Affinity purification and characterization of protein gene product 9.5 (PGP9.5) from retina

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Protein gene product 9.5 (PGP9.5) is a cytosolic protein that is highly expressed in vertebrate neurons, which is now included in the ubiquitin C-terminal hydrolase subclass (UCH) on the basis of primary-structure homology and hydrolytic activity on the synthetic substrate ubiquitin ethyl ester (UboEt). Some UCHs show affinity for immobilized ubiquitin, a property exploited to purify them. In this study we show that this property can also be applied to PGP9.5, since a protein has been purified to homogeneity from bovine retina by affinity chromatography on a

ubiquitin–Sepharose column that can be identified with: (a) PGP9.5 with respect to molecular mass, primary structure and immunological reactivity; (b) the known UCHs with respect to some catalytic properties, such as hydrolytic activity on UboEt, (which also characterizes PGP9.5), K_m value and reactivity with cysteine and histidine-specific reagents. However, it differs with respect to other properties, e.g. inhibition by UboEt and a wider pH range of activity.

INTRODUCTION

The neuron-specific protein gene product 9.5 (PGP9.5) is a cytosolic 24.8 kDa protein [1]. It is widespread and highly expressed in vertebrate neurons and neuroendocrine cells [2], where it constitutes 1–5% of the total soluble proteins [3]. The role of PGP9.5 is still debated [3]; however, on the basis of primary-structure homology and hydrolytic activity with the synthetic substrate, ubiquitin ethyl ester (UboEt), it is now considered to belong to the ubiquitin C-terminal hydrolase subclass (UCH; EC 3.1.2.15) [1,4], members of which are involved in the hydrolysis of esters and amides at the C-terminal glycine residue of ubiquitin [5–7] as well as in the recovery of ubiquitin from ubiquitin–protein conjugates [8]. The best characterized of these hydrolases are those from erythrocytes and thymus. A property of these enzymes is their affinity for immobilized ubiquitin, and this has been exploited for their purification to homogeneity by means of ubiquitin–Sepharose affinity columns [6–8]. In view of the homology in the primary structure, it might be hypothesized that PGP9.5 shares with the UCHs the affinity for immobilized ubiquitin. To assess the validity of this hypothesis we attempted to purify PGP9.5 using the aforesaid affinity-chromatographic techniques instead of the protocol developed by Doran et al. [2], based on standard chromatography. If successful, a new property of PGP9.5 would be detected and the characterization of the catalytic properties of the molecule made easier.

MATERIALS AND METHODS

Purification of PGP9.5

PGP9.5 was purified according to the small-scale UCH preparation described by Pickart and Rose [6]; however, gradient-elution chromatography on a Mono Q column was used instead of two-step ion-exchange chromatography on DE-52.

Step 1

Bovine retinas were separated immediately after slaughter, washed five times in ice-cold 0.9% NaCl and homogenized at 4 °C in 20 mM Tris/HCl, pH 7.4, containing 1 mM EDTA, 1 mM PMSF, 1 µg/ml leupeptin and 20 mM 2-mercaptoethanol, using a Potter-Elvehjem apparatus equipped with a motor-driven Teflon pestle. The homogenate was centrifuged at 2000 g for 15 min at 4 °C; the resulting supernatant was ultracentrifuged at 250 000 g for 60 min at 4 °C; the clear supernatant (supernatant 2) was processed in step 2.

Step 2

After dialysis against 30 vol. of 25 mM Tris/HCl, pH 7.4, containing 20 mM 2-mercaptoethanol and 1 mM EDTA (buffer A), 30 mg of total proteins from supernatant 2 was applied to a Mono Q HR5/5 FPLC anion-exchange column (Pharmacia-Biotech, Uppsala, Sweden) pre-equilibrated in the same buffer at a flow rate of 0.5 ml/min; bound proteins were eluted by linearly increasing the KCl concentration in buffer A from 0 to 0.5 M over 60 min. The eluate was monitored at 280 nm and peaks were collected manually.

Step 3

Each peak from step 2 was briefly dialysed against 50 mM Tris/HCl, pH 7.2, containing 0.1 mM EDTA and 0.2 mM dithiothreitol (DTT) and subjected to affinity chromatography on a 2.5 ml ubiquitin–Sepharose column as described by Pickart and Rose [6]. The eluate (25 ml) was collected in 1 ml fractions and analysed for protein composition by SDS/PAGE (12% gel). The ubiquitin–Sepharose column was prepared from commercial bovine erythrocyte ubiquitin (Sigma, St. Louis, MO, U.S.A.) and activated CH-Sepharose 4B (Pharmacia-Biotech) as described by Ciechanover et al. [9].

Abbreviations used: DTT, dithiothreitol; TFA, trifluoroacetic acid; UboEt, ubiquitin ethyl ester; UCH, ubiquitin C-terminal hydrolase; PTH, phenylthiohydantoin.

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Figure 1 HPLC analysis of commercial ubiquitin (0.8 μ g) (A) and of 10 μ l of the synthetic substrate UbOEt preparation before (B) and after (C) incubation with an aliquot of purified PGP9.5 for 10 min in the presence of 35 mM phosphate buffer, pH 7.5, 10 mM DTT and 1 mM EDTA

Peak I is ubiquitin, peak II is the 74-amino acid tryptic hydrolysis product of ubiquitin formed during the transpeptidation reaction, usually present in small amounts (about 10%) in the UbOEt preparation, and peak III is UbOEt. UbOEt was synthesized from commercial ubiquitin (Sigma) by a transpeptidation reaction catalysed by TosPheCH₂Cl-treated trypsin (0.7 mg/ml) at pH 7.6 in the presence of 1.6 M glycyl-glycine ethyl ester (Sigma) and 7 mg/ml ubiquitin and purified by gel-filtration and cation-exchange chromatography, as described by Wilkinson et al. [13].

Step 4

Fractions from step 3 were concentrated to 0.3 ml by centrifugal ultrafiltration on a Centricon 10 concentrator (Amicon, Danvers, MA, U.S.A.) and subjected to gel-filtration chromatography on a Superose 6 (Pharmacia-Biotech) column equilibrated with 20 mM Tris/HCl, pH 7.4, containing 100 mM NaCl and 20 mM 2-mercaptoethanol. The column was operated at a flow rate of 0.4 ml/min; the eluate was monitored at 220 nm and peaks were collected manually, added to 10% glycerol and stored frozen.

Western blotting

An aliquot of the purified protein from step 4 was subjected to SDS/PAGE (12% gel) and electrophoretically transferred to nitrocellulose; blots were assayed for reactivity towards commercial monoclonal antibody 13-C4 directed against human PGP9.5 (Ultraclone) as previously described [10].

Determination of primary structure

N-Terminal sequencing

An aliquot of the purified protein from step 4 was desalted by reverse-phase HPLC on a Nucleosil C₄ column (250 mm $\times$ 4.6 mm) equilibrated at a flow rate of 1 ml/min with aq. 0.1% (v/v) trifluoroacetic acid (TFA) and developed with a linear 60 min gradient from 0 to 100% of 75% acetonitrile/25% water containing 0.085% TFA. The eluate was monitored at 220 nm. The protein was eluted as a single peak of 1 ml, concentrated to 50 μ l under a stream of nitrogen and applied to a Polybrene filter disc for sequencing with an Applied Biosystem 475-A protein sequencer.

Purification of tryptic peptides

Purified protein from step 4 (3 nmol) was reductively alkylated as described by Matsudaira [11]; the carboxymethylated protein was hydrolysed overnight with 5 μ g of Tos-Phe-CH₂Cl-treated trypsin (Sigma) in the presence of 1% NH₄HCO₃, pH 7.8, containing 1 mM CaCl₂ and 0.02% Tween 20 (Sigma) in a final volume of 1 ml [12]. Tryptic peptides were purified by reverse-phase HPLC on a Toso-Haas 80-TS C₁₈ column (250 mm $\times$ 4.6 mm) equilibrated with aq. 0.1% TFA at a flow rate of 1 ml/min and developed with a linear 90 min gradient from 0 to 100% of 75% acetonitrile/25% water containing 0.085% TFA. The eluate was monitored at 215 nm; peaks were collected manually and subjected to a second chromatographic step using a Beckman Ultrapore C₈ column (250 mm $\times$ 4.6 mm) developed with the same mobile phase. The purified peptides were concentrated under a stream of nitrogen and sequenced as described above.

Assay of UCH activity

UCH activity was assayed as described by Wilkinson et al. [13] using UbOEt as substrate. Samples contained, in a final volume of 175 μ l, 10 mM DTT, 1 mM EDTA, 35 mM phosphate buffer, pH 7.4, various amounts of purified PGP9.5 from step 4 and 8 μ M UbOEt unless otherwise specified. All assays were performed at 37 $^{\circ}$ C. At the indicated times, the reaction was stopped with HClO₄ at a final concentration of 0.2%. After centrifugation, aliquots of the supernatant were loaded on a Merck Select-B reverse-phase column equilibrated at a flow rate of 1 ml/min with 50 mM NaClO₄ and 42% acetonitrile in 0.07% HClO₄ and subjected to isocratic HPLC analysis. Ubiquitin

formed during the hydrolytic reaction and the residual UbOEt were detected at 220 nm (Figure 1). Areas under the respective peaks were integrated automatically and the amount of ubiquitin formed was calculated from a standard calibration curve.

Kinetic measurements

Reciprocal initial velocities of the hydrolytic reaction were plotted versus reciprocal substrate concentrations and the experimental data were fitted by a non-linear regression program (Marquardt–Levenberg algorithm) to the general equation for substrate inhibition [14]:

$$v = \frac{VA}{K + A + A^2/K_i}$$

pH-dependence of the catalytic activity

The dependence of the cleavage of UbOEt on pH was assayed from pH 4.0 to 5.5 in 35 mM acetate buffer, from pH 5.5 to 8.0 in 35 mM phosphate buffer and from pH 8.0 to 10.0 in 35 mM Tris/HCl in the presence of 3.5 ng of the purified protein, 1 μ M UbOEt, 10 mM DTT and 1 mM EDTA.

Effect of group-specific reagents

Thiol groups were alkylated at room temperature by treating an aliquot of the purified protein (1.8 μ g) with 10 mM iodoacetic acid as described by Wilkinson et al. [13].

The selective modification of histidine residues was performed as described by Miles [15] on 1.8 μ g of the purified protein in the presence of 10 mM diethyl pyrocarbonate, for 5 min at room temperature. UCH activity was examined in the presence of 3.5 ng of the carboxymethylated or carbethoxylated protein and 1 μ M UbOEt as described above.

Purification of UCH from bovine erythrocytes

UCH was purified according to the small-scale procedure described by Pickart and Rose [6].

Protein quantification

The protein content of samples was determined by the method of Peterson [16].

RESULTS

Enzyme purification

The anion-exchange chromatography of the proteins contained in supernatant 2 resulted in an 11-peak profile (Figure 2). After affinity chromatography of each peak on a ubiquitin–Sepharose column (step 3), a ubiquitin-binding protein, which was apparently homogeneous on SDS/PAGE, was detected in peak V (Figure 3, lane a). The protein showed a molecular mass close to 25 kDa on SDS/PAGE and around 30 kDa on gel-filtration chromatography under native conditions (step 4). Moreover the protein cross-reacted in Western blots with a monoclonal antibody directed against human PGP9.5 (Figure 4).

As a positive control of the correct functioning of the ubiquitin–Sepharose column used in the purification of the 25 kDa protein from retina, the column was used to purify, by the method of Pickart and Rose [6], proteins from bovine erythrocytes known to bind ubiquitin immobilized on this solid support. The electrophoretic pattern of the purified proteins matched that described

Figure 2 Anion-exchange chromatography on Mono Q HR5/5 of supernatant 2 from retina homogenate

The dialysed supernatant was fractionated as detailed in the Materials and methods section. The eluate was monitored at 280 nm and the peaks were collected manually.

in the literature [6,17], with two main bands of about 100 and 27 kDa (Figure 3, lane b).

Structural analysis

Automated N-terminal degradation of the purified protein revealed the presence of only one N-terminal residue and showed that the sequence of the first 14 amino acids fully matched that derived from human PGP9.5 cDNA [1]; the alignment of the two N-terminal sequences is given below:

25 kDa protein: Met-Gln-Leu-Lys-Pro-Met-Glu-Ile-Asn-Pro-Glu-Met-Leu-Asn

Human PGP9.5: Met-Gln-Leu-Lys-Pro-Met-Glu-Ile-Asn-Pro-Glu-Met-Leu-Asn

The sequence of some of the internal peptides, aligned with the deduced amino acid sequence of human PGP9.5, is reported in Figure 5. Only two substitutions could be observed out of 83 unambiguously identified amino acids: at position 187 Thr was substituted for Ala and at position 195 Gln for Lys. At position 97 bovine PGP9.5, like its human equivalent, has a histidine residue, which is highly conserved among species and considered as a potential active-site residue [1].

Enzyme kinetics

The hydrolytic reaction rate at a substrate concentration of 8 μ M and pH 7.4 resulted in a linear plot when tested as a function of enzyme concentration and time of incubation. These results indicate that the enzyme was stable in the conditions selected for the assay and that no inhibition was exerted by the reaction products (ubiquitin and ethanol). When the substrate concen-

Figure 3 SDS/PAGE of the purified protein from step 3 (lane a) and of the ubiquitin–Sepharose column eluate from the erythrocyte preparation (lane b)

Standard proteins were: myosin subunit, 205 kDa; β -galactosidase subunit, 116 kDa; phosphorylase *b* subunit, 97.5 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase subunit 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor from soya bean, 20.1 kDa; α -lactalbumin, 14.2 kDa. The gel was stained with Brilliant Blue G-colloidal (Sigma) according to the manufacturer's instructions.

Figure 4 Western-blot analysis of the 25 kDa purified protein

Aliquots of the purified protein (15 μ l) were subjected to SDS/PAGE (12% gel) and electrotransferred overnight on to nitrocellulose. After transfer, the membrane was blocked for 6 h with 5% instant non-fat dry milk. The membrane was then washed and incubated overnight with monoclonal antibody 13-C4 (Ultraclone) against PGP9.5. The washed membranes were then incubated with the appropriate secondary antibody conjugated with horseradish peroxidase, and detection of the immunocomplexes was achieved by incubating the washed membranes with 3,3'-diaminobenzidine tetrahydrochloride (Sigma). Prestained molecular-mass markers (Bio-Rad) were: phosphorylase *b* (106 kDa), BSA (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soya bean trypsin inhibitor (27.5 kDa) and lysozyme (18.5 kDa); according to the manufacturer, the addition of the dye causes the standard proteins to migrate differently ($\pm 10\%$) with respect to their true molecular mass.

trations were varied from 8 to 1 μ M, the reaction rate progressively increased, pointing to a substrate-inhibition kinetic pattern (Figure 6). To confirm these observations the initial reaction rate was investigated over a range of UbOEt concentrations spanning from 8 to 0.2 μ M. Figure 7 shows the Line-

26 kDa protein	Q-I-E-E-L-K-G-Q-E-V-X-P
human PGP 9.5	Q-I-E-E-L-K-G-Q-E-V-S-P pos. 66–77
26 kDa protein	Q-T-I-G-N-X-X-G-T-I-G-L-I-H-A-V-A-N-N-Q-D-K
human PGP 9.5	Q-T-I-G-N-S-C-G-T-I-G-L-I-H-A-V-A-N-N-Q-D-K pos. 84–105
26 kDa protein	N-E-A-I-Q-A-A-H-D-A-V-A-Q-E-G-Q-X-R
human PGP 9.5	N-E-A-I-Q-A-A-H-D-A-V-A-Q-E-G-Q-C-R pos. 136–153
26 kDa protein	N-N-V-D-G-H-L-Y-E-L-D-G-R
human PGP 9.5	N-N-V-D-G-H-L-Y-E-L-D-G-R pos. 166–178
26 kDa protein	M-P-F-P-V-N-H-G-T-X-X-E-D-X-L-L-Q-D-A-A
human PGP 9.5	M-P-F-P-V-N-H-G-A-S-S-E-D-T-L-L-K-D-A-A pos. 179–198
26 kDa protein	F-X-A-V-A-L-X
human PGP 9.5	F-S-A-V-A-L-C pos. 214–220

Figure 5 Sequence of some internal peptides of affinity-purified bovine PGP9.5 aligned with the derived human sequence

Amino acid substitutions are underlined. The HPLC system for phenylthiohydantoin (PTH)-amino acid identification was unable to differentiate PTH-serine from PTH-carboxymethyl-cysteine; for this reason X stands for C or S.

Figure 6 Hydrolytic reaction rates at different UbOEt concentrations

Each point is the result of three experiments. Substrate concentrations were 8 μ M ($\square$), 4 μ M ($\blacktriangle$), 2 μ M (∇) and 1 μ M ($\bullet$). Ub, ubiquitin.

Figure 7 Lineweaver–Burk plot of UbOEt hydrolysis at pH 7.4

The line represents a least-squares best fit of the data to the substrate-inhibition equation.

Figure 8 Effect of pH on the hydrolytic activity of PGP9.5

The buffers used were acetate (■), phosphate (▲) and Tris/HCl (□). Because of the alkali-sensitivity of the ester bond, assays at pH 8.0–10 were run in parallel with blanks in which PGP9.5 was omitted. Spontaneous hydrolysis of UbOEt became apparent at pH 9.0, and thus from this point the amount of ubiquitin (Ub) formed in the blank was subtracted from the amount formed in the assay.

Table 1 Effect of 10 mM iodoacetic acid and 10 mM diethyl pyrocarbonate on PGP9.5 UCH activity

Addition	UCH activity ($\mu\text{mol}/\text{min}$ per mg)	Inhibition (%)
None	3.6	—
Iodoacetic acid	0	100
Diethyl pyrocarbonate	0	100

weaver–Burk double-reciprocal plot of the measured rates; the experimental data were fitted to the general substrate-inhibition equation resulting in a K_m value of $0.5 \mu\text{M}$. The specific activity of the enzyme, measured at a substrate concentration of $1 \mu\text{M}$, was $3.6 \mu\text{mol}/\text{min}$ per mg of protein.

When the hydrolytic reaction rate was measured as a function of pH, the activity of PGP9.5 was found to be almost constant from pH 5.0 to 8.5 with two inflection points at pH 5.5 and 8.5 (Figure 8) which would indicate the involvement of histidine and cysteine residues in the catalytic function of PGP9.5 [18]. To confirm this hypothesis, PGP9.5 was treated with either iodoacetic acid or diethyl pyrocarbonate; as shown in Table 1, both reagents completely inhibited the hydrolytic reaction.

DISCUSSION

In this study we describe the purification from bovine retina of a protein that interacts with ubiquitin immobilized on Sepharose. The protein can be identified with PGP9.5 on the basis of structural characteristics such as primary-structure homology, (the first 14 amino acids at the N-terminus and several internal peptides), apparent molecular mass and cross-reactivity with a monoclonal antibody directed against human PGP9.5. In addition, since PGP9.5 has been reported to hydrolyse the synthetic substrate UbOEt [4] and the purified protein proved to do the same, its catalytic properties were investigated further; they reflect those of the known UCHs, with respect to the K_m value and dependence on cysteine and histidine residues for hydrolytic activity. These similarities possibly reflect the ability of the protein to bind to immobilized ubiquitin, a property demonstrated in this study, and can thus be extended to PGP9.5 in

addition to the UCHs from circulating cells. However, in other respects the UCH activity of PGP9.5 differs from that characterizing circulating cells [13]; it shows a wider pH range of activity and is inhibited by the synthetic substrate UbOEt but not by the reaction product ubiquitin, raising the question of whether these properties are connected with the neuronal context in which PGP9.5 performs its task. With reference to the inhibition by UbOEt on the hydrolytic activity of PGP9.5, recent evidence emphasizes that esterification at the ubiquitin C-terminus greatly decreases the hydrolytic activity of some UCHs from circulating cells (isopeptidase T); these hydrolases are specifically involved in disassembling polyubiquitin chains through cleavage of the isopeptide bond between the C-terminal glycine of a ubiquitin molecule and the side chain amino groups of other ubiquitins [19].

The ability of PGP9.5 to interact with immobilized ubiquitin is a previously unknown property of this protein, which supports the view that it and circulating-cell UCHs, in addition to and possibly in relation to their deduced primary-structure homology [1,4], have an overall conformational similarity with respect to the domains by which all these proteins recognize ubiquitin. On the other hand, this property should accelerate and facilitate purification of PGP9.5 to homogeneity, as the results of this study have proven; this finding may help us to explore whether the UCH activity of PGP9.5 plays a physiological role and is involved in the onset of neuronal degenerative diseases characterized by abnormal accumulation of PGP9.5 and ubiquitin [20,21].

Finally, to solve the apparent discrepancy between the reported blocked N-terminus of human PGP9.5 [22] and the free N-terminus observed in this study, PGP9.5 was affinity-purified from bovine brain and subjected to automated N-terminal degradation: the bovine brain protein, like the human equivalent, showed a blocked N-terminus. The N-terminal modification of PGP9.5 can thus be considered to be tissue rather than species-specific and does not interfere with the docking of the protein to immobilized ubiquitin.

We are indebted to Dr. A. Conti for his technical help in automated protein sequencing, and Dr. S. Ghidella and Mrs. L. Chiappino for their technical assistance. This study was supported by a grant from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica, Italy.

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Received 24 January 1996/25 March 1996; accepted 21 May 1996