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Prolyl 4-hydroxylase from *Volvox carteri*

A low-M_r enzyme antigenically related to the α subunit of the vertebrate enzyme

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Prolyl 4-hydroxylase was isolated in a highly purified form from a multi-cellular green alga, *Volvox carteri*, by a procedure consisting of ion-exchange chromatography and affinity chromatography on poly(L-hydroxyproline) coupled to Sepharose. Two other affinity-column procedures were also developed, one involving 3,4-dihydroxyphenylacetate and the other 3,4-dihydroxyphenylpropionate linked to Sepharose. The K_m values of the *Volvox* enzyme for the co-substrates and the peptide substrate, as well as the inhibition constants for selected 2-oxoglutarate analogues, were similar to those of the enzyme from *Chlamydomonas reinhardii*, except that the K_m for 2-oxoglutarate with the *Volvox* enzyme was 6-fold greater. The temperature optimum of the *Volvox* enzyme was also 10 °C higher. The apparent M_r of the *Volvox* enzyme by gel filtration was about 40000, being similar to that reported for the *Chlamydomonas* enzyme but markedly lower than that of the vertebrate enzymes. A similar apparent M_r of about 40000 was also found for prolyl 4-hydroxylase from the green alga *Enteromorpha intestinalis*, whereas the enzyme from various vascular plants gave an apparent $M_r > 300000$. SDS/polyacrylamide-gel electrophoresis demonstrated in the highly purified *Volvox* enzyme the presence of a major protein band doublet with a M_r of about 65000 and a minor doublet of M_r about 55000–57000. A polyclonal antiserum, prepared against the M_r-65000 doublet, stained in immunoblotting the M_r-65000 doublet as well as the α subunit, but not the β subunit, of the vertebrate prolyl 4-hydroxylase. An antiserum against the β subunit of the vertebrate enzyme stained in immunoblotting a M_r-50000 polypeptide in a partially purified *Volvox* enzyme preparation, but did not stain either the M_r-65000 or the M_r-55000–57000 doublet of the highly purified enzyme. The data thus suggest that the active *Volvox carteri* prolyl 4-hydroxylase is an enzyme monomer antigenically related to the α subunit of the vertebrate enzyme.

INTRODUCTION

The extracellular matrices of plants and animals contain unique proteins in which proline residues are post-translationally modified to 4-*trans*-hydroxyproline. These proteins include the collagens and collagen-like proteins, which are essential for the maintenance of animal tissue structure and play important roles in cell–cell interaction and the regulation of development. In plants, hydroxyproline is found primarily in a family of cell-wall glycoproteins including the extensins, arabinogalactan proteins and lectins [1]. Although the functions of these wall components are not well understood, they have been shown to mediate recognition between plant cells [2,3], and numerous studies suggest their involvement in plant development, growth and defence [4–8]. Collagen biosynthesis has been extensively investigated (see [9–11]), whereas the biosynthesis of hydroxyproline-rich plant glycoproteins is not well defined.

The 4-hydroxylation of peptidyl proline is catalysed by prolyl 4-hydroxylase (EC 1.14.11.2), which in vertebrates is a tetramer (M_r 250000) composed of two α (M_r 64000) and two β (M_r 60000) subunits (see [10–12]). Prolyl 4-hydroxylase has also been identified in a variety of plant cells [13–21]. Both the plant and animal enzymes require Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate. The peptidyl substrates of the two enzymes, however, are distinctly different. The vertebrate enzymes require an Xaa-Pro-Gly triplet, whereas the plant enzyme acts on the poly(L-proline) type II helix [15,16]. Several extensins contain numerous repetitive Ser-Hyp-Hyp-Hyp-Hyp sequences [22], and cell-wall glycoproteins containing as much as 62% hydroxyproline have been reported [7].

Recent data strongly implicate the α subunit of the vertebrate prolyl 4-hydroxylase as the locus of the hydroxylation reaction [23–26]. The β subunit has been found to possess an independent enzymic activity and can function either in the prolyl 4-hydroxylase tetramer or as a protein disulphide-isomerase [27,28]. The hydroxylation of proline, however, is only catalysed by the $\alpha_2\beta_2$ tetramer. Prolyl 4-hydroxylase purified from a higher plant, the French bean *Phaseolus vulgaris*, is an oligomer ($M_r > 300000$) of M_r 65000 subunits [19]. Nothing is known about the enzymic active site of this plant enzyme.

In our previous studies we found that the catalytic properties of prolyl 4-hydroxylase in the green alga *Chlamydomonas reinhardii* were very similar to those of the vertebrate enzyme, although it recognized the synthetic plant substrate, poly(L-proline) [21]. The active enzyme, however, had a M_r considerably lower (40000) than that of the enzyme from vertebrates or higher plants, as determined by gel filtration. As the green algae (Chlorophytes) are considered to have diverged early in the evolutionary history of higher plants, and only a short evolutionary distance from the initial separation of plants from animals [29], the algal prolyl 4-hydroxylase may represent an earlier monomeric form of the enzyme.

Whether the enzyme of the unicellular *C. reinhardii* is an isolated case, or whether prolyl 4-hydroxylases of multicellular green algae such as *Volvox* also have a characteristic low M_r, is not known. Prolyl 4-hydroxylase from *Volvox* has not previously been investigated, although this organism possesses an abundant extracellular matrix rich in hydroxyproline [30].

The purpose of the present work was to isolate and characterize the prolyl 4-hydroxylase from *Volvox carteri*, and investigate whether or not this enzyme shares antigenic determinants with the vertebrate enzyme.

MATERIALS AND METHODS

Materials

2-Oxo[1-^{14}C]glutarate was purchased from Amersham International, Amersham, Bucks., U.K., and was adjusted to a specific radioactivity of 60000 d.p.m./0.1 μmol by mixing with the unlabelled compound (from Fluka, Buchs, Switzerland). Poly(L-proline) and poly(L-hydroxyproline) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Sources for the analogues of 2-oxoglutarate were as described elsewhere [31,32]. Ser-Pro and Ala-Pro were purchased from Accurate Chemicals, Westbury, U.S.A. Poly(L-proline)–Sepharose and poly(L-hydroxyproline)–Sepharose were prepared as previously described [12]. 3,4-Dihydroxyphenylacetate–Sepharose and 3,4-dihydroxyphenylpropionate–Sepharose were prepared by coupling to aminohexyl–Sepharose 4B (Pharmacia) by the carbodi-imide reaction using *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodi-imide (0.1 M).

Algal cultures and preparation of extracts

Volvox carteri f. *nagariensis* strain HK 10, obtained from David L. Kirk, Department of Biology, Washington University, St. Louis, MO, U.S.A., was cultured in standard *Volvox* medium supplemented with 0.5 mM-urea [33]. The spheres were collected on 90 μm-mesh nylon cloth mounted in a Millipore filtration assembly and homogenized with five strokes in a Dounce-type homogenizer with a loose-fitting pestle in *Volvox* culture medium. The broken spheres were collected by centrifugation (5 min at 1000 *g*), resuspended in a solubilization buffer [50 mM-Tris/HCl, pH 7.8 at 4 °C, 300 mM-KCl, 20 mM-$MgCl_2$, 0.05 mM-dithiothreitol, 0.1% (v/v) Triton X-100], sonicated in an ice bath (Microprobe, setting 4) for 3 × 5 s at 1 min intervals, and the supernatant (15000 *g* for 20 min) was stored at −70 °C. Extracts of the wall-less mutant of *Chlamydomonas reinhardii*, CW 15, were prepared as previously described [21].

Plant tissues and preparation of extracts

The green alga *Enteromorpha intestinalis* was collected at Santa Barbara, CA, U.S.A., and *Helianthus tuberosum* (Jerusalem artichoke) rhizomes were a gift from William Coleman, Santa Barbara, CA, U.S.A. The plants were finely chopped with a razor blade and homogenized in the solubilization buffer (above) at 4 °C, and the supernatant (15000 *g* for 30 min) stored at −70 °C. *Persea americana* (avocado) fruit, purchased locally, was homogenized at 4 °C in solubilization buffer (above), but in the absence of Triton X-100. The homogenate was centrifuged (15000 *g*, 20 min) and the large lipid phase was discarded. The remaining solution was then adjusted to 0.1% Triton X-100, re-homogenized, and the supernatant stored at 4 °C.

DEAE-cellulose chromatography

The *V. carteri* 15000 *g* supernatant was dialysed against 50 mM-Tris/HCl (pH 7.0 at 4 °C)/50 mM-KCl/0.05 mM-dithiothreitol (solution A) and then applied to a column of DEAE-cellulose equilibrated with the same dialysis buffer. The flow-through was collected, concentrated in an Amicon PM10 filter, and stored at −70 °C.

Affinity chromatographies

Poly(L-hydroxyproline)–Sepharose (50 ml) was equilibrated in solution A and mixed with the concentrated DEAE-cellulose flow-through (130 ml). 3,4-Dihydroxyphenylacetate–Sepharose (3 ml) and 3,4-dihydroxyphenylpropionate–Sepharose (3 ml) equilibrated in solution A were mixed with 2.5 ml of the enzyme solution. After gentle agitation at 4 °C overnight, the beads were allowed to settle and the supernatants collected for assay of the remaining enzyme activity. The affinity matrices were then poured into columns and washed with solution A. The two smaller columns were each eluted with solution A (10 ml) containing 0.5 M-3,4-dihydroxybenzoate. Poly(L-hydroxyproline)–Sepharose was eluted with solution A (10 ml) containing 4 mg of poly(L-hydroxyproline)/ml and subsequently with 6 M-urea (30 ml). The urea eluate was dialysed extensively against solution A.

Isoelectric focusing

The pI was determined with a model 8101 isoelectric-focusing apparatus (LKB, Stockholm, Sweden). The DEAE flow-through (20 ml, containing 4 mg of protein) was dialysed against 1% (w/v) glycine and focused in a solution containing 1% carrier ampholytes (Bio-Rad) (pH 4–6 and 6–8 in equal volumes) stabilized in a 5–50% (w/v) sucrose gradient. A constant electric field (2.4 W) was applied to the column at 4 °C, for 24 h. The fractions were then assayed for pH and enzyme activity.

Hydrophobic chromatography

The DEAE flow-through (3 ml, containing 0.9 mg of protein) was applied to a column (4 ml) of phenyl–Sepharose CL-4B (Pharmacia), equilibrated in solution A, and column was washed with the equilibration buffer. Enzyme activity was eluted with a solution of the equilibration buffer containing 60% (v/v) ethylene glycol.

Gel-filtration chromatography

Three gel-filtration columns were used; Bio-Gel (Bio-Rad) A 0.5 m (66 cm × 2 cm), Bio-Gel A 1.5 m (90 cm × 2.5 cm) and Sephacryl S-300 (Pharmacia) (95 cm × 1.5 cm). DEAE-flow-through (*C. reinhardii*, *V. carteri*) or 15000 *g*-supernatant (*Enteromorpha*, *P. americana*, *H. tuberosum*) samples (5 ml) were dialysed against solution A and applied to the columns, which were equilibrated with the same buffer. The columns were calibrated with standards of known M_r.

Antibody production

The algal prolyl 4-hydroxylase was fractionated by SDS/polyacrylamide-gel electrophoresis and the M_r-65000 band cut from the gels. The gel slices were homogenized in 0.14 M-NaCl/10 mM-sodium phosphate, pH 7.4, and samples containing approx. 15 μg of protein

Table 1. Purification of prolyl 4-hydroxylase from *Volvox carteri*

Purification step	Protein (mg)	Total activity (nmol of CO_2/s)	Specific activity (nmol of CO_2/s per g)	Recovery (%)	Purification (fold)
15000 *g* supernatant	350	15.0	43	100	1
DE52 DEAE-cellulose	52	11.3	218	75	5
Poly(L-hydroxyproline)–Sepharose					
After elution with poly-(L-hydroxyproline)	0.002	82.8×10^{-3}	41.4×10^{3}	0.6	963
After elution with 6 M-urea	0.009	45.9×10^{-3}	5.1×10^{3}	0.3	119

were injected into a rabbit at multiple sites both intradermally and subcutaneously at 2-week intervals. The antigen was mixed with Freund's complete adjuvant for the initial injection, and thereafter four times with Freund's incomplete adjuvant. The polyclonal antiserum against protein disulphide-isomerase isolated from chick embryos was a gift from Dr. Juha Koivu, Department of Medical Biochemistry, University of Oulu, Finland.

Polyacrylamide-gel electrophoresis and immunoblotting

Electrophoresis of prolyl 4-hydroxylase under non-denaturing conditions was carried out at 4 °C in 8%-acrylamide separating gels and 4% stacking gels. Purified chick-embryo prolyl 4-hydroxylase (20 μg), prepared as described previously [12], was treated with 1 mM-dithiothreitol at 21 °C for 3 h before gel electrophoresis, to separate the α and β subunits. *Volvox carteri* DEAE flow-through (250 μg) and dithiothreitol-treated chick-embryo prolyl 4-hydroxylase were applied to the gel after mixing with glycerol (25%, v/v) and Bromophenol Blue. The lanes containing the chick-embryo enzyme were stained by silver [34] and those containing the *V. carteri* enzyme were cut into 0.5 cm slices. The slices were homogenized in solution A, incubated at 4 °C for 1 h, and the supernatants collected by centrifugation (1000 *g*, 5 min) and assayed for enzyme activity. SDS/polyacrylamide-gel electrophoresis (10% separating gel, 4.5% stacking gel) was performed as described previously [35], and the gels were blotted on to nitrocellulose paper [36]. Immunological staining of the blots was done with horseradish-peroxidase-conjugated goat anti-rabbit IgG and 4-chloro-1-naphthol (Bio-Rad).

Other assays

Prolyl 4-hydroxylase was assayed as described previously [21], except that *V. carteri* enzyme assays were carried out at 40 °C. Protein concentrations were determined by the method of Bradford [37].

RESULTS

Purification of *Volvox carteri* prolyl 4-hydroxylase

A 70 litre volume of *Volvox carteri* culture medium was harvested to yield approx. 100 ml of packed spheres. The 15000 *g* supernatant fraction, prepared after sonication of the broken spheres, was the starting material for enzyme purification (Table 1). DEAE-cellulose chromatography under conditions in which the enzyme did not bind yielded a 5-fold purification, with substantial recovery of activity. The enzyme bound efficiently to poly(L-hydroxyproline)–Sepharose, as judged by the decrease (60–90%) in the activity of the enzyme solution after overnight incubation with the affinity material. In addition, the poly(L-hydroxyproline)–Sepharose beads, after incubation, showed prolyl 4-hydroxylase activity (results not shown). The efficiency of elution of enzyme activity with poly(L-hydroxyproline) was low, rarely exceeding 1%. Subsequent elution with 6 M-urea caused the release of more of the purified enzyme, however, with decreased specific activity. The purified enzyme was a close doublet, with an apparent M_r of 65000 when analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 1). A weak doublet at M_r 55000–57000 was also observed. The purification was calculated to be 963-fold, although this value may be low, as it has not been corrected for losses in enzyme activity owing to dilution.

Two additional affinity columns, 3,4-dihydroxyphenylacetate–Sepharose and 3,4-dihydroxyphenylpropionate–Sepharose, bound prolyl 4-hydroxylase with high efficiency. The eluted proteins from both columns were identical when analysed on SDS/polyacrylamide gels (Fig. 1), and appeared as a single band at M_r 68000, and doublets at M_r 65000 and 55000–57000. The eluting ligand, 3,4-dihydroxybenzoate, which is an inhibitor of prolyl 4-hydroxylase, was not removed by dialysis, preventing satisfactory activity assays of the eluted material.

Properties of prolyl 4-hydroxylase from *Volvox carteri*

V. carteri spheroids yielded a prolyl 4-hydroxylase preparation (15000 *g* supernatant) with a specific activity of 30–40 nmol/s per g. This enzyme activity was comparable with that in similar extracts of *C. reinhardii* [21]. The enzyme focused as a single sharp peak at pI 5.4 in a preparative isoelectric-focusing column. In the absence of $(NH_4)_2SO_4$, the enzyme bound quantitatively to phenyl-Sepharose, suggesting that the protein is highly hydrophobic. Only 30% of the activity could be eluted by a solution containing 60% ethylene glycol, and this could only be achieved by elution as a single step. Very little activity was recovered by gradient elution with increasing concentrations of ethylene glycol.

The *V. carteri* enzyme hydroxylated poly(L-proline) (K_m 10 μM), as has been previously observed for plant prolyl 4-hydroxylases [15]. Proline or the dipeptides Ser-Pro or Ala-Pro could not serve as substrates for this algal enzyme, although hydroxylation of Ser-Pro has been reported for the enzyme from a higher plant [38]. The hydroxylation required Fe^{2+} (K_m 20 μM), 2-oxoglutarate (K_m 170 μM), O_2 and ascorbate (K_m 140 μM), as previously reported for the animal (see [9–12]) and plant enzymes

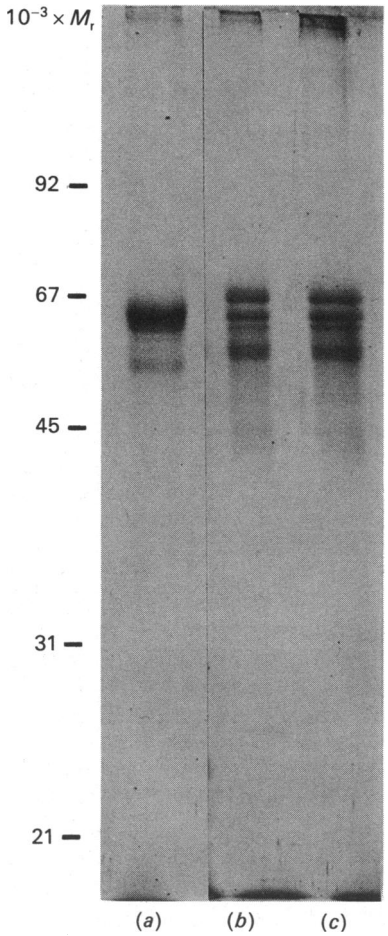

Fig. 1. Prolyl 4-hydroxylase from *Volvox carteri* isolated by affinity chromatographies

(*a*) Prolyl 4-hydroxylase purified by affinity chromatography on poly(L-hydroxyproline)–Sepharose. The active enzyme was eluted with poly(L-hydroxyproline), fractionated by SDS/polyacrylamide-gel electrophoresis and stained with Coomassie Blue. (*b*) and (*c*) Prolyl 4-hydroxylase purified by affinity chromatography on 3,4-dihydroxyphenylacetate–Sepharose (*b*) and 3,4-dihydroxyphenylpropionate–Sepharose (*c*). The proteins eluted with 3,4-dihydroxybenzoate were fractionated by SDS/polyacrylamide-gel electrophoresis and stained with Coomassie Blue. The positions of the M_r standards are indicated.

[13,15,21]. The K_m values for the co-substrates and cofactors were comparable with those of the *C. reinhardii* enzyme [21], with one exception. The K_m of 2-oxoglutarate was 5–6 times the value reported for *C. reinhardii*. The optimal pH and temperature were 6.6–6.8 and 40 °C respectively.

Both 3,4-dihydroxyphenylacetate (K_i 40 μM) and 3,4-dihydroxyphenylpropionate (K_i 24 μM) were competitive inhibitors with respect to 2-oxoglutarate and ascorbate (results not shown), as has been previously observed with the vertebrate prolyl 4-hydroxylase [32]. Poly(L-hydroxyproline) showed weak inhibitory activity (K_i 3 mM). The K_i values for the 2-oxoglutarate analogues pyridine-2,5-dicarboxylate (0.02 mM) and pyridine-2,4-dicarboxylate (0.008 mM) were the same as those reported for the *C. reinhardii* enzyme.

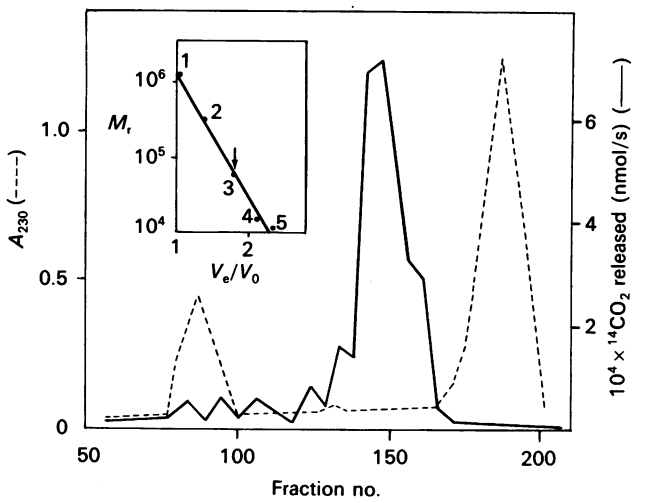

Fig. 2. Gel-filtration chromatography of prolyl 4-hydroxylase from *Volvox carteri*

A sample (4 ml) of 15000 *g* supernatant containing 6.8 mg of protein was chromatographed on Bio-Gel A 1.5 m. Fractions (2.35 ml) were collected, and were analysed for protein (----) and enzyme activity (100 μl portions; ——). The column was calibrated with the following standards: 1, Blue Dextran; 2, chick-embryo prolyl 4-hydroxylase; 3, ovalbumin; 4, poly(L-hydroxyproline), M_r 11000; 5, Phenol Red. Arrow indicates elution of *V. carteri* prolyl 4-hydroxylase.

Apparent M_r: comparison with other algal, higher-plant and vertebrate prolyl 4-hydroxylases

Gel filtration of the 15000 *g* supernatant from *V. carteri* on Bio-Gel A 1.5 m gave an apparent M_r of approx. 40000 (Fig. 2). In order to compare the behaviour of other algal and higher-plant prolyl 4-hydroxylases in gel filtration on various materials, extracts containing prolyl 4-hydroxylase activity from the green alga *Enteromorpha intestinalis* and the vascular plants *Helianthus tuberosum* and *Persea americana* were also chromatographed. Both the vascular-plant prolyl 4-hydroxylases were eluted from Bio-Gel A 0.5 m in the excluded fraction, with an apparent $M_r > 300000$. This is in agreement with the M_r previously reported for the enzyme from the vascular plant *Phaseolus vulgaris* (French bean) [19]. The enzymes from the green algae, on the other hand, were eluted with an apparent M_r of 40000 from Bio-Gel A 1.5 m, Bio-Gel A 0.5 m and Sephacryl S-300.

To compare directly the relative size of the active prolyl 4-hydroxylase from *V. carteri* with the dissociated α and β subunits of the vertebrate prolyl 4-hydroxylase, both enzymes were analysed by native polyacrylamide-gel electrophoresis (Fig. 3). The α and β subunits of purified chick prolyl 4-hydroxylase, dissociated by treatment with dithiothreitol, co-migrated with the active prolyl 4-hydroxylase from *V. carteri*.

Immunological cross-reactivity of algal prolyl 4-hydroxylase with the α subunit of the human enzyme

A polyclonal antiserum prepared against the M_r-65000 algal polypeptide recognized a M_r-65000 protein on immunoblots of partially purified prolyl 4-hydroxylase from *V. carteri* and *C. reinhardii* fractionated on SDS/

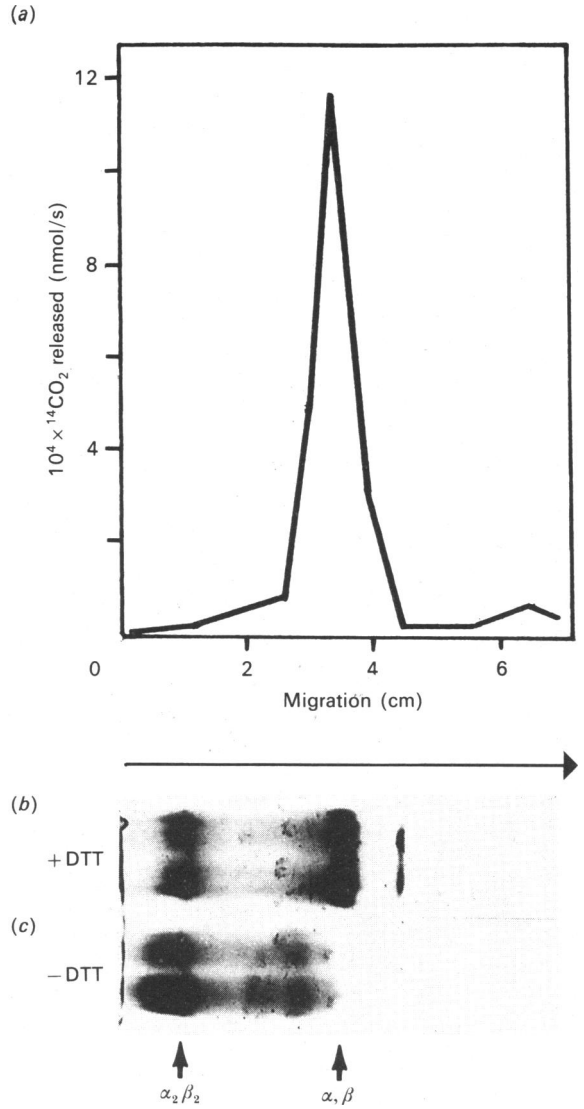

Fig. 3. Native-gel electrophoresis of prolyl 4-hydroxylase from *Volvox carteri*

(*a*) A sample (800 μl, containing 224 μg of protein) of *Volvox carteri* prolyl 4-hydroxylase partially purified by DEAE-cellulose DE52 chromatography (see the Materials and methods section) was mixed with 200 μl of glycerol containing a tracking dye and fractionated by native polyacrylamide-gel electrophoresis at 4 °C. After the tracking dye had migrated 7 cm (4 h at 20 mA), the gel lanes were cut into 0.5 cm slices, eluted into buffer, and samples were assayed for enzyme activity. The horizontal arrow indicates that the activity trace and the stained gel are the results of electrophoresis covering the same distance. (*b*) Prolyl 4-hydroxylase purified from chick embryos (20 μg) was treated with dithiothreitol (DTT; 1 mM final concn.) at room temperature for 4 h to dissociate the subunits, and fractionated by native polyacrylamide-gel electrophoresis as described above. The gel was stained with silver. (*c*) A sample identical with that in (*b*), but without dithiothreitol treatment. Vertical arrows indicate the positions of the tetramer ($\alpha_2\beta_2$) and dissociated α and β monomers.

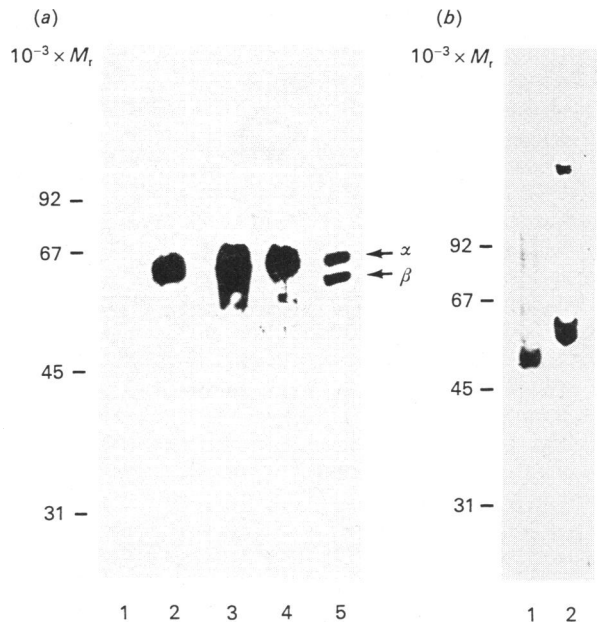

Fig. 4. Immunostaining of prolyl 4-hydroxylase from different sources by antiserum against *V. carteri* prolyl 4-hydroxylase and against chick-embryo protein disulphide-isomerase

(*a*) Immunoblots showing the reaction of pre-immune serum (lane 1) and polyclonal antiserum against algal prolyl 4-hydroxylase (lanes 2–4) with protein samples fractionated by SDS/polyacrylamide-gel electrophoresis. The protein sample in lanes 1 and 2 contained partially purified *C. reinhardii* prolyl 4-hydroxylase (DE52 DEAE-cellulose flow-through); lane 3, *V. carteri* prolyl 4-hydroxylase purified by affinity chromatography on poly(L-hydroxyproline)–Sepharose; lane 4, purified human prolyl 4-hydroxylase. Lane 5 shows an identical sample of purified human prolyl 4-hydroxylase fractionated on the same gel and stained with Coomassie Blue to show the positions of the α and β subunits. (*b*) Partially purified *V. carteri* prolyl 4-hydroxylase (lane 1) and purified chick prolyl 4-hydroxylase (lane 2) incubated with antiserum against protein disulphide-isomerase (prolyl 4-hydroxylase β subunit). For each set of blots, the positions of the M_r standards are indicated.

polyacrylamide gel (Fig. 4). However, only the denatured enzyme exposed the antigenic determinants, whereas no reactivity with the native algal enzyme was detected. When immunoblots of purified human prolyl 4-hydroxylase fractionated by SDS/polyacrylamide-gel electrophoresis were incubated with the anti-(algal prolyl 4-hydroxylase) serum, the α subunit showed strong cross-reactivity (Fig. 4). Incubation of an immunoblot of partially purified *V. carteri* prolyl 4-hydroxylase in polyclonal antiserum prepared against chick protein disulphide-isomerase (prolyl 4-hydroxylase β subunit) showed weak cross-reactivity with an algal protein of M_r 50000 (Fig. 4), but no cross-reactive protein of M_r 55000–57000 could be detected.

DISCUSSION

Recently we investigated the prolyl 4-hydroxylase from a lower plant, the green alga *Chlamydomonas reinhardii* [21], and observed that the M_r of the active enzyme was much lower than the values for the higher-plant [19] and vertebrate [10] enzymes as determined by gel filtration. In all other respects, i.e. cofactor, substrate

and co-substrate requirements, it closely resembled the higher-plant enzyme. Moreover, inhibition studies with 2-oxoglutarate analogues indicated that the catalytic sites of the algal and vertebrate enzymes have structural similarities [21].

The existence of a small, possibly monomeric, form of prolyl 4-hydroxylase in a lower plant presents intriguing possibilities for the study of the structure of the catalytic active site and the evolution of peptidyl proline hydroxylation. It was therefore significant to establish whether the enzyme of *C. reinhardii* was exceptional in this respect, or whether this low-M_r form of the enzyme was also found in other green algae. The alga selected for study was *Volvox carteri*, a colonial relative of *C. reinhardii*.

Prolyl 4-hydroxylase extracted from *V. carteri* had by gel filtration an apparent M_r of about 40000, thus identical with that of *C. reinhardii*. The value was not altered by chromatography at 21 °C, and thus the low M_r observed was apparently not the result of subunit dissociation at 4 °C owing to any type of weakened hydrophobic interactions. Since *Volvox* is a multicellular green alga, the low-M_r enzyme apparently is not restricted to unicellular species. However, *Volvox* and *Chlamydomonas* both belong to the same order (Volvocales) of green algae. To establish whether this form of prolyl 4-hydroxylase is limited to this order, an extract from *Enteromorpha intestinalis*, a green alga belonging to the order Ulvales, was analysed. *Enteromorpha* prolyl 4-hydroxylase also had an apparent M_r of 40000. In contrast, the M_r of > 300000 for the higher-plant enzyme reported by Bolwell [19] was confirmed by extraction and analysis of prolyl 4-hydroxylase from two additional higher plants, *Persea americana* and *Helianthus tuberosum*, under conditions identical with those employed for the algal enzymes. These data indicate that the green algae have a unique low-M_r form of prolyl 4-hydroxylase.

The enzyme from *V. carteri* was purified by anion-exchange chromatography followed by affinity chromatography on poly(L-hydroxyproline)–Sepharose. The active enzyme appeared as a close doublet of M_r 65000 on fractionation by SDS/polyacrylamide-gel electrophoresis. An additional doublet (M_r 55000–57000) was also observed, but the stoichiometry of the M_r-65000 and -55000–57000 doublets varied, as estimated by Coomassie Blue staining. The two other affinity columns developed in this study, 3,4-dihydroxyphenylacetate and 3,4-dihydroxyphenylpropionate coupled to Sepharose, both bound algal prolyl 4-hydroxylase with high efficiency. The reason for the difference between the apparent M_r of the algal enzyme determined by gel filtration and by SDS/polyacrylamide-gel electrophoresis is not yet understood. The conformation of the enzyme in its native configuration may retard its elution from gel-filtration columns, or the enzyme may have an affinity for the gel matrices. The behaviour of the algal enzymes on the gel-filtration columns, however, was independent of the matrix material employed.

Prolyl 4-hydroxylase from *C. reinhardii* was recently reported by Blankenstein *et al.* [20] to be composed also of a M_r-65000 doublet and a M_r-60000 component. The conclusion by Blankenstein *et al.* [20] that these represent subunits analogous to the α and β subunits of the vertebrate enzyme and assemble into an active tetramer of M_r 250000, however, is not supported by the present data. Prolyl 4-hydroxylase purified from the higher plant *Phaseolus vulgaris* is an oligomer with a subunit M_r of 65000 [19]. The M_r-60000 β subunit of the vertebrate prolyl 4-hydroxylase has recently been shown to also act as protein disulphide-isomerase [27,28], and an antiserum to the vertebrate protein disulphide-isomerase recognized a M_r-50000 protein in a partially purified algal preparation, but did not stain the M_r-55000–57000 or the M_r-65000 doublet.

Several factors, in addition to M_r value, related the M_r-64000 α subunit of the vertebrate enzyme with the M_r-65000 algal prolyl 4-hydroxylase. The α subunit of the vertebrate prolyl 4-hydroxylase appears to contribute a major part to the catalytic site of the enzyme, as it probably contains the peptide-binding region [23], 2-oxoglutarate-attachment site [24,25] and also a major part of the ascorbate-binding region [26]. Studies with analogues of 2-oxoglutarate suggest that the algal enzyme contains the same structural binding sites for this co-substrate as suggested for the vertebrate enzyme [21]. In the present study, the antiserum prepared against the M_r-65000 algal prolyl 4-hydroxylase was found to cross-react with the α subunit of the purified vertebrate enzyme on immunoblots, indicating that antigenic determinants on the algal enzyme are present on the α subunit. Any reactivity with the β subunit was below the level of detection by immunoblot analysis. A monoclonal antibody specific to the β subunit of human prolyl 4-hydroxylase was previously found to inhibit the activity of the *Chlamydomonas* enzyme [21], suggesting, however, that the algal enzyme monomer contains also sequences similar to those in the β subunit of human prolyl 4-hydroxylase. In order to define the full extent of the similarities and differences between the algal enzyme and the vertebrate α and β subunits, the amino acid sequences of these molecules must be compared.

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