The hydroxylase has the following characteristics: it requires O_2, Fe^{2+}, ascorbate and an α-keto acid for activity; it does not react with free proline; it is localized in the soluble cytoplasm; it readily hydroxylates prolyl residues of protocollagen isolated from chick embryos. A partial purification of the enzyme was achieved by the use of $(NH_4)_2SO_4$ and $Ca_3(PO_4)_2$ gel fractionations.

INTRODUCTION

Hydroxyproline has been detected in the protein hydrolyzates of higher[1,2] and lower[3] plants. In almost all cases in which it has been found, the hydroxyproline exists in the 4-*trans*-L-configuration[4]. Unlike animal cells, where this imino acid has been found specifically in only two proteins, collagen and elastin[5], preliminary studies indicate that small amounts of it appear in a wide variety of plant proteins[6,7]. In plants most of the cellular hydroxyproline occurs in the protein component of the primary cell wall, where it accounts for as much as one-fifth of the amino acid residues and is part of a glycoprotein postulated to be involved in cell wall changes during cell enlargement[8].

Although other biochemical routes are available for its synthesis[9,10], *in vivo* experiments suggest that in plants hydroxyproline is synthesized *via* the hydroxylation of peptidyl proline; a similar situation exists in animal cells[11]. Experimental results from this laboratory[12] have confirmed previous data on other plant systems[13–15] that there is a lag time between the incorporation of radioactive proline into acid-

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insoluble material and the appearance in such material of radioactive hydroxyproline. In the case of the carrot-root disks used in the study reported here, this lag time is 3–4 min.

Previous data on hydroxyproline synthesis in plants are consistent with a proline hydroxylation mechanism similar to that found in animal cells[11]. O_2 is the source of the hydroxyl group oxygen in both plant[16] and animal[17] systems. By the use of [3,4-*trans*-$^3H_2$]proline, it was shown in sycamore cells[18] and also in chick embryos[19] that the 4-*trans* hydrogen atom of proline is displaced by the incoming hydroxyl group during proline hydroxylation. Also, the chelating agent α,α'-dipyridyl specifically inhibits proline hydroxylation and this inhibition can be reversed by excess exogenous Fe^{2+}, as was shown in chick embryos[20] and carrot-root disks[21]. The results of the present report demonstrate the existence of a peptidyl proline hydroxylase in plant cells. The properties of this enzyme suggest that it is involved in proline hydroxylation *in vivo*.

MATERIALS AND METHODS

[3,4-$^3H_2$]Proline was purchased from New England Nuclear with a specific activity of 5.26 mC/mmole. Miracloth is a product of Chicopee Mills, Inc., New York. Chloramphenicol (B grade) and α,α'-dipyridyl were purchased from Calbiochem. Bio-Gel HTP, a $Ca_3(PO_4)_2$ gel, was purchased from Calbiochem and suspended in buffer (20 mg/ml) at least 18 h before use. 2,5-Diphenyloxazole (PPO) was purchased from New England Nuclear and *p*-dioxane, scintillation grade, from Matheson, Coleman and Bell.

Experimental organism

We performed our experiments on disks (1 cm in diameter and 1 mm thick) of phloem parenchyma from the storage roots of carrots (*Daucus carota* cv.). When such disks are incubated, they undergo a large number of physiological and metabolic changes collectively termed "aging"[22–24]. Disks were incubated at 30° for 24 h in water containing 50 μg of chloramphenicol per ml to prevent bacterial growth[25]. Generally 10 ml of liquid was used per g fresh weight of tissue. We have shown that disks aged in this way actively synthesize cell wall protein rich in hydroxyproline[12].

Substrates

Proline-rich substrate was made by incubating plant or animal cells in α,α'-dipyridyl, which inhibits proline hydroxylation but has no effect on protein synthesis[21]. For plant substrate, aged disks were incubated for 7 min at 30° in $1 \cdot 10^{-4}$ M α,α'-dipyridyl. [3,4-$^3H_2$]Proline was added at 10 μC/5 g fresh weight of tissue and the incubation continued for a further 20 min. The medium was removed, the disks chilled and rinsed with $1 \cdot 10^{-3}$ M proline. All further operations were carried out at 4°. The disks were homogenized in an equal volume of 0.4 M sucrose in a Sorvall Omnimixer for 30 sec. After filtration under suction through Miracloth, the filtrate was centrifuged at 40 000 $\times$ *g* for 30 min. The resultant pellet was suspended in 4.0 ml of 5% trichloroacetic acid and the suspension intermittently mixed for 20 min. Precipitated proteins were removed by centrifugation at 30 000 $\times$ *g* for 30 min. The supernatant was then extracted 3 times with ether to remove trichloroacetic acid and the remain-

ing ether was removed by an air stream. After pH adjustment to 7.0, the trichloroacetic acid-soluble fraction was dialyzed overnight at 4° against 2 l of 0.01 M KCl–0.001 M Tris (pH 7.0). Prior to use, this substrate (designated MS) could be assayed for radioactivity by pouring an aliquot over a B-4 membrane filter (Carl Schleicher and Schuell Co.), to which it adheres[12]. The MS substrate lost activity in the hydroxylase assay after freezing or storage at 4° for longer than a week. We used it for assaying proline hydroxylation because 80% of the radioactive proline residues incorporated into it are normally hydroxylated and its kinetic properties suggest it is an important precursor of cell wall protein[12]. In the presence of dipyridyl, hydroxylation never exceeded 5% and was usually less than 1%.

For animal substrate, protocollagen was prepared from a modification of the procedures of TAKEUCHI AND PROCKOP[26]. Instead of [^{14}C]proline, we used [3,4-$^{3}H_2$]-proline at 300 μC/30 tibiae from 7–9-day-old chick embryos. The specific activity of the protocollagen thus prepared was usually $2 \cdot 10^6$ disint./min per mg. We found that it could be frozen up to 2 weeks with no loss in activity as a hydroxylase substrate but after this time its activity rapidly decayed. Normally, the protocollagen thus prepared has 24% of its proline residues hydroxylated; in the presence of dipyridyl, hydroxylation never exceeded 1%.

Enzyme

All operations were carried out at 4°. Aged carrot disks were homogenized in a Sorvall Omnimixer in 1–1.5 vol. of 0.1 M KCl–0.3 M sucrose–0.001 M dithiothreitol–0.005 M Tris (pH 7.0). The homogenate was filtered through Miracloth and the filtrate was centrifuged for 40 000 × *g* for 40 min. Generally, this resultant supernatant was centrifuged again to sediment more particulate matter at the same speed and for the same time. As the bulk of the enzyme activity was in the supernatant of this centrifugation (see RESULTS, Table IV), it was used for routine enzyme assays. Generally, this extract contained 4 mg protein per g fresh weight of tissue.

Partial purification of the hydroxylase activity was achieved as follows: The supernatant of the above 40 000 × *g* centrifugation was brought to pH 7.0. To it was added, at constant pH and over 2.5 h, 0.43 g/ml of $(NH_4)_2SO_4$ bringing the solution to 65% ammonium sulfate saturation. Precipitated proteins were then collected by centrifugation at 20 000 × *g* for 20 min and dissolved in 0.1 M KCl–0.001 M Tris (pH 7.0) to a concentration of 10 mg protein per ml. This solution was dialyzed overnight against 4 l of 0.1 M KCl–0.001 M Tris (pH 7.0). After removal of undissolved proteins by centrifugation at 5000 × *g* for 5 min, the remaining solution was brought to pH 6.4. Sufficient $Ca_3(PO_4)_2$ gel (suspended in 0.05 M KCl–0.001 M Tris (pH 6.3)) was added to achieve a gel to protein ratio of 2. The resulting suspension was gently stirred for 30 min and the gel then removed by centrifugation. The supernatant was again treated with a similar batch of gel. Both gel pellets were eluted with 5.0 ml of 0.2 M phosphate (pH 7.4) by suspension and stirring for 1 h. After the gels were removed, the eluates were dialyzed against 4 l of 0.1 M KCl–0.001 M Tris (pH 7.0) overnight. The results obtained in the gel step varied from one enzyme preparation to the next. The results given (Table V) are those of a typical experiment.

Hydroxylase assay

We used the procedure of HUTTON *et al.*[27] developed for collagen proline hy-

TABLE I

VALIDITY OF TRITIATED WATER ASSAY

Enzyme assays were performed and the reactions terminated by chilling and making the mixtures 10% with respect to trichloroacetic acid. Protein precipitates were collected after 2 h and the supernatants were vacuum distilled to purify the tritiated water (^{3}HHO) which was formed. The residues and protein precipitates were hydrolyzed in 6 M HCl by autoclaving in a sealed vessel for 90 min. [^{3}H]Hydroxyproline was determined for several pooled reaction samples. Details of procedures in MATERIALS AND METHODS.

Sample	*^{3}H disint./ min in ^{3}HHO*	*^{3}H disint./ min in hydroxyproline*
1	6 060	6 900
2	14 450	11 500
3	37 950	37 500

droxylase. From *in vivo* experiments it was known that in the formation of 4-*trans*-hydroxyproline in plants, the 4-*trans* hydrogen atom is displaced by the incoming hydroxyl group[18]. Thus, by using proline labeled with tritium in the 3 and 4-*trans* positions, we could measure the tritium released into water as equivalent to the [3-^{3}H]hydroxyproline formed (see RESULTS, Table I). The standard hydroxylating system contained: substrate; enzyme, 4–5 mg protein; $1 \cdot 10^{-3}$ M Fe^{2+} as ferrous ammonium sulfate, $1 \cdot 10^{-4}$ M α-ketoglutarate; $1 \cdot 10^{-3}$ M sodium ascorbate, 0.075 M Tris (pH 7.5), in a volume of 4–6 ml. After the reaction was run in a 25-ml flask at 37° with gentle shaking for 30 min, it was terminated by chilling and the addition of concentrated trichloroacetic acid to 8% final concentration. After 4 h, precipitated proteins were removed by centrifugation and tritiated water was purified and counted as described by HUTTON *et al.*[27]. Counting efficiency was usually 25–30% as determined by external standardization.

O_2 and N_2 were varied by flow rates. When the reaction was not run in air, the reactants were equilibrated separately with the new gaseous environment by 3 min bubbling and at least 30 min in a closed vessel under constant gas flow. The reaction itself proceeded in a closed container under continuous gas flow.

Determination of protein and [^{3}H]hydroxyproline

Protein was determined according to LOWRY *et al.*[28] with crystallized bovine albumin as a standard. [^{3}H]Hydroxyproline was determined according to JUVA AND PROCKOP[29]. This method involves the oxidation of hydroxyproline to pyrrole; we found that the efficiency of this conversion varied from 26 to 60%.

RESULTS

For the hydroxylase assay we used to be valid, the radioactivity released into water (the 4-*trans* tritium of [3,4-*trans*-$^3H_2$]proline) should correspond to that retained by the synthesized hydroxyproline (the 3-*trans* tritium). The samples of Table I each represent the pooled mixtures of several individual assays. The data indicate that the tritiated water formed can be used to measure plant peptidyl proline hydroxylase activity.

TABLE II

In vitro HYDROXYLATION OF PEPTIDYL PROLINE BY PLANT CELL EXTRACT

A crude cell extract from carrot-root disks (4.5 mg protein) was assayed for hydroxylating activity on MS substrate (50 000 disint./min peptidyl [3,4-$^3H_2$]proline) as described under MATERIALS AND METHODS. Results expressed as ^3H disint./min in water purified by vacuum distillation (see Table I).

Condition	*^3H disint./min in ^3HHO*
No extract	272
Extract present	1012, 964 (duplicates)
Pre-boiled extract (100°, 5 min)	312

Table II shows the results of typical enzyme assays on 40 000 × *g* supernatant from homogenized carrot disks. Normally (no α,α′-dipyridyl), the substrate would have contained 20 000 disint./min of hydroxyproline. Thus, the efficiency of the *in vitro* system is approx. 3.5%. In other batches of enzyme and substrate the efficiency ranged from 1 to 8%. Hydroxylase activity with MS substrate was also demonstrated in etiolated pea stem, tobacco leaf and pith callus, and tomato shoot tissue.

The basic observation on this hydroxylating system—that it required for maximum activity the addition of Fe^{2+}, ascorbate and α-ketoglutarate (Table III)—is identical to the situation in animal cells[11]. Ascorbate could not be replaced by other cofactors such as NADH or NADPH. Other divalent cations (Mg^{2+}, Mn^{2+} and Zn^{2+}) could not replace Fe^{2+}, nor could Fe^{3+}; in the presence of Fe^{2+}, Cu^{2+} was inhibitory (all ions at $1 \cdot 10^{-3}$ M). However, in many of our preparations we found that α-ketoglutarate could be replaced by pyruvate ($1 \cdot 10^{-4}$ M) and oxaloacetate ($1 \cdot 10^{-4}$ M)

TABLE III

REQUIREMENTS FOR HYDROXYLATING ACTIVITY

"Complete" system: cell extract, 5.0 mg protein; MS substrate, 15 000–25 000 disint./min [3,4-$^3H_2$]proline; Fe^{2+}, $1 \cdot 10^{-3}$ M (as ferrous ammonium sulfate); α-ketoglutarate, $1 \cdot 10^{-4}$ M; ascorbate, $1 \cdot 10^{-2}$ M (as sodium salt); 0.075 M Tris–HCl (pH 7.2); shaking in air at 37° for 30 min. As the table summarizes several experiments, values are normalized as percentages of "complete" activity; in the experiments this activity was 740–1530 disint./min in ^3HHO after correction for background of 200–900 disint./min.

Condition	*Relative activity (% of complete system)*
Complete	100
$-Fe^{2+}$	14
− Ascorbate	2
− α-Ketoglutarate	7
pH 2.0, pH 10.0	0
4°	23
50°	11
− MS substrate + free [3,4-$^3H_2$]proline, $3 \cdot 10^5$ disint./min	0
+ nonradioactive MS substrate, 1.0 mg protein	8
+ $1 \cdot 10^{-3}$ M proline	92
+ $1 \cdot 10^{-3}$ M hydroxyproline	100

TABLE IV

SUBCELLULAR DISTRIBUTION OF HYDROXYLATING ACTIVITY

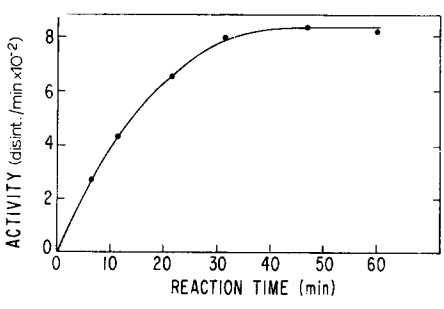
A homogenate of aged carrot-root disks was filtered through Miracloth. The residue was extracted with 0.5 M NaCl at 4° by slurrying; the filtrate was centrifuged at 40 000 × *g* for 40 min. The resulting pellet was suspended in 0.4 M sucrose buffered with 0.001 M Tris–HCl to pH 7.0. The salt extract, suspended pellet and 40 000 × *g* supernatant were then assayed for hydroxylating activity on MS substrate.

Source of enzyme activity	*Net mg protein*	*Net disint./min ^3HHO formed*	*% Total activity*
NaCl extract	8.0	3 000	4.8
40 000 × *g* pellet	200.0	6 060	9.6
40 000 × *g* supernatant	80.0	53 800	85.6

with little loss in hydroxylase activity. In a representative experiment, "complete" system produced 1960 disint./min hydroxyproline; "complete" without α-ketoglutarate gave 210 disint./min; "complete" without α-ketoglutarate but with added pyruvate and oxaloacetate gave 1580 disint./min, an 85% restoration of hydroxylase activity. Only bound proline could be hydroxylated in this system.

Table IV describes the subcellular distribution of the hydroxylase activity. Further experiments showed that all of the activity remained in the supernatant after the 40 000 × *g* supernatant was centrifuged at 105 000 × *g* for 2 h. But since this step afforded little purification, we routinely used the 40 000 × *g* supernatant for enzyme assays.

Some kinetic properties of the plant proline hydroxylating system are shown in Figs. 1–3. A double reciprocal plot of Fig. 3 ($1/v$ against $1/[S]$) was linear, giving an apparent K_m of approximately 30 000 disint./min substrate input. Because of the lack of a defined substrate, calculation of an exact K_m on the basis of moles of available hydroxylatable proline is not possible. Rough estimations indicate that the K_m is approx. $1 \cdot 10^{-6}$ moles of bound proline for MS substrate.



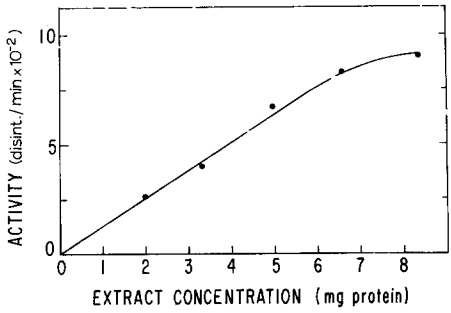
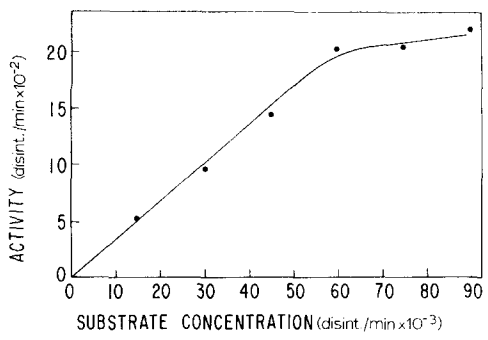


Fig. 1. Time-course of proline hydroxylase activity *in vitro*. Reaction mixtures contained: hydroxylase enzyme from 40 000 × *g* supernatant, 5.0 mg protein; MS substrate, 25 000 disint./min input; "complete" system, as outlined in MATERIALS AND METHODS. All enzyme activities are expressed as disint./min ^3H released into water from [3,4-$^3H_2$]proline in the substrate (see Table I).

Fig. 2. Effect of protein concentration in enzyme extract on hydroxylating activity. Reactions were run using "complete" system as outlined in MATERIALS AND METHODS; substrate input: 20 000 disint./min of MS substrate.



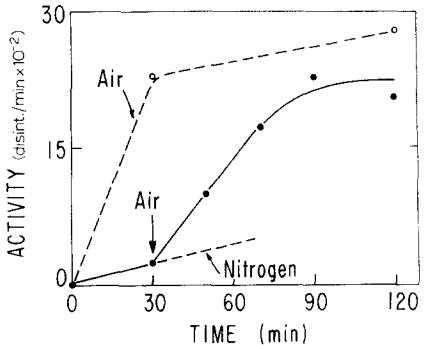


Fig. 3. Effect of substrate concentration on hydroxylase activity. Reaction mixtures contained: enzyme, 4.0 mg protein; MS substrate, variable, as stated; "complete" system, as described in MATERIALS AND METHODS. MS substrate contained 50 μg protein per 10 000 disint./min.

Fig. 4. O_2 requirement for proline hydroxylase activity. Reactions were performed in "complete" system, with 6.0 mg protein enzyme and 48 000 disint./min of MS substrate. A shaking water bath was fitted with an airtight cover with one entry jet. Assays performed under N_2 were done with continuous gas flow from a N_2 source after equilibration as outlined in MATERIALS AND METHODS. Assays performed in air were similarly treated, using an air jet as the gas source. The upper curve (open circles) represents enzyme activity in air. In the experiment shown in the lower curve (closed circles), the reaction was performed under N_2 for 30 min and then switched to air.

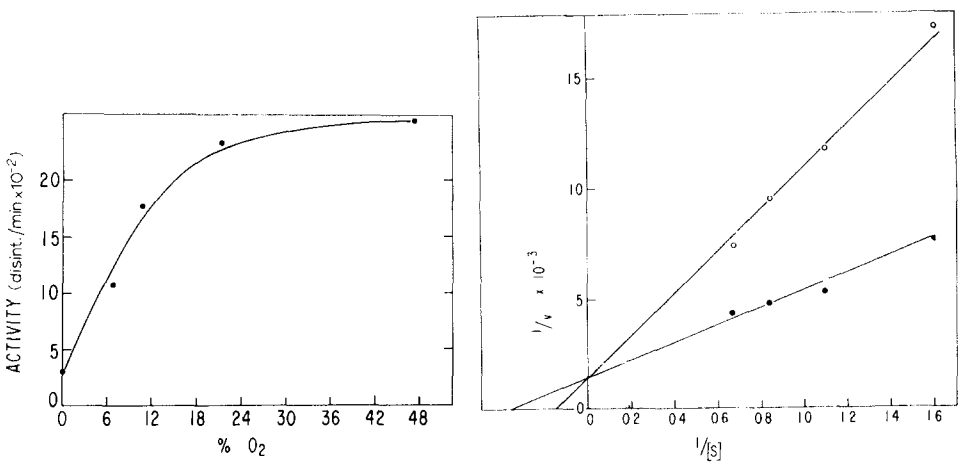


Fig. 5. Effect of variation in O_2 concentration on proline hydroxylase activity. Reaction mixtures contained: enzyme, 6.0 mg protein; MS substrate, 48 000 disint./min input; "complete" system as outlined in MATERIALS AND METHODS except that O_2 concentration was varied (as volume percentage) by regulating the relative flow rates of O_2 and N_2 sources in the apparatus outlined in Fig. 4.

Fig. 6. Effect of added plant (MS) substrate on plant enzyme hydroxylation of proline residues in protocollagen. Plant enzyme (40,000 × g supernatant, 2.75 mg protein) was presented with varying amounts of protocollagen (●—●) as substrate and enzyme assays were performed with "complete" system (see MATERIALS AND METHODS). This experiment was then repeated in the presence of nonradioactive plant substrate (approximately equivalent to 30 000 disint./min MS substrate) and the results are shown in ○--○. v = disint./min per 130 min. [S] = ml of substrate.

The experiments reported in Figs. 4 and 5 were designed to demonstrate the dependence of the hydroxylation reaction on O_2. In Fig. 4, N_2 greatly inhibited the reaction but this inhibition could be reversed by adding back air (O_2). An O_2 saturation curve for the plant proline hydroxylating system is given in Fig. 5.

Because MS substrate is heterogeneous and is not easily stored, before purifying and studying further properties of the hydroxylase, we desired a more homogeneous, easily prepared and stored substrate. Preliminary experiments showed that the plant system could hydroxylate the proline residues of protocollagen, a defined substrate used in assaying collagen proline hydroxylase. We likewise found activity by the latter enzyme on our plant substrate. In order to validate our future use of protocollagen with plant hydroxylase, we performed the experiment shown in Fig. 6. The results demonstrate competitive inhibition by plant on animal substrates for the plant enzyme.

TABLE V

PARTIAL PURIFICATION OF PEPTIDYL PROLINE HYDROXYLASE

Proline hydroxylase activity was purified from carrot-root disks as described in MATERIALS AND METHODS. Protocollagen ([3,4-$^3H_2$]proline) prepared from chick embryo tibiae acted as substrate with an input of 600 000 disint./min, 0.3 mg protein.

Enzyme fraction	*Protein content (mg)*	*Total activity (disint./min ^3HHO)*	*Specific activity (disint./min per mg)*	*Purification (-fold)*
40 000 × *g* supernatant	273	238 000	885	1
65% $(NH_4)_2SO_4$ pellet	44	146 080	3 320	3.7
$Ca_3(PO_4)_2$ (1)	1.8	32 200	17 900	20.2
$Ca_3(PO_4)_2$ (2)	2.1	43 790	20 850	23.6

A partial purification of the hydroxylase activity is shown in Table V. The degree of purification was fairly constant between preparations. The enzyme at all stages was unstable and lost activity upon freezing or prolonged (1 week) storage at 4°. The cofactor requirements and kinetic properties of purified preparations were similar to those of the initial supernatant. Peroxidase activity, which had been implicated in plant hydroxyproline synthesis[30] was in the supernatant of the 65% $(NH_4)_2SO_4$ step.

DISCUSSION

This report shows that plant hydroxyproline can arise in the same manner as does the imino acid in animal cells: by the hydroxylation of peptidyl proline catalyzed by a hydroxylase enzyme. Since both O_2 and an external reducing agent are required for the reaction to proceed, the enzyme may be classified as a mixed-function oxidase[31]. The properties of the enzyme *in vitro* are consistent with those expected from data obtained on hydroxyproline synthesis *in vivo*. O_2 (ref. 16) and Fe^{2+} (ref. 21), both shown to be involved in hydroxyproline synthesis in intact plant tissues, are likewise necessary for proline hydroxylation *in vitro*; α,α'-dipyridyl inhibits hydroxyproline synthesis in carrot disks[21] and also prevents hydroxylation of proline by

extracts of the same tissue; half of the radioactivity of proline is lost during hydroxylation *in vivo*, and this is also true in the *in vitro* hydroxylating system we have described here.

The observation that protocollagen can serve equally well as plant substrate for the plant hydroxylase is of interest as protocollagen has a more defined structure than the crude plant substrate used in this study. This cross-reactivity may point up the lack of specificity for this enzyme system *in vitro*, a property which is shared by collagen proline hydroxylase[32]. Moreover, since many plant proteins contain hydroxyproline[7], it is unlikely that a defined substrate of specific amino acid sequence exists for the proline hydroxylase *in vivo*. This would mean that there is probably not a defined peptidyl primary structure that the enzyme recognizes to determine which proline residues are to be hydroxylated. Indeed, not one primary sequence of a plant protein containing hydroxyproline has been determined, although considerable progress is being made[33].

Our observation that other α-keto acids (pyruvic and oxaloacetic acids) could replace α-ketoglutarate as a cofactor in many of our plant proline hydroxylase preparations is in contrast to the situation in animal cell enzyme work, where α-ketoglutarate is a specific requirement[11]. However, the efficacy of other α-keto acids is consistent with the hydroxylase mechanism proposed by LINDSTEDT *et al.*[34]. According to these authors, Fe^{2+} would activate O_2 onto the hydroxylatable site. The peroxide anion thus formed would, as a nucleophile, attack the α-carbon of an α-keto acid, thus decarboxylating the acid. In the case of collagen proline hydroxylase, the α-ketoglutarate is stoichiometrically decarboxylated during the proline hydroxylation reaction[35]. In the case of the plant enzyme, clearly more data are needed before such a mechanism can be proposed.

Experiments are in progress to further describe the properties of the plant peptidyl proline hydroxylase, to determine whether it is a rate-limiting step in hydroxyproline biosynthesis under physiological conditions, and to determine the cytological site of proline hydroxylation *in situ*.

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