Evidence for 4-Hydroxyproline in Viral Proteins

CHARACTERIZATION OF A VIRAL PROLYL 4-HYDROXYLASE AND ITS PEPTIDE SUBSTRATES

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4-Hydroxyproline, the characteristic amino acid of collagens and collagen-like proteins in animals, is also found in certain proline-rich proteins in plants but has been believed to be absent from viral and bacterial proteins. We report here on the cloning and characterization of a prokaryotic algal virus, Paramecium bursaria Chlorella virus-1, of a 242-residue polypeptide, which shows distinct sequence similarity to the C-terminal half of the catalytic α subunits of animal prolyl 4-hydroxylases. The recombinant polypeptide, expressed in Escherichia coli, was found to be a soluble monomer and to hydroxylate both (Pro-Pro-Gly)₁₀ and poly(t-proline), the standard substrates of animal and plant prolyl 4-hydroxylases, respectively. Synthetic peptides such as (Pro-Ala-Pro-Lys)₉, (Ser-Pro-Lys-Pro-Pro)₉, and (Pro-Glu-Pro-Pro-Ala)₅ corresponding to proline-rich repeats coded by the viral genome also served as substrates. (Pro-Ala-Pro-Lys)₁₀ was a particularly good substrate, with a Km of 20 μM. The prolines in both positions in this repeat were hydroxylated, those preceding the alanines being hydroxylated more efficiently. The data strongly suggest that P. bursaria Chlorella virus-1 expresses proteins in which a number of proline residues become hydroxylated by a viral prolyl 4-hydroxylation with many unique properties. Thus the occurrence of 4-hydroxyproline in proteins is probably not restricted to certain animal and plant proteins.

EXPERIMENTAL PROCEDURES

Identification of the PBCV-1 Prolyl 4-Hydroxylase-like Polypeptide—A sequence homology search in GenBank™ using the Basic Local Alignment Search Tool (12) indicated the presence in the PBCV-1 genome (accession number U42580) of an open reading frame encoding a 242-amino acid polypeptide that shows a distinct amino acid sequence similarity to the C-terminal half of the catalytic α subunits of animal prolyl 4-hydroxylases. In addition, the genome contains many open reading frames for proteins with proline-rich repeats. The recombinant viral polypeptide, expressed in E. coli, was found to be a soluble monomer and to hydroxylate (Pro-Pro-Gly)₁₀, poly(t-proline), and several synthetic peptides corresponding to proline-rich repeats coded by the viral genome. The data strongly suggest that PBCV-1 expresses proteins in which a number of proline residues become hydroxylated by a viral prolyl 4-hydroxylase with many unique properties. Thus the occurrence of 4-hydroxyproline in proteins is probably not restricted to certain animal and plant proteins.

4-Hydroxyproline is the characteristic amino acid of collagens and more than 10 other animal proteins with collagen-like sequences. This amino acid plays a central role in all collagens, as the hydroxy groups of the 4-hydroxyproline residues are essential for the formation of the collagen triple helix at body temperature. 4-Hydroxyproline is also found in certain proline-rich plant proteins, but it has been believed to be absent from viral and bacterial proteins (for reviews, see Refs. 1–4).

The formation of 4-hydroxyproline is catalyzed by prolyl 4-hydroxylases that act on proline residues in peptide linkages. The vertebrate enzymes are 240-kDa α₁β₂ tetramers, in which the catalytic sites are located in the α subunits and the β subunits are identical to the enzyme and chaperone protein disulfide isomerase. They require Fe²⁺, 2-oxoglutarate, O₂, and ascorbate and hydroxylate X-Pro-Gly- sequences (for reviews, see Refs. 5 and 6). Prolyl 4-hydroxylases from higher plants may resemble the vertebrate enzymes in their structure (7), whereas prolyl 4-hydroxylases from multicellular and unicellular green algae are 60-kDa monomers (8, 9). Plant prolyl 4-hydroxylases require the same co-substrates as the animal enzymes, but they differ from the latter in that they hydroxylate proline residues in poly(t-proline) and poly(t-proline)-like sequences, while the repeating X-Pro-Gly- triplets are either very poor substrates or not hydroxylated at all (2, 8).

We report here that the genome of Paramecium bursaria Chlorella virus-1 (PBCV-1; Refs. 10 and 11) encodes a 242-amino acid polypeptide that shows a distinct amino acid sequence similarity to the C-terminal half of the catalytic α subunits of animal prolyl 4-hydroxylases. In addition, the genome contains many open reading frames for proteins with proline-rich repeats. The recombinant viral polypeptide, expressed in E. coli, was found to be a soluble monomer and to hydroxylate (Pro-Pro-Gly)₁₀, poly(t-proline), and several synthetic peptides corresponding to proline-rich repeats coded by the viral genome. The data strongly suggest that PBCV-1 expresses proteins in which a number of proline residues become hydroxylated by a viral prolyl 4-hydroxylase with many unique properties. Thus the occurrence of 4-hydroxyproline in proteins is probably not restricted to certain animal and plant proteins.

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Cloning and Expression in E. coli of the PBCV-1 Prolyl 4-Hydroxylase-like Polypeptide—PCR primers 5′-CCGCCATATGAGGGTTT-GAAACCCAGGAT-3′ and 5′-CCGGTCTGAGTCCATTTAAACAGCAG-GATTTT-3′ were synthesized based on the viral DNA sequence and used to obtain a 621-base pair PCR product flanked by NdeI and XhoI restriction sites from the viral genomic DNA. This PCR product coding for the amino acids Glu-36–Lys-242 of the viral prolyl 4-hydroxylase-like polypeptide was cloned to pET21a for expression in E. coli BL21(DE3) strain (Novagen). The cells were grown at 37 °C to an optical density of 0.55 at 600 nm, incubated at 28 °C for 30 min, and expression was

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induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 0.8 mM. The cells were harvested 3 h after induction, suspended in a phosphate buffer, pH 7.9, sonicated until the sample was no longer viscous, centrifuged at 38,000 × g for 30 min, and the soluble and insoluble fractions were analyzed by 12% SDS-PAGE.

Protein Purification—The recombinant PBCV-1 polypeptide was purified by applying the soluble fraction of the cell lysate to a Ni^{2+}-chelate affinity column (Invitrogen); the unbound material was removed by washing with a solution of 50 mM NaCl, 0.5 M Tris, pH 7.9; and the recombinant polypeptide was eluted by increasing the imidazole concentration to 0.5 M. The fractions were analyzed by 12% SDS-PAGE and those containing the polypeptide were pooled and concentrated with Macrosep 10K concentrators (Filtron). The apparent molecular weight of the purified protein was estimated by applying it to a calibrated HiLoad 16/60 Superdex S-200 column (Amersham Pharmacia Biotech). The residues present in the various subunits that precede the alignment region are not shown. The human α1 and α2 subunits, respectively, and the C. elegans and Drosophila a subunits are indicated by bars.

Assays—Prolyl 4-hydroxylase activity was assayed by a method based on the hydroxylation-coupled decarboxylation of 2-oxo-[1-^{14}C]glutarate (19). In some experiments the (Pro-Ala-Pro-Lys)5 substrate was used instead of the PBCV-1 substrate. Assays were performed as described previously (20).

**RESULTS AND DISCUSSION**

The PBCV-1 Genome Encodes a Prolyl 4-Hydroxylase-like Polypeptide—A sequence homology search indicated that the genome of PBCV-1 (Refs. 10 and 11; GenBank™ accession number U42580) contains an open reading frame encoding a 242-amino acid polypeptide that shows a distinct sequence similarity to the C-terminal half of the catalytic α subunits of prolyl 4-hydroxylases from various animal sources (Fig. 1). A putative signal sequence is located at its N terminus, the most likely first amino acid of the processed viral polypeptide being glutamate (Fig. 1), based on the computational parameters of von Hejne (18). Thus the length of the signal peptide is probably 32 residues and that of the processed polypeptide 210 amino acids. The sequence of the processed viral polypeptide is 20% identical to residues 294–504 in the 517-residue a subunit of human type I prolyl 4-hydroxylase (15) and 15–23% identical to the corresponding residues in the α subunits of the human type II prolyl 4-hydroxylase (14) and the C. elegans (15) and D. melanogaster (16) prolyl 4-hydroxylases (Fig. 1). The two histidines and one aspartate that bind the Fe^{2+} atom at the catalytic site (20–22) and the lysine that binds the C-5 carboxyl group of 2-oxoglutarate (20) are all conserved in the PBCV-1 sequence (His-152, Asp-154, His-221, and Lys-231 in Fig. 1). Since the last mentioned residue in all other 2-oxoglutarate dioxygenases, including the closely related enzyme lysyl hydroxylase (23), is an arginine (21, 24, 25), we regarded it as possible that the viral polypeptide might be a prolyl 4-hydroxylase. The fifth critical residue at the catalytic site of the vertebrate prolyl 4-hydroxylases, a histidine that is probably involved in the binding of the C-1 carboxyl group of 2-oxoglutarate to the Fe^{2+} atom and in the decarboxylation of this cosubstrate (20), is replaced in the PBCV-1 sequence as in the Drosophila a subunit sequence by an arginine (Arg-239 in Fig. 1). However, the PBCV-1 sequence shows no similarity to the peptide substrate binding domain present between residues 140–240 in the a subunits of animal prolyl 4-hydroxylases (26).

The Recombinant PBCV-1 Prolyl 4-Hydroxylase-like Polypeptide Is a Soluble Monomer—To express the viral polypeptide in *E. coli*, the PBCV-1 DNA sequence coding for amino acids Glu-36–Lys-242 was synthesized by PCR, cloned into the pET-15b vector with an N-terminal histidine tag, and transformed into the BL21(DE3) host strain. Expression of the polypeptide was induced with IPTG, and the cells were incubated at 28 °C for 3 h. The cells were then harvested, suspended in a Tris-HCl buffer, pH 7.9, containing 5 mM imidazole, sonicated, and the soluble and insoluble fractions were analyzed by 12% SDS-PAGE and Coomassie Blue staining (Fig. 2, lanes 2 and 3). The expressed recombinant polypeptide was mainly found in the soluble fraction (Fig. 2, lane 2) and could be purified using a Ni^{2+}-chelate affinity column and imidazole elution (Fig. 2, lane 4). Gel filtration in a calibrated Superdex S-200 column indicated that the recombinant polypeptide had an apparent molecular weight of about 30,000 (details not shown). As the calculated molecular weight of the recombinant polypeptide with the N-terminal histidine tag and the thrombin cleavage site is 27,195, the recombinant polypeptide was apparently a monomer.

The Recombinant PBCV-1 Polypeptide Hydroxylates Both (Pro-Pro-Gly)_{10} and Poly(L-proline)—To study whether the vi-
polypeptide purified by a Ni²⁺-affinity chromatography fraction of the cell sonicates, and lane 4 shows the recombinant polypeptide purified by a Ni²⁺-chelate affinity column. The samples were analyzed by 12% SDS-PAGE and Coomassie Blue staining. Molecular weight markers are shown in lane 1.

Viral Prolyl 4-Hydroxylase

![Graph](image)

**Figure 2. Analysis of the expression of the PBCV-1 prolyl 4-hydroxylase-like polypeptide in E. coli by SDS-PAGE under reducing conditions.** A recombinant pET-15b vector coding for amino acids Glu-36–Lys-242 of the PBCV-1 prolyl 4-hydroxylase-like polypeptide was transformed into the *E. coli* BL21(DE3) host strain. The expression was induced, and the cells were harvested as described under "Experimental Procedures." Lanes 2 and 3 show the soluble and insoluble fractions of the cell sonicates, and lane 4 shows the recombinant polypeptide purified by a Ni²⁺-chelate affinity column. The samples were analyzed by 12% SDS-PAGE and Coomassie Blue staining. Molecular weight markers are shown in lane 1.

The polypeptide had any prolyl 4-hydroxylase activity, 10 μg of the purified protein was assayed as a possible enzyme by a method based on the hydroxylation-coupled decarboxylation of 2-oxo-[1-14C]glutarate (19). When 0.5 mg/ml of (Pro-Pro-Gly)₁₀ was used as the peptide substrate, the amount of 14CO₂ generated was 6450 cpm, whereas various negative controls gave less than 500 cpm. Poly(L-proline), Mᵣ = 40,000, a competitive inhibitor of animal prolyl 4-hydroxylases (5, 6), also acted as a substrate, giving 6850 cpm under the above conditions. The pH optimum of the hydroxylation reaction was 7.0 (details not shown).

The viral enzyme, like the animal and plant prolyl 4-hydroxylases, required Fe²⁺, 2-oxoglutarate, O₂, and ascorbate (details not shown). The Kₘ values for the cosubstrates Fe²⁺, 2-oxoglutarate, and ascorbate were very similar to those of human type I prolyl 4-hydroxylase (Table I), suggesting that the cofactor binding sites of these enzymes may be similar. However, the Kₘ value of the viral enzyme for the peptide substrate (Pro-Pro-Gly)₁₀ was about 150-fold (Table I), and the Kₘ values for poly(L-proline), Mᵣ = 13,000 and 40,000 (Table I), were also much higher than those of 23 and 7 μM reported for the viral enzyme (16), the human enzyme (26), and the viral enzyme for poly( L-proline) was more than 1 order of magnitude higher than those reported for plant enzymes (2, 8, 9). The best peptide substrates of the viral enzyme, (Pro-Ala-Pro-Lys)₁₀, but only at a very low rate (8). The hydroxylation of poly(L-proline) is a property of plant prolyl 4-hydroxylases (2), whereas poly(L-proline) is a competitive inhibitor of the animal enzymes (6), but the Kₘ values of the viral enzyme for poly(L-proline) were more than 1 order of magnitude higher than those reported for plant enzymes (2, 8, 9). The best peptide substrates of the viral enzyme, (Pro-Ala-Pro-Lys)₁₀ and (Ser-Pro-Lys-Pro-Pro)₁₀, correspond to sequences coded by the viral genome. The Kₘ values for the authentic viral polypeptides may be even lower, as the Kₘ values decreased with an increase in the chain length of the substrates and as the actual viral repeat sequences range up to (Pro-Ala-Pro-Lys)₁₀ and (Ser-Pro-Lys-Pro-Pro)₁₀.

### Table I

| Substrate                  | Kₘ (μM) | Vₘax (cpm/μg) |
|----------------------------|---------|---------------|
| (Ser-Pro-Lys-Pro-Pro)₁₀    | 20      | 1800          |
| (Pro-Glu-Pro-Pro-Ala)₁₀    | 1000    | 400           |
| Lys-Pro-Ala                | 8900    | 400           |
| Pro-Ala-Pro-Lys            | 4800    | 400           |
| Pro-Ala-Pro-Lys            | 950     | 4600          |
| Pro-Ala-Pro-Lys            | 310     | 13,500        |
| Pro-Ala-Pro-Lys            | 50      | 10,300        |
| Pro-Ala-Pro-Lys(Pro)₁₀     | 20      | 11,900        |

a Ref. 20.  
b cl, competitive inhibitor.

### Table II

| Substrate                  | Kₘ (μM) | Vₘax (μM) |
|----------------------------|---------|-----------|
| (Ser-Pro-Lys-Pro-Pro)₁₀    | 20      | 1800      |
| (Pro-Glu-Pro-Pro-Ala)₁₀    | 1000    | 400       |
| Lys-Pro-Ala                | 8900    | 400       |
| Pro-Ala-Pro-Lys            | 4800    | 400       |
| Pro-Ala-Pro-Lys            | 950     | 4600      |
| Pro-Ala-Pro-Lys            | 310     | 13,500    |
| Pro-Ala-Pro-Lys            | 50      | 10,300    |
| Pro-Ala-Pro-Lys(Pro)₁₀     | 20      | 11,900    |

### Table III

| Substrate                  | Kₘ (μM) | Vₘax (μM) |
|----------------------------|---------|-----------|
| (Ser-Pro-Lys-Pro-Pro)₁₀    | 20      | 1800      |
| (Pro-Glu-Pro-Pro-Ala)₁₀    | 1000    | 400       |
| Lys-Pro-Ala                | 8900    | 400       |
| Pro-Ala-Pro-Lys            | 4800    | 400       |
| Pro-Ala-Pro-Lys            | 950     | 4600      |
| Pro-Ala-Pro-Lys            | 310     | 13,500    |
| Pro-Ala-Pro-Lys            | 50      | 10,300    |
| Pro-Ala-Pro-Lys(Pro)₁₀     | 20      | 11,900    |

### Table IV

| Substrate                  | Kₘ (μM) | Vₘax (μM) |
|----------------------------|---------|-----------|
| (Ser-Pro-Lys-Pro-Pro)₁₀    | 20      | 1800      |
| (Pro-Glu-Pro-Pro-Ala)₁₀    | 1000    | 400       |
| Lys-Pro-Ala                | 8900    | 400       |
| Pro-Ala-Pro-Lys            | 4800    | 400       |
| Pro-Ala-Pro-Lys            | 950     | 4600      |
| Pro-Ala-Pro-Lys            | 310     | 13,500    |
| Pro-Ala-Pro-Lys            | 50      | 10,300    |
| Pro-Ala-Pro-Lys(Pro)₁₀     | 20      | 11,900    |

4-hydroxyproline in the (Pro-Ala-Pro-Lys)₁₀ peptide was verified by amino acid analysis of the peptide purified from the hydroxylation reaction mixture by reverse phase HPLC (details not shown).

The substrate requirements of the viral enzyme thus differed distinctly from those of both animal and plant prolyl 4-hydroxylases. The hydroxylation of (Pro-Pro-Gly)₁₀ is a property similar to that of animal prolyl 4-hydroxylases. Although the Kₘ of 2900 μM is much higher than the Kₘ of the human type I and type II enzymes (26), the Kₘ of 20 μM of the C. elegans enzyme (27) and 260 μM of the *D. melanogaster* enzyme (16), the Vₘax of the viral enzyme for (Pro-Pro-Gly)₁₀ was similar to its Vₘax values for poly(L-proline) and the best polypeptide substrates. Some plant prolyl 4-hydroxylases also hydroxylate (Pro-Pro-Gly)₁₀, but only at a very low rate (8). The hydroxylation of poly(L-proline) is a property of plant prolyl 4-hydroxylases (2), whereas poly(L-proline) is a competitive inhibitor of the animal enzymes (6), but the Kₘ values of the viral enzyme for poly(L-proline) were more than 1 order of magnitude higher than those reported for plant enzymes (2, 8, 9). The best peptide substrates of the viral enzyme, (Pro-Ala-Pro-Lys)₁₀ and (Ser-Pro-Lys-Pro-Pro)₁₀, correspond to sequences coded by the viral genome. The Kₘ values for the authentic viral polypeptides may be even lower, as the Kₘ values decreased with an increase in the chain length of the substrates and as the actual viral repeat sequences range up to (Pro-Ala-Pro-Lys)₁₀ and (Ser-Pro-Lys-Pro-Pro)₁₀.
probable that the occurrence of 4-hydroxyproline in proteins is not restricted to certain animal and plant proteins.

The function of 4-hydroxyproline residues in all collagens and collagen-like proteins in animals is to stabilize their triple-helical structures (3, 6, 30). The functions of these residues in plant proteins are less well characterized but are also likely to involve stabilization of structures (4). The 4-hydroxyproline residues in plant proteins are often O-glycosylated, and the glycosylation is probably important for the structural role of the proteins in plant cells (1, 4). The functions of the 4-hydroxyproline residues in viral proteins are likely to be similar to those in animal and plant proteins, but work will be needed to elucidate these functions and to determine whether 4-hydroxyproline residues in viral proteins serve as attachment sites for carbohydrate units.

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FIG. 3. Analysis of the hydroxylation of the proline residues in (Pro-Ala-Pro-Lys)₅ by the PBCV-1 prolyl 4-hydroxylase. The hydroxylation reaction was carried out with 80 µg/ml of (Pro-Ala-Pro-Lys)₅ as the substrate in the standard prolyl 4-hydroxylase reaction mixture under conditions that gave a high extent but not complete hydroxylation of the substrate. The peptide substrate was purified from the reaction mixture by HPLC and subjected to N-terminal sequencing. The columns indicate the degree of hydroxylation of the various proline residues in the hydroxylated peptide. P = proline; A = alanine; K = lysine.