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Biochemical and Biophysical Research Communications 325 (2004) 1406–1411
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Synthetic human prion protein octapeptide repeat binds to the proteinase K active site

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Received 18 October 2004

Abstract

Proteinase K is widely used in tests for the presence of infectious prion protein causing fatal spongiform encephalopathies. To investigate possible interactions between the enzyme and the functionally important N-terminal prion domain, we crystallized mercury-inhibited proteinase K in the presence of the synthetic peptides GGGWGQPH and HGGGW. The octapeptide sequence is identical to that of a single octapeptide repeat (OPR) from the physiologically important OPR region. Here, we present the first direct evidence for the complex formation between a proteolytic enzyme and a segment of human prion molecule. The X-ray structures of the complexes at 1.4 and 1.8 Å resolution, respectively, revealed that in both cases the segment GGG is strongly bound as a real substrate at the substrate recognition site of the proteinase forming an antiparallel β-strand between the two parallel strands of Asn99–Tyr104 and Ser132–Gly136. The complex is stabilized through an extended H-bonding network.

Keywords: Prion protein; Scrapie isoform; Proteinase K; Octapeptide repeats; X-ray; Substrate recognition

The cellular prion protein is a synaptic glycoprotein expressed in the central nervous system, in lymphatic tissue, and at neuromuscular junctions [1]. It is distributed in the brain of mammals where it is attached to the cell membrane by a phosphatidylinositol anchor ([2] and references therein). The physiological function of this protein is largely unknown. A number of evidences support the involvement of PrP^{C} in copper homeostasis and metabolism [3], in cell adhesion [4], transmembrane signalling, and regulation of the cholinergic receptor expression ([2] and references therein). The conversion of the normal, cellular form of the prion protein, containing a highly helical domain, into the scrapie isoform with a high β-sheet content, leads to the invariably fatal neurodegenerative diseases, transmissible spongiform encephalopathies, known also as "prion diseases" [5]. PrP^{Sc} acts as a template for the refolding of the cellular prion into the scrapie protein, which is facilitated by another, still unknown, "X-protein" [5]. A characteristic feature of the infectious form is the resistance to the proteolytic action of proteinase K, which is widely used in

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doi:10.1016/j.bbrc.2004.10.184

the clinical and laboratory practice for identification of the scrapie agent [6].

The NMR structure of the recombinant human prion protein, rhPrP (23–230), includes a globular domain (residues 125–228) and an N-terminal disordered tail [7]. The fragment between residues 53 and 85 consists of repeats of the sequence GGGWGQPH and can be defined as an "octapeptide repeat (OPR) region." This region plays an important role in the physiological function of prion proteins. In a model investigation, a four-octapeptide fragment cooperatively binds four Cu(II) ions coordinated by histidine residues from the OPR [8]. The octapeptide region of PrP at physiological Cu(II) concentration adopts a compact structure [8]. PrP^{C} binds copper in vivo and the N-terminal domain exhibits five to six metal ion binding sites [9]. Higher copper levels enhance the infectivity of the scrapie prion protein which effect can be used in a chelator-based therapy of prion diseases [10]. Copper reversibly stimulates endocytosis of PrP^{C} from the cell surface which suggests that the cellular prion could serve as a recycling receptor for uptake of copper ions [11]. These evidences support a hypothesis that PrP^{C} has a role in the copper metabolism. It is likely that Cu(II) is involved in the neurodegenerative diseases ([10] and references therein).

The octapeptide repeats in prion proteins are also a region capable of binding to heparin and heparan sulphate (HS) [12]. HS is expressed in neural and other cell types. It modulates the activity of growth factors and cytokines [13]. Cellular glycosaminoglycans play a role in the biogenesis of PrP^{Sc} and may be involved in the development of prion diseases [14].

In an attempt to investigate interactions between prion protein and proteinase K, used in tests to distinguish between the normal, cellular PrP^{C}, and the infectious forms, PrP^{Sc}, we started a program for X-ray investigations of complexes between the enzyme and the PrP or its segments. Here, we report the complex formation between the mercury-inhibited enzyme and: (a) a peptide with a sequence identical to that of a single octapeptide repeat, GGGWGQPH, from the physiologically important N-terminal domain of the human prion protein and (b) with a pentapeptide representing a segment of OPR. X-ray data revealed the prion OPR region and especially the sequence GGG as a site for binding to proteinase K.

Materials and methods

Proteinase K and synthesis of the prion protein octapeptide repeat and the pentapeptide segment. Proteinase K was delivered from Sigma–Aldrich (St. Louis, USA). Two millilitres of a proteinase solution (30 mg/ml) in 50 mM Tris/HCl buffer containing 10 mM $CaCl_2$, pH 6.5, was incubated overnight with a sevenfold molar excess of $HgCl_2$ in the same buffer. The mixture was dialysed overnight against the buffer mentioned above. The mercury complex of proteinase K was further used for crystallization in the presence of the prion protein octapeptide repeat or its pentapeptide segment.

The octapeptide repeat GGGWGQPH and the segment HGGGW were synthesized on an Eppendorf (Biotronik ECOSN P) solid phase peptide synthesizer (Hamburg, Germany) using a TentaGel S RAM-Gln(trt) Fmoc resin (Rapp Polymere, Tübingen, Germany). The amino acids were incorporated with their α-amino functional group protected by 9-fluorenylmethoxycarbonyl (Fmoc) group. The side chain of tryptophan was protected by *tert*-butyloxycarbonyl- and that of histidine by a trityl residue. Coupling was performed using a four-fold excess of the protected amino acids and the coupling reagent 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) plus 1.2 ml diisopropylethylamine (DIEA) as a 12.5% solution in DMF over the resin loading. Before coupling the protected amino acids, the Fmoc groups were removed from the last amino acid of the growing fragment using 25% piperidine in DMF. After cleavage of the N-terminal Fmoc group, the imino function of the proline residue was acetylated using a mixture of acetic anhydride (0.47 ml), diisopropylethylimine (0.87 ml) in DCM (5 ml) for 1 h at room temperature. Then, the respective peptide was removed from the resin under simultaneous cleavage of the amino acid side chain protecting groups by incubation in a mixture of 12 ml trifluoroacetic acid (TFA, 12 ml), ethanedithiol (0.6 ml), thioanisole (0.3 ml), anisole (0.3 ml), water (0.3 ml), and triisopropylsilane (0.1 ml) for 1 h. The mixture was filtered, washed with TFA, and the combined filtrates were precipitated with anhydrous diethyl ether. The remaining carbaminic acid at the tryptophan moiety was destroyed by incubation of the crude peptide with 20 ml of a mixture of glacial acetic acid (25%) and water for 20 min. After lyophilization, the crude products were further purified by HPLC on a Nucleosil 100 C18 (7 μm) 250 × 10 mm column, monitoring the elution (3.5 ml/min) at 214 nm and using as eluents A: 0.07% TFA/H_2O and B: 0.059% TFA/80% CH_3CN. A gradient from 10% to 90% B was applied within 32 min. The peptides were assayed for purity (>98%) by analytical HPLC, amino acid analyses, and matrix-assisted laser desorption ionization mass spectrometry.

Crystallization of the complexes between the mercury inhibited proteinase K and (a) the synthetic prion protein octapeptide repeat or (b) the segment HGGGW. The crystallization was performed by the hanging-drop vapour diffusion method. The hanging drops were 6 μl in volume. Mercury inhibited proteinase K was dissolved to a final concentration of 20 mg/ml in 50 mM Tris/HCl buffer containing 10 mM $CaCl_2$, pH 6.5. Water solutions of the synthetic octa- or pentapeptides were prepared at a concentration of 10 mg/ml. Two microlitres of the enzyme solution was mixed with 2 μl of the octa- or pentapeptide solutions and 2 μl of the reservoir solution consisting of 50 mM Tris/HCl, 1 M $NaNO_3$, 10 mM $CaCl_2$, and 0.02% NaN_3, pH 6.5. The drops were equilibrated against 1 ml of the reservoir solution. Crystals suitable for a high resolution X-ray analysis were grown within 1 week at 15 °C.

X-ray data collection, refinement, and model building. Diffraction data were collected from a flash-frozen crystal at 100 K with a synchrotron radiation ($\lambda = 0.802$ Å) at the consortium's beamline X13 at HASYLAB/DESY–Hamburg. The images were processed using the *DENZO* (SCALEPACK) program suite [15]. The structure of the native proteinase K, previously refined to 0.98 Å (PDB code 1IC6), was used as a starting model for the initial calculations and structure refinement of the proteinase complexes. The program *REFMAC* 5 was used. All data in the resolution limit of 20–1.4 Å for the proteinase–octapeptide complex and in the limit of 20–1.6 Å for the proteinase–pentapeptide complex were included and 5% of them were applied for R_{free}. According to the fit of $2F_0 - F_c$ and $F_0 - F_c$ maps, the models were modified using the program TURBO-FRODO [16] running on a Silicon Graphics O_2 workstation. The two mercury ions were placed according to the $F_0 - F_c$ maps in two partially occupied metal ion binding sites. In a next step the peptides were placed in $F_o - F_c$ and $2F_o - F_c$ maps. In the process of refinement, including the minimization of individual *B*-factors, water molecules were added according to

Table 1
Data collection and refinement statistics

Parameter	Complex of proteinase K with	
	GGGWGQPH	HGGGW
Space group	$P4_32_12$	$P4_32_12$
Unit-cell parameters		
a (Å) = b (Å)	67.71	67.76
c (Å)	106.89	107.30
Crystal volume per Dalton (V_M) (Å^3 Da)	2.2	2.2
Data collection and refinement		
Wavelength used (Å)	0.802	0.802
Resolution range (Å)	20–1.4	20–1.8
Total number of reflections used	673,964	250,901
No. of unique reflections	49,871	24,325
Average I/σ	10 (2)	9 (2)
R_{merge}[a]	0.121 (0.805)	0.095 (0.325)
Completeness (%)	99.8 (98.5)	98.6 (99.4)
Refinement statistics		
R_{value}/R_{free}	0.19/0.21	0.16/0.24
No. of protein amino acids	279	279
No. of amino acids from the prion segment	7	5
Modelled water molecules	349	342
Mercury binding sites	2	2
Average atomic B-factor (Å^2)	15.3	18.5
r.m.s. deviations from ideal values		
Bond distances (Å)	0.010	0.017
Bond angle (°)	1.289	1.533
Ramachandran plot: non-Gly residues in (%)		
Most favoured regions	88.6	88.1
Additionally allowed regions	11.4	11.9
Generously allowed regions	0	0

Values in parentheses are for the high resolution bin.
[a] $R_{merge} = \sum|I - \langle I\rangle|\sum I$, where $\langle I\rangle$ is the average intensity and summation is done for all reflections.

the $F_o - F_c$ maps. The data collection and refinement statistics are presented in Table 1. The final models have been analysed using the program PROCHECK [17].

Atomic coordinates. Coordinates of the refined models of proteinase K complexed with the synthetic human prion protein fragments have been deposited in the Protein Data Bank with the entry code 1XQT.

Results and discussion

The susceptibility of prion proteins to proteinase K digestion is widely used in laboratories and clinics for distinguishing between the normal and infectious forms. The enzyme hydrolyses PrP^C while the scrapie form is proteinase resistant [5]. It is of high interest to investigate in more detail the interactions of proteinase K with prion proteins and to identify the regions in the enzyme and substrate involved in these interactions. As it was mentioned before, prion molecule consists of a C-terminal domain with a predominant α-helical structure and a disordered N-terminal tail [7]. The helical domain represents a highly ordered compact structure and probably is less susceptible to proteolysis, at least in the beginning of the hydrolytic attack, than the tail. The N-terminal domain of the prion molecule becomes more structured after binding copper ions [8]. In any case, this part of the molecule should be hydrolysed easier and, in our opinion, offers more possibilities for interactions with proteinases. The tail is dominated by octapeptide repeats which represent the most conserved segment in the mammalian prion proteins [18,19]. The N-terminal domain of the prion protein molecule is physiologically important as it was described before. We have chosen a single octapeptide repeat and its pentapeptide segment as representative structural motifs to study proteinase K–prion protein interactions. A mercury-inhibited enzyme with a minimal structural change in the region of the catalytic site was used to avoid proteolytic digestion of GGGWGQPH or HGGGW and, at the same time, to preserve the substrate recognition site empty for binding of substrates or their analogues.

The crystal of proteinase K complexed with a single OPR diffracted to 1.4 Å resolution and belonged to the space group $P4_32_12$ with unit cell dimensions of $a = b = 67.71$ Å and $c = 106.89$ Å. The structure has been refined to an R-factor of 19% for all the data in the resolution range 20–1.4 Å and contains 279 protein amino acids, seven residues of the OPR fragment, two mercury binding sites, and 349 water molecules. The data collection parameters and refinement statistics are summarized in Table 1. Non-protein electron density was visible in the region of the substrate recognition site

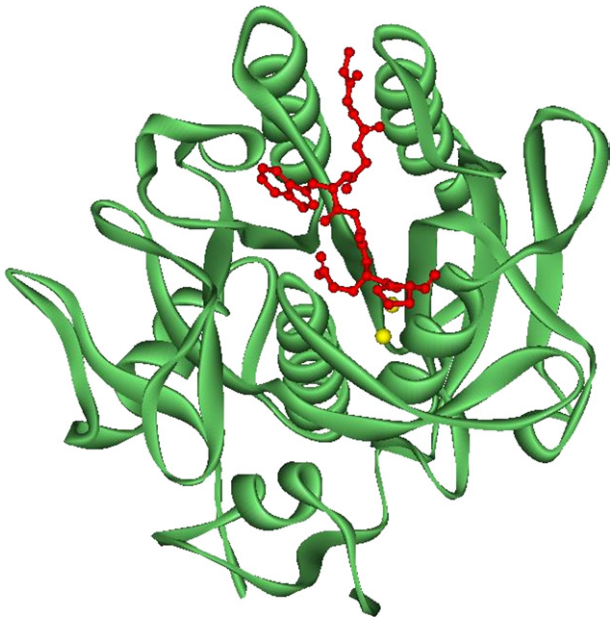

Fig. 1. Three-dimensional structure of the complex between proteinase K and synthetic prion protein OPR, ribbon representation. The proteinase molecule is coloured in green, the mercury binding sites in yellow, and the OPR in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and the prion protein octapeptide repeat was built into the electron density. The three-dimensional structure of the complex (ribbon representation) is shown in Fig. 1. The N-terminal part of the OPR fragment, consisting of the first three residues, is strongly bound as a real substrate forming an antiparallel β-strand between the two parallel strands of Asn99–Tyr104 and Ser132–Gly136 from the enzyme active site. The binding sites for the next four residues are partially occupied. Weak density was observed for the C-terminal histidine. The complex is stabilized through an extended hydrogen bonding network including protein and OPR atoms as well as a number of water molecules (Table 2). These molecules play an important role for the complex stabilization bridging atoms of the octapeptide repeat with the substrate recognition site (Fig. 2). The side chain of $Trp4^{OPR}$ is located in the hydrophobic pocket of the S1 substrate binding subsite which possesses a high affinity for aromatic residues. S1 is a large hydrophobic cavity which can adopt every side chain of a peptide substrate. There are no steric limitations to the size of the bulky indole group.

The crystal of the complex between proteinase K and the pentapeptide segment HGGGW diffracted to 1.8 Å and belonged to the space group $P4_32_12$ with unit cell

Table 2
Intermolecular contacts stabilizing the complex between the human prion protein octapeptide repeat and proteinase K

Prion OPR	Distance (Å)	Proteinase K or water molecules	Distance (Å)	Proteinase K or water molecules	Distance (Å)	Proteinase K
G1BN	2.96	OW320				
		OW320	2.88	Y104AN		
G1BO	2.78	OW320				
G2BO	2.86	G102AN				
G2BO	3.05	OW340				
		OW340	2.67	S101AOG		
G3BN	3.21	G134AO				
G3BO	3.24	G134AN				
W4BN	2.70	OW327				
		OW327	2.70	OW341		
				OW341	3.20	G100AO
W4BN	2.88	OW324				
		OW324	2.75	G134AO		
W4BNE1	3.05	OW333				
		OW333	3.22	G160AN		
W4BO	2.99	OW343				
		OW343	2.87	N161AND2		
W4BO	2.99	OW335				
		OW335	3.29	OW319		
				OW319	2.77	N161AN
G5BN	3.06	OW341				
		OW341	3.20	G100AO		
G5BN	2.79	OW336				
		OW336	2.97	S132AO		
Q6BOE1	3.07	N161AND2				
Q6BNE2	3.02	S224AOG				

OW means water molecule; A, atom from proteinase K; and B, atom from the prion protein segment.

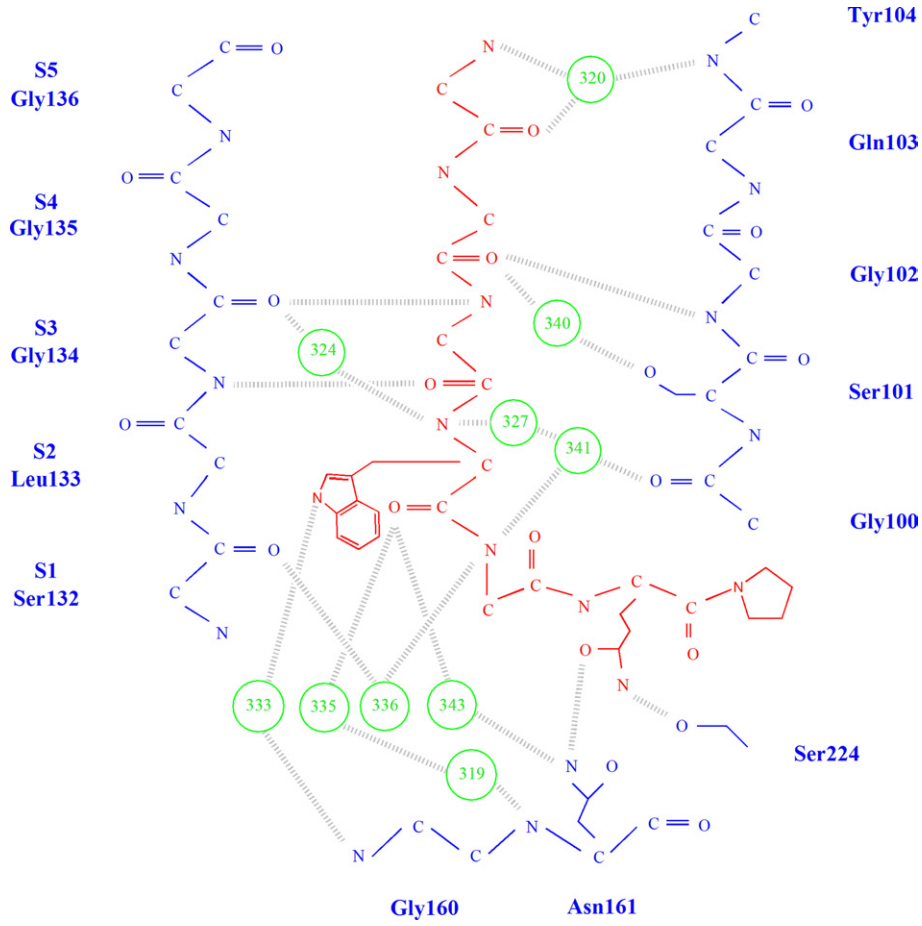

Fig. 2. A scheme of the proteinase K—prion protein octapeptide repeat interactions. Residues from the proteinase substrate binding site are coloured in blue and those from the prion segment in red. Water molecules are shown as green circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dimensions of $a = b = 67.76$ Å and $c = 107.30$ Å. The structure has been refined to an R-factor of 16% for all the data in the resolution range 20–1.8 Å and contains 279 protein atoms, five residues of the prion fragment, two mercury binding sites, and 342 water molecules. Data collection parameters and refinement statistics are shown in Table 1. Again, non-protein electron density was visible in the substrate recognition site and the pentapeptide was built into the electron density. This prion segment binds to the two active site β-strands in the same manner as the human prion protein octapeptide repeat. The GGG sequence is strongly bound forming antiparallel β-strand between the two parallel active site strands.

The SH-group of Cys73 is located below the active site His69 and the binding of Hg^{2+} inactivates the enzyme. There are two alternate binding sites for the mercury atom with an occupancy of approximately 0.5 each, bridged by the SG atom of the free cysteine which covalently links both sites. The binding of Hg^{2+} disturbs the catalytic triad. The catalytic site Asp39 OD1, His69 NE2, and His69 ND1 atoms are involved in coordinations with the mercury atom which leads to loss of catalytic activity. However, the geometry of the substrate recognition site is preserved.

The present results reveal a new function of the octapeptide repeat region as a site for binding of prion protein molecule to a proteolytic enzyme. The X-ray structures of the complexes are direct evidences for a proteinase–prion complex formation in which part of the PrP molecule binds at the enzyme active site as a real substrate. We plan further experiments with complexes of proteinase K and the whole prion molecule or its segments for better characterization of the enzyme–prion interactions.

Acknowledgments

The authors thank the Deutsche Luft- und Raumfahrtagentur (DLR, Projektträger Gesundheitsforschung, Grant 01KO0206) and the RiNA GmbH Berlin for financial support. This work was supported in part by the Bulgarian National Foundation for Scientific Re-

search, Grant X-1301. Ch. Betzel thanks the Deutsche Forschungsgemeinschaft for the financial support during a visit at the Institute of Organic Chemistry, Bulgarian Academy of Sciences, under contract 436 BUL 113/115/8-1.

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