Characterization of a low-relative-molecular-mass prolyl 4-hydroxylase from the green alga *Chlamydomonas reinhardtii*

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Prolyl 4-hydroxylase was partially purified and characterized from the unicellular green alga, *Chlamydomonas reinhardtii*. This enzyme differed from all the animal and plant prolyl 4-hydroxylases studied so far in that its M_r was only about 40000 by gel filtration, being thus less than one-sixth of those determined for the vertebrate and higher-plant enzymes. The algal enzyme did not hydroxylate to any significant extent chick-embryo protocollagen or triple-helical (Pro-Pro-Gly)₁₀, whereas a low hydroxylation rate was found with denatured (Pro-Pro-Gly)₁₀. Poly(L-proline), which is an effective inhibitor of the vertebrate enzymes but acts as a substrate for some higher-plant enzymes, was a good substrate. In the absence of poly(L-proline) the enzyme catalysed an uncoupled decarboxylation of 2-oxoglutarate. Studies of the K_m values for the co-substrates and cofactors and the specificity of the 2-oxoglutarate requirement, as well as inhibition studies with selected 2-oxoglutarate analogues, suggested that the catalytic site of the algal enzyme is similar to, but not identical with, those of the vertebrate enzymes. The existence of distinct similarities was further demonstrated by an inhibition of the algal enzyme activity with a monoclonal antibody to the β -subunit of human prolyl 4-hydroxylase. The amount of prolyl 4-hydroxylase activity in the algal cells was not altered by signals which recognize the presence or absence of the cell wall, as determined in studies on experimental cell-wall regeneration and wall-less mutants.

INTRODUCTION

Both the extracellular matrix of plant cells and the extracellular supporting elements of animal tissues, collagen and elastin, contain large amounts of 4-hydroxyproline. The synthesis of hydroxyproline in plants and animals requires post-translational hydroxylation of peptidyl-proline catalysed by prolyl 4-hydroxylase (proline:2-oxoglutarate dioxygenase, EC 1.14.11.2). The vertebrate enzyme has been purified and extensively characterized (Prockop *et al.*, 1976; Kivirikko & Myllylä, 1980, 1982, 1985). Prolyl 4-hydroxylase has also been detected in several plant species (Sadava & Chrispeels, 1971; Sadava & Volcani, 1977; Tanaka *et al.*, 1980, 1981; Cohen *et al.*, 1983; Sauer & Robinson, 1985), and has been purified from the French bean *Phaseolus vulgaris* (Bolwell *et al.*, 1985a). The enzyme isolated from higher plants has many features in common with the vertebrate enzyme, including the requirement for Fe²⁺, 2-oxoglutarate, O₂ and ascorbate, as well as the M_r .

Hydroxyproline residues have a structural function in collagen, where the hydroxyl groups stabilize the triple helix (Prockop *et al.*, 1976). Plant proteins are unique in that the hydroxyproline residues are frequently O-glycosylated and may form intermolecular cross-links which function in cell-wall assembly and stability. Hydroxyproline-rich glycoproteins may also play an important role in plant development (Cassab *et al.*, 1985), growth (Cleland & Karlsnes, 1967; Hoson & Wada, 1980), defence (Chrispeels *et al.*, 1974; Esquerre-Tuguey *et al.*, 1979; Bolwell *et al.*, 1985b) and cell-cell recognition (Homan, 1982). The enzyme responsible for the post-translational hydroxylation of proline is therefore a candidate for an important regulation.

Basic to an understanding of how prolyl hydroxylase functions in the plant cell is characterization of the plant

enzyme and its regulation during cell-wall biosynthesis. Such studies are easiest to perform with a plant in which protoplasts can be readily obtained in quantities suitable for enzyme extraction, and which rapidly and synchronously resynthesize cell walls. This can be achieved in the unicellular green alga *Chlamydomonas reinhardtii*, which possesses a hydroxyproline-rich cell wall (Roberts, 1974; Solter *et al.*, 1980). The extracellular matrix can be easily solubilized, and the resulting protoplasts will rapidly resynthesize cell walls. This algal species has the added advantage that mutants are available that lack cell walls (Davies & Plaskitt, 1971).

Prolyl hydroxylase has previously been detected in the diatom *Nitzschia angularis* (Sadava & Volcani, 1977); however, the properties of the algal enzyme have not been defined or compared with prolyl hydroxylase found in higher plants and vertebrates. In the present study we have characterized this enzyme from *C. reinhardtii*, and measured its activity in the cells in the absence of intact cell walls and during cell-wall biosynthesis.

MATERIALS AND METHODS

Materials

The synthetic peptide (Pro-Pro-Gly)₁₀·9H₂O was purchased from the Protein Research Foundation, Minoh, Osaka, Japan, and poly(L-proline) was from Sigma Chemical Co., St. Louis, MO, U.S.A. Sources for the analogues of 2-oxoglutarate were as described by Majamaa *et al.* (1984). [¹⁴C]Proline-labelled protocollagen was prepared from freshly isolated chick-embryo tendon cells as described in detail elsewhere (Kivirikko & Myllylä, 1982). 2-Oxo[1-¹⁴C]glutarate was purchased from Amersham International, Amersham, Bucks., U.K., and was adjusted to a specific radioactivity of

60 000 d.p.m./0.1 μ mol by mixing with the unlabelled compound (from Fluka, Buchs, Switzerland).

Cultures and production of gametes

The wild-type strain 137 c (mating type + and mating type -) of *Chlamydomonas reinhardtii* was obtained from Dr. M. Margulies, of the Radiation Biology Laboratory, Smithsonian Institute, Washington, DC, U.S.A. The wall-less mutant CW 15 (mating type +) was obtained from the Chlamydomonas Genetics Center, Department of Botany, Duke University, Durham, NC, U.S.A. The vegetative cells were grown under constant illumination in a phosphate-buffered medium as described previously (Solter & Gibor, 1977) supplemented with 0.2% (w/v) sodium acetate. Gametes were induced in liquid culture by using overnight incubation in growth medium without a nitrogen source, in accordance with the method of Kates & Jones (1964).

Preparation of cell-wall-degrading enzyme and cell-wall-lysis assay

Equal numbers of opposite mating-type gametes at a concentration of approx. 2×10^8 cells/ml were mixed and allowed to mate for 1 h. The cells were then removed from the suspension by centrifugation (1000 *g* for 10 min) and the supernatant, containing crude cell-wall-degrading enzyme, was stored at -20 °C. Cell-wall lysis was assayed by protoplast solubility in 0.02% Triton X-100 as previously described (Kaska & Gibor, 1982).

Preparation of cell extracts

Vegetative cells or gametes were harvested by centrifugation (1000 *g*) and resuspended in cell-wall-degrading enzyme prepared as described above for 30–60 min at room temperature until wall lysis was complete. The resulting protoplasts were washed twice with growth medium by centrifugation (1000 *g*), and the final cell pellet was treated with 2 vol. of cold solubilization buffer [50 mM-Tris/HCl, pH 7.8 at 4 °C, 300 mM-KCl, 20 mM-MgCl₂, 0.05 mM-dithiothreitol, 0.1% (v/v) Triton X-100]. Cells of the wall-less mutant, CW 15, were harvested by centrifugation (1000 *g*) and resuspended in 2 vol. of cold solubilization buffer. After incubation for 1 h at 4 °C, the solutions were centrifuged (15000 *g* for 20 min) and the supernatants stored at -70 °C.

Partial purification of prolyl 4-hydroxylase

The 15000 *g* supernatant was dialysed against several changes of 50 mM-Tris/HCl, pH 7.0, containing 0.05 mM-dithiothreitol at 4 °C, and applied to a column of DEAE-cellulose (38 cm $\times$ 1 cm) previously equilibrated with the dialysis buffer. The enzyme bound and was eluted with the dialysis buffer containing NaCl in a linear gradient from 0 to 2 M (total volume 200 ml). The active fractions were pooled, and 5 ml portions were applied to a Bio-Gel A 1.5 m (Bio-Rad) gel-filtration column (90 cm $\times$ 1 cm) equilibrated in 50 mM-Tris/HCl, pH 7.0 at 4 °C, containing 0.05 mM-dithiothreitol, 100 mM-KCl and 10 mM-MgCl₂. The column was calibrated with standards of known *M_r*.

Measurement of pI

The partially purified enzyme (1 ml) after DEAE-cellulose chromatography was dialysed against 0.025 M-imidazole/HCl, pH 7.4, and applied to a 3 ml

chromatofocusing column (PBE 94; Pharmacia) packed in a 5 ml glass pipette. The column was eluted with 42 ml of Polybuffer 74/HCl in accordance with the instructions provided; 1.5 ml fractions were collected, in which pH and enzyme activity were measured.

Assay of prolyl 4-hydroxylase activity

Proline-hydroxylation-coupled stoichiometric decarboxylation of 2-oxo[1-¹⁴C]glutarate was measured by a modification of a standard procedure (Kivirikko & Myllylä, 1982). Poly(L-proline), of *M_r* 7000 (600 μ g/ml), was used as the substrate, Tris/HCl buffer was replaced by 50 mM-Hepes buffer, pH 6.8, and the concentration of FeSO₄ was increased to 200 μ M. The assay tubes were incubated at 30 °C for 1 h with gentle shaking.

Inhibition of prolyl 4-hydroxylase with analogues of 2-oxoglutarate

Inhibitors were prepared in stock solutions, which were neutralized before addition to the assay mixtures containing partially purified enzyme. The substances to be tested were added at three constant concentrations and the concentration of 2-oxoglutarate was varied. The *K_i* values were calculated from the secondary transforms of the primary plots. The values reported are the means of those obtained in three experiments for each inhibitor.

Immunological cross-reactivity

Monoclonal antibodies (IgG) to human prolyl 4-hydroxylase (Höyhty *et al.*, 1984) or non-immune serum were added to the assay mixtures in various concentrations. Monoclonal IgG was prepared from cell culture media by Protein A-Sepharose affinity chromatography (see Höyhty *et al.*, 1984). Inhibition was calculated as percentage of control, and the results presented are means of two separate experiments.

Protein and 4-hydroxyproline determinations

Protein content of intact cells was measured, after solubilization of the cells in 1 M-NaOH, by the method of Lowry *et al.* (1951). Protein concentrations of soluble fractions were determined by the method of Bradford (1976). The quantity of 4-hydroxyproline (Kivirikko *et al.*, 1967) and the radioactivity of 4-hydroxy[¹⁴C]proline (Juva & Prockop, 1966) were measured after acid hydrolysis (6 M-HCl) overnight at 120 °C. These assays do not measure the isomer 3-hydroxyproline (Berg, 1982).

Preparation of isolated flagella

Flagella of vegetative cells and gametes were isolated as previously described (Kaska & Gibor, 1982).

RESULTS

Preparation of prolyl 4-hydroxylase

Solubilization of cells in the cold yielded a prolyl 4-hydroxylase preparation (15000 *g* supernatant) with a specific activity of 10–30 nmol/s per g. Sonication did not increase the amount of enzyme activity extracted, or the solubility. The enzyme bound to DEAE-cellulose, but only after dialysis (Fig. 1a), as the presence of only 50 mM-KCl in the equilibration buffer was sufficient to prevent binding of the enzyme to DEAE-cellulose, DEAE-Sephadex or DEAE-Trisacryl. About 54% of the applied activity was recovered in the fractions eluted

Fig. 1. DEAE-cellulose and gel-filtration chromatography of prolyl 4-hydroxylase from *C. reinhardtii*

(a) A sample (5 ml) of 15000 *g* supernatant, containing 60 mg of protein, was dialysed and chromatographed on DEAE-cellulose as described in the Materials and methods section. The column was washed with the dialysis buffer and eluted with NaCl in a continuous gradient from 0 to 0.2 M. Fraction (2 ml) were collected and analysed for protein (A_{230}) and enzyme activity by the ${}^{14}\text{CO}_2$ -release assay. (b) The active fractions from DEAE-cellulose chromatography were pooled, and a portion (5 ml) was chromatographed on Bio-Gel A 1.5 m. Fractions (1.85 ml) were collected and analysed for protein and enzyme activity as in (a). Inset: the column was calibrated with the following standards; 1, Blue Dextran; 2, chick-embryo prolyl 4-hydroxylase; 3 and 4, bovine serum albumin (dimer and monomer); 5, ovalbumin; 6, soya-bean trypsin inhibitor; 7, cytochrome *c*. Arrow indicates elution of *C. reinhardtii* prolyl 4-hydroxylase.

between 80 mM- and 120 mM-NaCl, resulting in a 7-fold purification.

Chromatofocusing confirmed that the enzyme carried a net negative charge at neutral pH. The enzyme bound to the PBE column and was eluted in a continuous pH gradient between pH 5.2 and 5.6.

Gel filtration of the enzyme after DEAE-cellulose chromatography on Bio-Gel A-1.5 m gave an apparent M_r of 40000 (Fig. 1b). This value is much lower than those of about 250000 and over 300000 determined for prolyl 4-hydroxylase of vertebrates (Pänkäläinen *et al.*, 1970; Berg & Prockop, 1973; Tuderman *et al.*, 1975) and higher plants (Bolwell *et al.*, 1985a) respectively. Additional experiments demonstrated that this difference was apparently not due to any artefact, as chick-embryo prolyl 4-hydroxylase was eluted from the same column with an apparent M_r of about 350000, as previously reported for the vertebrate enzyme by gel filtration.

Hydroxyapatite chromatography revealed that, although the enzyme was bound and detectable activity could be eluted in a continuous phosphate gradient, phosphate buffer markedly inactivated the enzyme.

Requirements for the peptide substrate

Initial experiments demonstrated that no synthesis of 4-hydroxy[${}^{14}\text{C}$]proline could be detected when [${}^{14}\text{C}$]proline-labelled chick-embryo procollagen was incubated with the enzyme in the presence of all the other reactants needed in the reaction. By contrast, synthesis of substantial amounts of 4-hydroxyproline was found with native poly(L-proline) as the substrate. Formation of ${}^{14}\text{CO}_2$ from 2-oxo[1- ${}^{14}\text{C}$]glutarate was found to be stoichiometric with the synthesis of 4-hydroxyproline, and thus the measurement of ${}^{14}\text{CO}_2$ could be used to assay the enzyme activity, as has previously been used for the assays of vertebrate prolyl hydroxylases (Kivirikko &

Myllylä, 1982). This assay is more rapid and sensitive than those based on measurement of the quantity of the 4-hydroxyproline synthesized.

A small but definite hydroxylation rate was found at 30 °C or 37 °C with heat-denatured (Pro-Pro-Gly)₁₀ as the substrate. This rate with 0.6 mg of (Pro-Pro-Gly)₁₀/ml was about 17% of that found with 0.6 mg of poly(L-proline) (*M_r* about 7000)/ml. The results obtained by measurement of ¹⁴CO₂ formation or hydroxyproline synthesis were identical. By contrast, when the reaction was carried out at 15 °C, a temperature at which most of (Pro-Pro-Gly)₁₀ is in a triple-helical conformation (Berg *et al.*, 1970; Kivirikko *et al.*, 1972), the rate with 0.6 mg of (Pro-Pro-Gly)₁₀/ml was in various experiments only 0–4% of that found with 0.6 mg of poly(L-proline) (*M_r* about 7000)/ml under the same conditions. If the poly(L-proline) was denatured by heating, no hydroxylation could be detected.

The *K_m* for poly(L-proline) decreased with an increasing chain length, when expressed as μ M concentration of the peptide (Table 1). However, when expressed as μ M concentration of proline residues, the value remained essentially unchanged with an increasing chain length.

Cofactors, co-substrates and optimal reaction conditions

The algal prolyl 4-hydroxylase was found to require Fe²⁺, 2-oxoglutarate, O₂ and ascorbate, as previously reported for vertebrate (Prockop *et al.*, 1976; Kivirikko & Myllylä, 1980, 1982, 1985) and higher-plant (Tanaka *et al.*, 1980; Cohen *et al.*, 1983; Bolwell *et al.*, 1985a; Sauer & Robinson, 1985) enzymes. The presence in the assay solution of bovine serum albumin, catalase and dithiothreitol stimulated the enzyme activity by about 300%, 100% and 10% respectively. These compounds are also added to the assay mixture of other prolyl hydroxylases (see the above references). No change in the enzyme activity was observed in the presence or absence of 0.01% Triton X-100. The pH and temperature optima were 6.8–6.9 and 30 °C respectively, which agree with those reported for the higher-plant enzymes (Tanaka *et al.*, 1980; Cohen *et al.*, 1983; Sauer & Robinson, 1985).

Table 1. Comparison of *K_m* values of substrates and cofactors for prolyl 4-hydroxylases from various sources

The values for *C. reinhardtii* are means of three determinations. Values for higher plants, see references Tanaka *et al.* (1980, 1981), Cohen *et al.* (1983) and Sauer & Robinson (1985). Values for chick, see reference Kivirikko & Myllylä (1982). Abbreviations: NR, not reported; NS, not a substrate.

Substrate or cofactor	<i>K_m</i> (μ M)		
	<i>C. reinhardtii</i>	Higher plants	Chick
Poly(L-proline)			
<i>M_r</i> 7000	23	40, 230	NS
<i>M_r</i> 19000	11	NR	NS
<i>M_r</i> 31000	7	5	NS
Fe ²⁺	28	NR	4
2-Oxoglutarate	30	90, 250	22
Ascorbate	240	NR	300

The *K_m* for Fe²⁺ with the algal prolyl 4-hydroxylase was about 7 times that reported for the chick enzyme (Table 1). The *K_m* values for 2-oxoglutarate and ascorbate were comparable with those of the chick enzyme (Table 1).

The specificity of the 2-oxoglutarate requirement was studied by replacing the 2-oxo[1-¹⁴C]glutarate with various 2-oxo acids and assaying the quantity of 4-hydroxyproline formed. No hydroxyproline synthesis was detected with 0.8 mM-2-oxoadipate, 2 mM-2-oxosuccinate or 2 mM-2-oxovalerate. Under the same conditions 3.46 μ g of 4-hydroxyproline was synthesized in the presence of 80 μ M-2-oxoglutarate. Owing to the comparatively low sensitivity of the assay procedure, a reaction rate less than 3% of that found with 2-oxoglutarate cannot be excluded, however. In an additional experiment it was demonstrated that [1-¹⁴C]pyruvate is not able to replace the 2-oxo[1-¹⁴C]glutarate, either.

Ascorbate (optimal concentration 2 mM) could be completely replaced by equimolar concentrations of D(–)-isoascorbate or 5,6-isopropylidene ascorbate (results not shown).

The algal prolyl 4-hydroxylase catalysed an uncoupled decarboxylation of 2-oxoglutarate when the assay was performed in the absence of the poly(L-proline) substrate (Table 2). This reaction required the presence of Fe²⁺ and ascorbate, and the rate was about 10% of that observed in the presence of poly(L-proline) (Table 2).

Inhibition studies

Selected analogues of 2-oxoglutarate, all of which have previously been characterized as inhibitors of chick prolyl 4-hydroxylase, prolyl 3-hydroxylase and lysyl hydroxylase, were used to study the properties of the 2-oxoglutarate-binding site of *Chlamydomonas reinhardtii* prolyl hydroxylase. The *K_i* values found (Table 3) were fairly similar to those for the vertebrate collagen hydroxylases (Majamaa *et al.*, 1984, 1985, 1986). The most effective inhibitors contained groups capable of chelating the enzyme-bound iron and a carboxyl function corresponding to C-5 of 2-oxoglutarate.

In agreement with data on chick prolyl 4-hydroxylase

Table 2. Uncoupled decarboxylation of 2-oxoglutarate catalysed by *Chlamydomonas reinhardtii* prolyl 4-hydroxylase

The enzyme reaction was carried out as follows: 'Complete' mixture included 2-oxoglutarate, enzyme, substrate, Fe²⁺ and ascorbate as in a standard condition; '– Substrate', substrate omitted from the reaction mixture; '– Substrate, – Fe²⁺', substrate and Fe²⁺ omitted; '– Substrate, – ascorbate', substrate and ascorbate omitted; '– Substrate, – Fe²⁺, – ascorbate', substrate, Fe²⁺ and ascorbate omitted from the reaction mixture.

Composition of assay mixture	¹⁴ CO ₂ released (d.p.m./h)	Decarboxylation (%)
Complete	16680	100
– Substrate	1708	10
– Substrate, – Fe ²⁺	0	0
– Substrate, – ascorbate	187	2
– Substrate, – Fe ²⁺ , – ascorbate	0	0

Table 3. Inhibition constants for selected 2-oxoglutarate analogues

The K_i values were determined by using the $^{14}\text{CO}_2$ -release assay (see the Materials and methods section).

Compound	K_i (mM)
2-Oxoadipate	0.2
2-Oxosuccinate	0.4
Pyruvate	20
Glutarate	1.5
Succinate	1.6
Benzene-1,3-dicarboxylate	11
Pyridine-2,4-dicarboxylate	0.008
Pyridine-2,5-dicarboxylate	0.02
Pyridine-2-carboxylate	0.05
3,4-Dihydroxybenzoate	0.07

(Tuderman *et al.*, 1977), Zn^{2+} was an effective inhibitor of the *Chlamydomonas* enzyme. The K_i was $5\text{ }\mu\text{M}$, and the mode of inhibition was competitive with respect to Fe^{2+} .

Immunological cross-reactivity with human prolyl 4-hydroxylase

A monoclonal antibody, 5B5-A, prepared against the β -subunit of human prolyl 4-hydroxylase (Höyhty *et al.*, 1984) inhibited the activity of the algal enzyme by about 50% (Fig. 2). This antibody also inhibits the human enzyme activity by about 50% and is thought to recognize a determinant at or near the active site. Another monoclonal antibody, 3G3-E, which likewise recognizes the β -subunit of the human enzyme but does

Fig. 2. Inhibition of *C. reinhardtii* prolyl 4-hydroxylase by monoclonal antibody to human prolyl 4-hydroxylase

Two monoclonal antibodies to the β -subunit of human prolyl 4-hydroxylase and non-immune serum were added to the enzyme assay mixture in various concentrations. Inhibition was determined as percentage of control, and the results presented are the means of two separate experiments. ●, Monoclonal antibody 5B5-A; ○, monoclonal antibody 3G3-E; ×, non-immune serum.

Table 4. Hydroxyproline content and prolyl 4-hydroxylase activity of *Chlamydomonas reinhardtii*

Abbreviations: Wt veg, wild type, vegetative cells; Wt gam, wild type, gametes; ND, not determined.

Cell type/organelle	4-Hydroxyproline ($\mu\text{g}/\text{mg}$ of protein)	Prolyl 4-hydroxylase activity (nmol of CO_2/s per g)
Wt veg, intact	10.9	28
Wt veg, wall removed	1.5	28
Wall-less mutant CW 15	1.7	27
Wt gam, wall removed	ND	31
Wt veg, isolated flagella	1.9	ND
Wt gam, isolated flagella	2.1	ND

not inhibit the human enzyme activity (Höyhty *et al.*, 1984), gave no inhibition of the algal enzyme (Fig. 2). Similarly, no inhibition was found with a non-immune serum.

4-Hydroxyproline content and specific activity of prolyl 4-hydroxylase in vegetative cells, gametes and cell-wall-less mutant CW 15

The intact vegetative cells contained about $11\text{ }\mu\text{g}$ of 4-hydroxyproline/mg of protein (Table 4). Wall removal resulted in an 85% decrease in this hydroxyproline content, but the remaining 15% could not be removed by repeated exposure to the wall-degrading enzyme. The wall-less mutant (CW 15) had a similar hydroxyproline content to wild-type cells from which walls had been removed (Table 4). Flagella isolated from vegetative cells and gametes contained similar amounts of hydroxyproline.

Although the wall-less mutant contained only 15% of the 4-hydroxyproline present in wild-type cells, the activity of prolyl 4-hydroxylase in the mutant was comparable with that found in vegetative cells and gametes (Table 4). This finding indicates that the absence of the cell walls in the mutant is not due to a deficiency in prolyl 4-hydroxylase.

To determine whether prolyl 4-hydroxylase activity could be regulated in wild-type cells by signals recognizing the presence or absence of the cell wall, vegetative cells were treated repeatedly with wall-dissolving enzyme at 4 h intervals during a 12 h period. Cell-wall removal was monitored by solubility of the protoplasts in 0.02% Triton X-100, and cell-wall resynthesis was measured by increase in 4-hydroxyproline content. After an initial lag of 1 h, cell-wall resynthesis proceeded at a constant rate continuously throughout the 12 h period (Fig. 3). Hydroxyproline was formed at a rate of about $1.0\text{ }\mu\text{g}/\text{h}$ per mg of protein. Enzyme activity, however, remained relatively constant during the 12 h of induced cell-wall synthesis. A slight overall increase in activity was observed, but this did not exceed 150% of the initial value (Fig. 3).

DISCUSSION

The post-translational 4-hydroxylation of peptidyl-proline which occurs in many plant and animal cells,

Fig. 3. Prolyl 4-hydroxylase activity during induced cell-wall synthesis

Vegetative cells (approx. 10^{10} cells) were collected by centrifugation (1000 g) and treated with wall-degrading enzyme (25 ml) for 15 min. Cell-wall lysis was complete, as determined by solubility of the protoplasts (0.5 ml sample) in 0.02% Triton X-100. The protoplasts were collected by centrifugation (1000 g), washed with growth medium and resuspended in 1.5 litres of growth medium. At intervals, 100 ml samples were centrifuged (1000 g) and the cells resuspended in 10 ml of growth medium. Protoplast solubility in 0.02% Triton X-100 was determined by using a 0.5 ml sample of this suspension. The release of chlorophyll was measured spectrophotometrically (A_{660}). The remainder of the suspension was divided into portions (1 ml) and the cells were collected by centrifugation (15000 g for 20 min). For determination of prolyl 4-hydroxylase activity, two cell pellets were pooled and treated with solubilization buffer (0.5 ml) at 4 °C. Enzyme activity was determined by the $^{14}\text{CO}_2$ -release assay. The remaining cell pellets were analysed for protein and 4-hydroxyproline contents as described in the Materials and methods section. At approx. 4 h intervals (indicated by arrowheads), the entire cell suspension was centrifuged (1000 g) and the cells were treated with wall-degrading enzyme (25 ml) for 15 min. After washing, the cells were resuspended in a volume of growth medium sufficient to maintain a constant cell density throughout the experiment.

and the appearance of these modified proteins almost exclusively in the extracellular matrix, has led to speculation concerning the similarities with regard to the enzymology of these phenomena (Robinson *et al.*, 1985). To study this reaction in the plant and to compare the results with the reaction catalysed by vertebrate prolyl 4-hydroxylases, prolyl 4-hydroxylase from the unicellular green alga *C. reinhardtii* was characterized. This plant species, cultured readily in light on a simple salt medium, produces a wall-degrading enzyme during mating which facilitates production of protoplasts. Prolyl 4-hydroxylase was extracted from these protoplasts by lysis in 0.1% Triton X-100 at 4 °C. No homogenation or sonication were required, and the specific activity of the crude extract (15000 g supernatant) was 20–60 times higher than that reported for the homogenate of *Phaseolus vulgaris* (Bolwell *et al.*, 1985a), and more than 5000 times higher than for homogenates of *Lolium multiflorum* (Cohen *et al.*, 1983) and maize (Sauer & Robinson, 1985). Homogenates of *Vinca rosea* cells contained no measurable enzyme activity, which could be detected only after subsequent fractionation (Tanaka *et al.*, 1980). The observation that cells of the wall-less mutant CW 15 contain enzyme activity equivalent to that of wild-type cells enables further simplification of the extraction procedure, by obviating cell-wall removal before lysis.

The M_r of the algal prolyl 4-hydroxylase, about 40000, was distinctly lower than those of the vertebrate (Pänkäläinen *et al.*, 1970; Berg & Prockop, 1973; Tuderman *et al.*, 1975) and higher-plant enzymes (Bolwell *et al.*, 1985a) which both exceeded 300000 in

gel filtration. The vertebrate prolyl 4-hydroxylases are tetramers ($\alpha_2\beta_2$), with inactive subunits of M_r about 64000 and 60000 (Prockop *et al.*, 1976; Kivirikko & Myllylä, 1980, 1982, 1985). It is possible that the lower- M_r algal prolyl 4-hydroxylase is a new enzyme, unrelated to those of higher plants and animals. Another possibility is that in lower plants, such as algae, an earlier monomer form of prolyl 4-hydroxylase has been retained, and that the active site has been conserved during evolution of higher eukaryotes. Although neither of these possibilities is ruled out, our data suggest a strong similarity between the hydroxylation reactions catalysed by the higher-plant, animal and algal enzymes. The pI of the algal enzyme is also similar to those reported for the two subunits of the chick enzyme (Kedersha *et al.*, 1985).

The basic feature distinguishing the algal and higher-plant from the animal prolyl 4-hydroxylase reaction is the recognition of different peptidyl substrates. The vertebrate enzymes require an -Xaa-Pro-Gly- triplet in a non-triple-helical conformation, poly(L-proline) in type-II helix being an effective competitive inhibitor (Prockop *et al.*, 1976; Kivirikko & Myllylä, 1980, 1982, 1985). Some of the plant enzymes also hydroxylate -Xaa-Pro-Gly- polymers. For example, a prolyl hydroxylase from carrot discs has been reported to hydroxylate readily denatured chick-embryo procollagen (non-hydroxylated procollagen) (Sadava & Chrispeels, 1971). Prolyl 4-hydroxylases from *V. rosea* (Tanaka *et al.*, 1980), *L. multiflorum* (Cohen *et al.*, 1983), maize (Sauer & Robinson, 1985) and *P. vulgaris* (Bolwell *et al.*, 1985a) act on proline in the poly(L-proline) type-II helix, but the

V. rosea enzyme (Tanaka *et al.*, 1980) also acts on denatured polypeptide chains of (Pro-Pro-Gly)₁₀ at rates about 20% of those found with poly(L-proline). The present data indicate that the *C. reinhardtii* prolyl 4-hydroxylase appears to be particularly strict with respect to the requirement of a poly(L-proline) type-II substrate. No significant reaction was found with protocollagen, triple-helical (Pro-Pro-Gly)₁₀ or denatured poly(L-proline), and only a low reaction rate was found with denatured (Pro-Pro-Gly)₁₀.

The K_m values reported for poly(L-proline) and 2-oxoglutarate in the higher-plant prolyl 4-hydroxylation reactions show considerable variability (Tanaka *et al.*, 1980; Cohen *et al.*, 1983; Bolwell *et al.*, 1985a; Sauer & Robinson, 1985). However, the K_m values for 2-oxoglutarate and ascorbate determined here for the algal enzyme are very close to those for the vertebrate prolyl 4-hydroxylase (Kivirikko & Myllylä, 1980, 1982), and that for Fe²⁺ of the algal enzyme is about 7-fold greater. The algal enzyme also resembled the chick enzyme in that ascorbate could be replaced by D-(−)isoascorbate and 5,6-isopropylideneascorbate (Kivirikko & Myllylä, 1980; Majamaa *et al.*, 1986) and that it catalysed an uncoupled decarboxylation (Tuderman *et al.*, 1977; Myllylä *et al.*, 1984; de Jong & Kemp, 1984) of 2-oxoglutarate in the absence of the peptide substrate. However, the rate of the uncoupled reaction, about 10%, was distinctly higher than the rates of about 1% reported for chick-embryo prolyl 4-hydroxylase (Tuderman *et al.*, 1977; Myllylä *et al.*, 1984) or 4% for chick-embryo lysyl hydroxylase (Puistola *et al.*, 1980).

Additional similarities between the algal and chick-embryo prolyl 4-hydroxylases were found in the properties of the 2-oxoglutarate-binding site. Inhibition studies with analogues of 2-oxoglutarate suggested that the active centre of the algal enzyme contains the same structural binding sites for this co-substrate as have previously been suggested for the chick enzyme (Hanauske-Abel & Günzler, 1982; Majamaa *et al.*, 1984, 1985, 1986). Interestingly, pyridine-2,4-dicarboxylate was the most potent inhibitor of the algal enzyme, whereas pyridine-2,5-dicarboxylate is the most effective inhibitor of the chick prolyl 4-hydroxylase. In this respect the algal enzyme appears to be related to chick lysyl hydroxylase and prolyl 3-hydroxylase, which are likewise inhibited more effectively with pyridine-2,4-dicarboxylate than with the 2,5-dicarboxylate. The algal enzyme differed from chick prolyl 4-hydroxylase and lysyl hydroxylase in that 2-oxoglutarate could not be replaced in the hydroxylation reaction to any significant extent with 2-oxoadipate. The strict requirement for 2-oxoglutarate differs also from that found for the carrot disc enzyme, which has been reported to utilize readily also pyruvate and 2-oxosuccinate (oxaloacetate) (Sadava & Chrispeels, 1971).

Similarities in the active site of the animal and algal prolyl 4-hydroxylases were also suggested by immunocross-reactivity. Partial inhibition of the activity of both enzymes by a monoclonal antibody to the β -subunit of human prolyl 4-hydroxylase (Höyhty *et al.*, 1984) indicated that similar antigenic determinants were recognized in the region of the active site. Detailed comparison of the amino acid or cDNA sequences are required, however, before the degree of similarity between these enzymes can be determined.

Studies on the regulation of prolyl 4-hydroxylase in the

French bean *P. vulgaris* have shown specific induction of activity within 2 h in response to elicitors from a phytopathogenic fungus (Bolwell *et al.*, 1985b). The signalling mechanism which initiates synthesis and secretion of hydroxyproline-rich cell-wall glycoproteins, however, is unknown. To determine whether induction of prolyl 4-hydroxylase activity could be related to signals monitoring changes in the wall matrix, the activity was measured during repeated cell-wall removal from wild-type cells, and in a cell-wall-less mutant. *C. reinhardtii* provided a useful model system to study this response because cell-wall recovery is quantitative and rapid in wild-type cells. In addition, wall-less mutants are not available from other plants. In the algal system, prolyl 4-hydroxylase activity remained relatively constant during 12 h of continuous cell-wall resynthesis, and the formation of 4-hydroxyproline, after an initial 1 h lag, occurred at a constant rate of about 10 μ g/h per mg of protein. It was not possible by this method to detect induction of enzyme activity in living cells which occurred during the 30 min required for cell-wall removal. However, studies on intact cells killed by freezing before wall removal and enzyme extraction indicated that enzyme activity did not change during wall removal. These data, and the finding that the cell-wall-less mutant contained prolyl 4-hydroxylase activity equivalent to the wild-type cells, indicate that the enzyme is not regulated by signals which detect changes in the cell-wall matrix, and suggests that the regulation in higher plants results from more specific interactions between the plant cell and inducers generated by wounding or infection.

This work was supported in part by grants from the Medical Research Council of the Academy of Finland. D.D.K. had a fellowship from the Sigrid Jusélius Foundation to participate in this research at the Department of Medical Biochemistry, University of Oulu. V.G. is the recipient of a long-term fellowship from EMBO, Heidelberg, Germany.

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Received 24 July 1986; accepted 25 September 1986