Degradation of the Amyloid β-Protein by the Novel Mitochondrial Peptidasome, PreP**

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Recently we have identified the novel mitochondrial peptidase responsible for degrading presequences and other short unstructured peptides in mitochondria, the presequence peptidase, which we named PreP peptidasome. In the present study we have identified and characterized the human PreP homologue, hPreP, in brain mitochondria, and we show its capacity to degrade the amyloid β-protein (Aβ). PreP belongs to the pitrilysin oligopeptidase family M16C containing an inverted zinc-binding motif. We show that hPreP is localized to the mitochondrial matrix. In situ immuno-inactivation studies in human brain mitochondria using anti-hPreP antibodies showed complete inhibition of proteolytic activity against Aβ. We have cloned, overexpressed, and purified recombinant hPreP and its mutant with catalytic base Glu14 in the inverted zinc-binding motif replaced by Gln. In vitro studies using recombinant hPreP and liquid chromatography nanospray tandem mass spectrometry revealed novel cleavage specificities against Aβ(-1–42), Aβ(-1–40), and Aβ Arctic, a protein that causes increased protofibril formation an early onset familial variant of Alzheimer disease. In contrast to insulin degrading enzyme, which is a functional analogue of hPreP, hPreP does not degrade insulin but does degrade insulin B-chain. Molecular modeling of hPreP based on the crystal structure at 2.1 Å resolution of ATPreP allowed us to identify Cys90 and Cys527 that form disulfide bridges under oxidized conditions and might be involved in redox regulation of the enzyme. Degradation of the mitochondrial Aβ by hPreP may potentially be of importance in the pathology of Alzheimer disease.

Several human disorders are associated with the deposition of aggregated peptides. One of them is Alzheimer disease (AD)4

This work was supported by grants from The Swedish Research Council (to E. G.) and the Knut & Alice Wallenberg, Carl Trygger, and Magnus Bergwall foundations (to T. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This article was selected as a Paper of the Week.

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4 The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid β-protein; APP, amyloid precursor protein; IDE, insulin degrading enzyme; GST, glutathione S-transferase; IPTG, isopropyl β-D-thiogalactopyranosidase; PMSF, phenylmethylsulfonyl fluoride; oPH, ortho-phenanthroline; NEM, N-ethylmaleimide; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DTT, dithiothreitol; OM, outer membrane; IM, inner membrane; IMS, intermembrane space; Ma, matrix; LC-MS/MS, liquid chromatography nanospray tandem mass spectrometry; ROS, reactive oxygen species; SNP, single nucleotide polymorphism; NRD, di-basic convertase Nardilysin.

29096 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 281 • NUMBER 39 • SEPTEMBER 29, 2006

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strate peptide (Protein Data Bank accession code 2FGE). The AtPreP structure revealed a unique totally enclosed large cavity of 10,000 Å³ that opens and closes in response to peptide binding, revealing a novel catalytic mechanism for proteolysis. We have identified amino acid residues involved in catalysis and shown that amino acids located about 800 residues from the inverted zinc-binding motif contribute to the formation of the active site. As PreP-catalyzed proteolysis occurs in the cavity (like in the proteasome) we refer to the structure of PreP as peptidosome (15).

Interestingly, PreP is an organellar functional analogue of the human insulin degrading enzyme, IDE, that also belongs to the pitrilysin family. IDE has been implicated in AD as it cleaves Aβ before insoluble amyloid fibers are formed (16–18). Moreover, a genetic deletion of IDE in mice leads to significant elevation in brain Aβ levels (19, 20). Up-regulation of IDE in neurons prevented AD-type pathology in transgenic mice overexpressing brain tissue was thawed and homogenized in buffer A (250 mM mannitol, 0.5 mM EGTA, 5 mM HEPES, 0.1% bovine serum albumin, pH 7.4) by 10 strokes at 1500 rpm with a high torque motor-driven pestle. All centrifugation steps were carried out at 4 °C. Unbroken cells and cell nuclei were removed by two centrifugations at 15,000 g for 10 min. The mitochondrial pellet was obtained by centrifugation at 100,000 g for 30 min. Mitochondria were resuspended in 20 mM HEPES, pH 7.5, 50 mM KCl, 2 mM EGTA, and Complete™ protease inhibitor mixture (Roche Applied Science) plus 20% glycerol, flash-frozen in liquid N₂, and stored at −70 °C before use.

Preparation and Subfractionation of Rat Liver Mitochondria—Mitochondria were isolated from liver of 200-g body weight Sprague-Dawley male rats by differential centrifugation technique according to Lapidus and Sokolove (24). To obtain mitoplasts rat liver mitochondria were diluted 10 times with sonication buffer containing 20 mM HEPES-KOH, pH 7.5 (1 mg/ml final concentration) and incubated 20 min at 4 °C following centrifugation at 8000 × g for 5 min. The pellet containing mitoplasts was either used directly for determination of rat PreP localization or used for preparation of the inner membrane and matrix fractions, which were obtained after sonication of mitoplasts and subsequent centrifugation at 50,000 × g for 1 h. Supernatant containing outer membrane as well as soluble intermembrane space proteins were further centrifuged at 50,000 × g for 1 h. The pellet contained outer membrane, whereas soluble intermembrane space proteins were concentrated by precipitation with 50% saturated ammonium sulfate, centrifuged for 10 min at 15,000 × g, and suspended to 1 mg/ml in sonication buffer.

Overexpression and Purification of hPreP and Its hPreP-(E78Q) and hPreP(C90S) Mutants—An Escherichia coli overexpression strain, BL21(DE3), was transformed with the pGEX-6P-2 vector containing the predicted mature part (amino acids 27–1036 of hPreP (AAH05025, gi:13477137) fused to glutathione S-transferase (GST) and was grown at 30 °C in LB medium. After 4 h, 1 mM IPTG was added to the culture, and the incubation was continued for another 4 h. The cells were pelleted, resuspended in phosphate-buffered saline buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3), and lysed by addition of 0.5 mg/ml lysozyme and 10 μg/ml DNase I followed by sonication for 3 × 30 s. The lysate was centrifuged for 20 min at 15,000 × g and filtered through a 0.2-μm membrane. The supernatant was loaded onto a GSTrap FF 1-ml column (Amersham Biosciences) equilibrated with phosphate-buffered saline buffer. The hPreP was eluted after on-column cleavage with PreScission Protease according to the manufacturer’s instructions (Amersham Biosciences). The eluted hPreP was applied to a Superdex 200 HR 10/30 (Amersham Biosciences) equilibrated with 20 mM HEPES-KOH, 10 mM MgCl₂, pH 8.0. The protein content of eluted fractions from GSTrap FF and Superdex 200 HR10/30 was analyzed on 12% SDS-PAGE in the presence of 4 mM urea (Laemmli 1970) (25) and stained with Coomassie Brilliant Blue. Mutants of hPreP(E78Q) and hPreP(C90S) were constructed using a QuickChange site-directed mutagenesis kit (Stratagene). The construct was verified by DNA sequencing using a DYEnamic sequencing kit (Amersham Biosciences). The hPreP mutant (E78Q) for production antibodies was overexpressed in E. coli as GST-hPreP fusion, induced with IPTG in the presence of 10 μM zinc acetate, and purified as follows: anion exchange on a Superdex 200 HR 26/60 column performed to obtain pure fusion protein, then a second anion exchange step on a 6-ml ResourceQ15 column was performed to obtain pure fusion protein. Then the fusion protein was desalted into cleavage buffer (Amersham Biosciences) and reacted overnight at 4 °C with

**Experimental Procedures**

Preparation of Human Mitochondria—Post-mortem brain material (cortex) (four cases, age: 58–91 years; post-mortem delay: 14–24 h) was obtained from the Huddinge Brain Bank, Karolinska University Hospital, Huddinge, Sweden, and mitochondria were isolated as described by Ankarcrona et al. (23). Ethical approval was received from the local Ethical Committee at Karolinska Institutet Huddinge Universitetssjukhus. The analysis reveals several unique cleavage sites on Aβ-(1–40) and Aβ Arctic protein. Our findings contribute to studies of the mitochondrial component in AD.
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PreScission protease. The cleaved hPreP mutant was purified by anion exchange on the 6-ml ReSourceQ column and then was loaded onto a 26 × 60 Superdex200 gel filtration column. The gel filtration peaks were concentrated by ultrafiltration to 5 mg/ml.

Degradation Assays—The degradation assay for studying the proteolytic activity of recombinant hPreP contained 1 μg purified hPreP and following substrates: 1 μg of Aβ-(1–40) or Aβ-(1–42) or Aβ-(1–42) E22G in a degradation buffer containing 20 mM HEPES-KOH, pH 8.0, and 10 mM MgCl₂. For the inhibitory studies, 1 mM PMSF (phenylmethylsulfonyl fluoride), 20 mM oPh (ortho-phenanthroline), 1 mM NEM (N-ethylmaleimide), 100 units/ml aprotinin, 50 μg/ml bestatin were preincubated with 1 μg of hPreP on ice for 10 min before addition of the substrate. The samples were incubated for 1 h at 37 °C, and the reactions were stopped by addition of 2 × Laemmli sample buffer, analyzed on 10–20% Tris-Tricine gels (Bio-Rad), and stained with Coomassie Brilliant Blue. To investigate the degradation of insulin and insulin B-chain by hPreP, 1 μg of insulin or insulin B-chain was incubated with 1 μg of purified hPreP in the degradation buffer for 1 h at 37 °C. The reactions were stopped and analyzed as described above. Proteolytic activity of the PreP cysteine mutant was investigated under oxidizing conditions (2.5 mM K₃Fe(CN)₆) and reducing conditions (2.5 mM DTT). The protease was incubated under the respective conditions at room temperature for 5 min prior to the addition of Aβ. The proteolytic activity of the human mitochondrial matrix and the rat mitochondrial subfractions was tested by incubation of the samples with Aβ for 3 h at 37 °C. Proteolytic activity in the presence of protease inhibitors was tested after preincubation of the matrix fraction (20 μg) with the inhibitors for 10 min at 4 °C before addition of 1 μg of Aβ-(1–40). The reactions were stopped by adding 2 × Laemmli sample buffer, analyzed on 10–20% Tris-Tricine gels (Bio-Rad), and stained with Coomassie Brilliant Blue.

Intracellular Localization of hPreP—Human brain mitochondria were isolated as described elsewhere by Ankarcrona et al. (23). Human mitochondrial membrane and matrix were obtained by resuspending isolated mitochondria in a sonication buffer. The resuspended mitochondria were incubated on ice for 10 min followed by sonication 5 × 15 s on ice and centrifuged at 18,600 × g to discard the unbroken mitochondria. The supernatant was thereafter ultracentrifuged at 70,000 rpm (Beckman TL-100 Ultracentrifuge, TLA 100.2 rotor) for 45 min. Human brain cytosol, mitochondria, membrane, and matrix were analyzed on 12% SDS-PAGE in the presence of 4 M urea. Immunological cross-reactivity was analyzed by Western blot analysis using nitrocellulose membrane Hybond™ (Amersham Biosciences) and antibodies raised against hPreP, F₁ moiety of the ATP synthase from N. plumaginifolia, and Tim17 followed by detection with horseradish peroxidase-coupled secondary antibody and ECL (Amersham Biosciences). Intracellular localization of PreP in rat liver mitochondria, mitoplasts, and different mitochondrial subfractions, outer membrane (OM), inner membrane (IM), intermembrane space (IMS), and matrix (Ma), was studied by Western blot analysis with compartment-specific antibodies against porin (OM), Tim17 (IM), Omi (IMS), and GRP75 (Ma) using ECL detection system.

Immuno-inactivation—Immuno-inactivation studies were performed as follows: 6 μg of antibodies raised against hPreP or 18 μg of antibodies raised against presense of F₁ β subunit of the ATP synthase from Nicotiana plumaginifolia, pF₁,β (2–54), were preincubated with human mitochondrial soluble fraction at 4 °C for 30 min before addition of Aβ-(1–40). The samples were incubated for 3 h at 37 °C, and the reaction was stopped by addition of Laemmli sample buffer and analyzed on 12–20% SDS-PAGE in the presence of 4 M urea. For Western blot analysis, proteins were transferred onto nitrocellulose membrane Hybond™ (Amersham Biosciences) and incubated with anti-Aβ monoclonal antibody 6E10 (Signet Laboratories) at 1:1000. Immunoreactivity was detected with anti-mouse horseradish peroxidase secondary antibody and ECL (Amersham Biosciences).

Mass Spectrometric Analysis; High Performance Liquid Chromatography–MS/MS—The sample was concentrated and desalted using ZipTips (Millipore) and injected onto a 0.1 × 150-mm C18 column (YMC Co., Ltd., Kyoto, Japan) using a nano-LC injector (Valco Instruments) with a 1–μl loop. Peptides were eluted using a water/acetonitrile gradient supplemented with 0.2% formic acid: from 10–20% acetonitrile in 10 min, 20–35% acetonitrile in 30 min, and 35–50% acetonitrile in 15 min. The flow rate was 400 nl/min delivered by an Agilent 1100 nanopump (Agilent Technologies), and the column was coupled to an Agilent ion trap mass spectrometer (Agilent) fitted with a nanospray interface. Mass spectra were recorded from m/z 240 to 1800, and the three largest peaks in each spectrum were subjected to MS/MS analysis. The cycle time was 12 s. Peak areas were obtained from the selected ion traces corresponding to the calculated masses of the intact and cleaved peptide.

Homology Modeling of hPreP and Flexible Aβ Docking—The homology model of hPreP was based on the 2.1-Å crystal structure of AtPreP (Protein Data Bank accession code 2FGE (15)) using the internal coordinate mechanics energy optimization method (26, 27) and a sequence alignment generated by CLUSTALW (28). The model comprises all atoms including hydrogens and was created by the zero end gap dynamic programming algorithm, where the backbone and conserved side chains adopt the same conformation as the template. Loop regions were subjected to search against a data base of loop structures from the Protein Data Bank, and loops with the closest matching sequences and loop end positions are inserted into the homology model. The bound Aβ-(12–17) peptide was created by substituting the side chains of the unidentified peptide found in the AtPreP crystal structure followed by several rounds of energy optimization implemented by Internal Coordinate Mechanics to allow the peptide to adopt a conformation that fit the active site of the hPreP model. The coordinates of AtPreP have been deposited in the Protein Data Bank under the accession code 2FGE (15).

RESULTS

Degradation of Aβ Proteins by hPreP—The recombinant hPreP was cloned as a fusion protein with GST, overexpressed
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FIGURE 1. Expression, purification, and degradation of Aβ by the recombinant hPreP. A, expression and purification of the hPreP. The lanes show total cell lysate from uninduced E. coli and cells induced with IPTG. The overexpressed hPreP protein was purified on GSTrap FF column after cleavage with PreScission protease. B, degradation of Aβ-(1–40), Aβ-(1–42), and Arctic Aβ-(1–42) by purified hPreP analyzed on Tris-Tricine gels stained with Coomassie blue. C, degradation of insulin and insulin β-chain by hPreP. D, degradation of Aβ-(1–40) by hPreP in the presence of inhibitors. E, expression and purification of the mutant hPreP(E78Q). F, degradation of Aβ-(1–40) by hPreP and mutant hPreP(E78Q).

in E. coli, and purified to homogeneity on GSTrap FF column after cleavage with PreScission protease (Fig. 1A). hPreP has a molecular mass of 114 kDa. The “light” band at about 70 kDa corresponds to DnaK indicating that small quantity of this bacterial molecular chaperone co-purifies with hPreP. The recombinant hPreP shows proteolytic activity against different Aβ proteins. Fig. 1B shows SDS-PAGE of amyloid β-proteins degraded by hPreP. hPreP completely degraded both Aβ-(1–40) and Aβ-(1–42) as well as Aβ Arctic protein (1–42 E22G), a protein that causes an AD-like pathology, with an approximate activity of 0.053 μg of Aβ/min/μg of hPreP. To compare proteolytic activities of hPreP and IDE, the degradation of insulin and insulin B-chain by hPreP was investigated. hPreP degraded completely the insulin B-chain but was not capable of degrading insulin (Fig. 1C).

Investigation of the effect of different types of protease inhibitors on the degradation of Aβ-(1–40) by the recombinant hPreP showed that neither PMSF nor bestatin (i.e. serine or aminopeptidase type protease inhibitors) affected proteolysis (Fig. 1D), whereas NEM, a cysteine-type protease inhibitor, showed a small inhibitory effect of 10%, and the metalloprotease inhibitor oPh completely inhibited degradation of Aβ, demonstrating that hPreP is a thiol-sensitive metalloprotease. This is in agreement with studies on hMP1, which showed that hMP1 was efficiently inhibited by oPh and p-chloromercuriphenylsulfonic acid (22). Apyrase had no effect on the proteolytic activity showing that Aβ degradation is independent of ATP. hPreP contains an inverted zinc-binding motif, H75ILE78H79. The importance of the metal-binding motif for the proteolytic activity has been demonstrated by studying the recombinant hPreP mutant, hPreP(E78Q), in which the catalytic base Glu78 was changed to Gln (Fig. 1E). We have overexpressed and purified the mutant and showed that hPreP(E78Q) could not degrade (only 10% was degraded) the Aβ-(1–40) protein (Fig. 1F) confirming the importance of the inverted zinc-binding site for the proteolytic activity.

The degradation pattern of Aβ-(1–40) and Aβ Arctic was studied using liquid chromatography nanospray tandem mass spectrometry (LC-MS/MS) (Fig. 2). The base peak chromatogram shown in Fig. 2B also shows a schematic representation of the cleavage sites by hPreP (filled arrows) on Aβ-(1–40) and Aβ Arctic Fig. 2. A and C). The overall degradation resulted in the production of several 4–14 amino acid fragments that are
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unique for hPreP. Degradation of Aβ-(1–40) resulted in the cleavage after Gln15, Lys16, Phe19, Phe20, Ala30, Gly33, and Leu34 (Fig. 2A). The major cleavage product is the short very hydrophobic peptide, Leu17-Val18-Phe19-Phe20. hPreP degraded Arctic at cleavage sites that generally overlapped with those found for wild type Aβ. However, two new cleavage sites appeared, after Gly29 and Gly38 (gray arrows).

Mitochondrial Localization of hPreP and Its Function—The bioinformatic programs created to predict intracellular localization of proteins, Mitoprot, Predotar, and TargetP localize hPreP to mitochondria with a high score (0.9888 for MitoProt and 0.877 for TargetP). However, the predicted presequence of hPreP is much shorter (29 amino acids) than for the plant homologue AtPreP (85 amino acids). The yeast homologue of PreP, MOP112, is not predicted to be a mitochondrial protein but was shown to be located in the mitochondrial intermembrane space (29). To confirm the localization of hPreP to mitochondria, we isolated human brain mitochondria and the cytosolic fraction and tested immunological cross-reactivity with antibodies against hPreP and the F1 moiety of the ATP synthase. Western blot analysis verified mitochondrial localization of hPreP (Fig. 3A). Furthermore, hPreP was shown to be located in the soluble fraction of human brain mitochondria, no cross-reactivity was found in the membrane fraction probed with anti-Tim17 antibodies (Fig. 3B). The soluble fraction of human brain mitochondria showed proteolytic activity against Aβ that was almost completely inhibited by oPh and partially (20%) by NEM but not by PMSF or apyrase (Fig. 3C).

To investigate intramitochondrial localization of PreP we have used rat liver mitochondria due to a limited amount of human material. Rat PreP shows 85% sequence identity (93% similarity) to hPreP and is predicted to be a mitochondrial protein with a 28-amino acid-long presequence. Consistent with human and rat PreP, the predicted presequences of PreP from mouse, dog, and orangutan (Pongo pygmaeus) are estimated to be 29 amino acid residues in length. We have prepared highly purified rat liver mitochondria free from microsomal contamination and we have osmotically ruptured the outer mitochondrial membrane to obtain mitoplasts. Mitochondria were fractionated into OM, IM, IMS, and Ma. Western blot analysis of mitochondria and mitoplasts after proteinase K treatment clearly showed intramitochondrial localization of PreP. Analysis of the mitochondrial subfractions with compartment-specific antibodies against porin (OM), Tim17 (IM), Omi (IMS), and GRP75 (Ma) clearly showed that PreP was localized to the mitochondrial matrix (Fig. 3D). Insignificant amounts of porin in the IM might be due to the presence of "contact points"
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FIGURE 3. Intramitochondrial localization of PreP. A, localization of hPreP in human brain mitochondria. Cytosolic fraction and mitochondria were probed with antibodies against hPreP and F1-ATPase. B, intramitochondrial localization of hPreP. Total mitochondrial extract and soluble and membrane fractions were probed with antibodies against hPreP and Tim17. C, degradation of Aβ-(1–40) by human mitochondrial soluble fraction in the presence of protease inhibitors. D, intramitochondrial localization of PreP in rat liver mitochondria. Mitochondrial subfractions were probed with antibodies against GRP-75 (Ma), anti-Omi (IMS), Tim17 (IM), and porin (OM). E, degradation of Aβ-(1–40) by subfractions of rat liver mitochondria analyzed with Aβ antibodies.

between the OM and the IM. The “light” band in the IMS does not have the same molecular mass as porin. Cross-reactivity detected with anti-Omi in the Ma might reflect residual electrostatic association of Omi with mitoplasts and its dissociation to the Ma fraction during preparation.

Degradation activity measurements of Aβ-(1–40) with the mitochondrial subfractions further confirmed matrix localization of PreP (Fig. 3E).

The involvement of hPreP in Aβ proteolysis in organello was verified by immuno-inactivation assay using human brain mitochondria. When the soluble human brain mitochondrial fraction was preincubated with antibodies against hPreP, the degradation of Aβ-(1–40) was completely abolished (Fig. 4), whereas in the presence of antibodies against the F1β subunit, protease activity was detected. These results show that hPreP is the protease responsible for degradation of Aβ in mitochondria under conditions when Aβ accumulates in mitochondria as reported in recent studies (8, 9).

Structural Model of hPreP with Aβ Hexapeptide—The sequence of hPreP is 31% identical to AtPreP (11), and the protein has a similar function, thus their structures are likely to be similar. A model of hPreP was generated by homology with the program ICM (26, 27) using the 2.1 Å resolution structure of AtPreP as a template (15). In a homology model the main chain of the core protein is normally correct as is the position of most residues. However, the conformation of loops and side chains are harder to predict since they often differ between closely related proteins such as the PreP family. PreP comprises four topologically similar domains organized in two halves connected by a hinge region, which enclose a large proteolytic chamber wherein the active site resides (Fig. 5A). The active site is highly conserved with very few amino acid substitutions compared with AtPreP. The N-terminal domain contains the inverted zinc-binding motif and the distal glutamate at position 176, H75ILE78H79X(97)E176. Tyr878 in the C-terminal domain separated by 800 residues from the zinc-binding motif completes the active site. A few small differences between human and plant PreP are found in the S3 and S1′ pockets. The crystal structure of AtPreP comprises a six-residue peptide in the active site, which binds in an extended conformation forming a short antiparallel β-strand to the enzyme. We modeled the human substrate Aβ-(12–17) based on this peptide, and its position was refined to fit to the active site of the hPreP model (Fig. 5B). However, it should be noted that hPreP degrades several different substrates at multiple sites, so the model simply illustrates where the substrate is located during proteolysis.

Interestingly, in the homology model of hPreP two cysteine residues at positions Cys90 in the first domain and Cys527 at the hinge region are in close proximity to each other. These cysteines are not present in the AtPreP structure. Measurement of the proteolytic activity of hPreP in the presence of K4Fe(CN)6, shows complete inhibition under oxidizing conditions, indicating that these cysteines can form a disulfide bridge locking the enzyme in a closed conformation and thus hinder substrate binding (Fig. 5C). To confirm involvement of the listed cysteines in disulfide bridge formation, we have produced a mutant, in which Cys90 was changed to Ser, hPreP(C90S). The cysteine mutant, in contrast to the wild type hPreP, was active under oxidizing conditions showing that Cys90 was indeed involved in formation of the disulfide bridge locking the enzyme in a closed inactive conformation (15) (Fig. 5D). This finding is interesting and might be of physiological importance as it implies a possible inhibition of the enzyme under conditions of elevated ROS production in mitochondria.
Our findings demonstrate for the first time that the newly identified mitochondrial metalloendopeptidase, called hPreP peptidasome, is present in the mitochondrial matrix of human brain mitochondria and that the enzyme, in addition to its previously identified function, degradation of the mitochondrial presequences and other unstructured peptides, is also putatively responsible for the degradation of the amyloid β-protein. Accumulation of Aβ in mitochondria under pathological conditions associated with AD has been recently reported (8, 9). hPreP is a metalloendopeptidase that belongs to pitrilysin subfamily M16C. The pitrilysin M16A subfamily includes several peptide-degrading oligopeptidases, such as IDE, di-basic convertase Nardilysin (NRD), and bacterial pitrilysin (protease III), that are monomers of similar size as PreP (about 100 kDa). Members of the M16B subfamily are heterodimers formed by two 50-kDa subunits, e.g. the mitochondrial processing peptidase that accepts bigger substrates cutting off targeting peptides from precursor proteins. IDE controls levels of insulin and degrades a wide range of other physiological peptides including glucagon, transforming growth factor-β, β-endorphin, and amylin (30). NRD convertase is processing a number of neuropeptides including opioid peptides. This indicates that both IDE and pitrilysin have also been shown to cleave Aβ protein (17, 31, 32) therefore leading us to the investigation of Aβ degradation by hPreP.

hPreP efficiently degrades different Aβ proteins including Aβ-(1–40), Aβ-(1–42), and Aβ Arctic. Screening of the hPreP cleavage sites on the Aβ protein shows that in most cases hydrophobic and small uncharged amino acids and also a positively charged amino acid are found in P1 and P2 positions. It is in agreement with our previous subsite specificity study for cleavage of mitochondrial presequences by AtPreP showing that the enzyme has preference for basic amino acids in the P1 position and small uncharged amino acids or serines in the P2 position (10). Substrates to IDE show little or no sequence similarities, but the enzyme also exhibits preference for basic and/or large hydrophobic residues (33, 30). Comparison of our results with the previously described study for IDE (34) shows, however, that only two cleavage sites produced by hPreP overlapped with those found for IDE, Phe19↓Phe20 and Phe20↓Ala21 (34). All the other cleavage sites on Aβ-(1–40) seem to be unique for hPreP (Gln15↓Lys16, Lys16↓Leu17, Gln18↓Lys19, and Ala20↓Glu21) (34). Additionally, two new cleavage sites appeared on Aβ Arctic (Gly22↓Ala30 and Gly29↓Val30). Interestingly, the main difference in subsite specificity between hPreP and IDE on Aβ proteins is that hPreP
has several cleavage sites after Gly⁷⁹ in a very hydrophobic C-terminal Aβ-(29–42) segment of Aβ. This segment is prone to aggregation adopting exclusively a β-sheet conformation (35). Despite the fact that both PreP and IDE belong to the pitarlsin protease family, the overall sequence similarity of hPreP and IDE is very low and might explain different recognition patterns. There is only a 28-amino acid residue stretch in IDE around the zinc-binding motif that shows 38% sequence identity (55% similarity) to hPreP. Another difference, evident between hPreP and IDE, is that hPreP is not capable of degrading insulin (cf. Fig. 1C), while the insulin B-chain was completely degraded. Insulin A- and B-chains are linked by two disulfide bonds, and an additional disulfide is formed within the A-chain. The A-chain consists of 21 amino acids and the B-chain of 30 amino acids. As PreP degrades peptides up to 70 amino acid residues, the size of insulin is appropriate for degradation but the folding status seems to limit accessibility to the active site. Neither the de novo peptide Ala-α₂, consisting of 66 amino acids that is tightly folded into a three helical bundle structure was cleaved by PreP (11). This further confirms that PreP is inactive against folded small proteins (10).

It can be also mentioned that hPreP is encoded by a single gene PITRM1 that is located in chromosome region 10p15.2 and contains 30 exons. Also IDE is located on chromosome 10 (10q) and genetic association between single nucleotide polymorphisms (SNPs) in IDE and late-onset of AD has been reported (36). Interestingly, preliminary SNP searches in the public data bases (ENSEMBL and NCBI) of the hPreP gene, PITRM1, revealed several SNPs in the vicinity of the active site.⁵ The relevance of this finding for hPreP proteolytic activity is at present unknown, but localization of SNPs in exons, close to the active site, makes these mutants interesting for further studies.

What is the intramitochondrial localization of hPreP and what is its function within the organelle? hPreP is a soluble protein with an isoelectric point of 6.57. Our immunological cross-reactivity studies clearly show that hPreP is localized to the mitochondrial matrix. Matrix localization is also found in plants.⁶ Interestingly, the yeast homologue of PreP, MOP112 has been shown to be located in the intermembrane space (29). On the other hand, the first genome-wide screen of the Cym1 gene corresponding to the MOP112 protein in complexes localized to the mitochondrial matrix (37). Comparison of the N-terminal sequences of human, other mammalian, and yeast PreP shows differences that may explain varying intramitochondrial localization. Whereas the mammalian presequences are predicted to be 28–29 amino acids long and clearly classified as the mitochondrial targeting peptides (MitoProt), the yeast PreP is not predicted to be a mitochondrial protein, and it contains only a short, seven-amino-acid long N-terminal extension in comparison with the predicted mammalian mature forms of PreP. Proteolysis is crucial for maintaining mitochondrial morphology and function as it removes misfolded or damaged proteins and peptides and protects organelles from the toxicity of potentially harmful peptides.

Mitochondrial ATP-dependent proteases, the AAA proteases (ATPases associated with a number of cellular activities), Lon and ClpP generate peptides of 5–20 amino acid residues, whereas the Oma1 protease produces fragments of ~20 kDa (38, 39). These peptides are subsequently degraded to smaller peptides or free amino acids by the ATP-independent proteases, such as the matrix hPreP or intermembrane space yeast MOP. Also the cleaved targeting peptides, accumulated Aβ, and other toxic peptides will be degraded by PreP. It indicates thus that PreP is a general mitochondrial peptidase clearing the organelle from different peptides susceptible for proteolysis. Immuno-inactivation studies showed that PreP is an Aβ degrading protease in mitochondria (cf. Fig. 4). Our previous immuno-inactivation studies with S. oleracea mitochondria showed that PreP is also responsible for degradation of targeting peptides in mitochondria (11). Interestingly, an isoform of IDE with an N-terminal mitochondrial targeting sequence generated by translation at an in-frame initiation codon upstream of the canonical translation start has been recently identified, localized to mitochondria in cell lines, and shown to degrade mitochondrial targeting peptides (40). Previously, IDE has been reported to be present in other subcellular compartments including peroxisomes, endosomes, and the nucleus (41). Relative levels of IDE expression in different organelar compartments have not been reported.

The recently solved crystal structure at 2.1 Å resolution of AtPreP allowed molecular modeling of hPreP and identification of amino acids involved in hPreP catalyzed proteolysis, as discussed above. Furthermore, the structure reveals closed conformation of the enzyme forming an enclosed chamber in which the proteolysis occurs, which explains how these type of unspecific proteases discriminate between substrate peptides and larger folded proteins. The chamber is formed by two enzyme halves that come together and the volume of the chamber limits the substrate size to peptides and prevents unintentional degradation of proteins. Based on the structure and mutagenesis experiments with double cysteine mutants whose activity is inhibited under oxidizing conditions, we have proposed a novel mechanism for PreP proteolysis involving hinge-bending motions in an opening and closing cycle that is controlled by substrate binding (15). Unexpectedly, we have found that hPreP contains two native cysteine residues at position Cys⁹⁰ in the first domain and position Cys⁴²⁷ at the hinge region in close proximity to each other. Notably, these cysteine residues are conserved in all known mammalian PreP sequences as well as in the yeast PreP homologue. Oxidation of hPreP resulted in the complete inhibition of the proteolytic activity against the Aβ protein, indicating formation of a disulfide bridge between the cysteines that locks the enzyme in a closed conformation and hinder substrate binding. Indeed, the cysteine mutant, hPreP(C90S), was active under oxidizing conditions showing that Cys⁹⁰ was involved in formation of the disulfide bridge locking the enzyme in a closed inactive conformation (15). Furthermore, importance of the status of cysteine residues for the proteolytic activity of hPreP may explain partial inhibitory effect of NEM on hPreP activity. As NEM is a cysteine-modifying agent, its substitution may cause steric hindrance during proteolysis (15). Importance of the redox status of cysteine res-

⁵ C. Graff, unpublished results.
⁶ P. F. Pavlov, unpublished results.
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ides for the proteolytic activity implies a possible inhibition of the enzyme in mitochondria under conditions with elevated ROS production that would additionally increase mitochondrial dysfunctions. The physiological consequences of this finding will be studied further.

In summary, mechanisms through which intracellular Aβ impairs cellular properties causing neuronal dysfunctions are not fully understood. Besides an immense number of studies showing extracellular accumulation of Aβ, it has been shown that Aβ also accumulates in the brain mitochondrial matrix of AD patients and of transgenic mice overexpressing Aβ precursor protein (8, 9) and causes perturbations of mitochondrial functions. Recent studies in which Aβ was incubated with isolated rat brain and muscle mitochondria show the accumulation of monomeric Aβ within the mitochondria, an increase in mitochondrial membrane viscosity with a concomitant decrease in ATP/O ratio, respiratory chain complexes inhibition, a potentialization of ROS production, and cytochrome c release (42). On the basis of the results discussed above we propose that the newly identified mitochondrial pepidase, hPreP, functions as a peptide scavenger in the mitochondrial matrix clearing mitochondria from toxic peptides and protecting them against pathogenic peptide intruders. We speculate that age-dependent reduction of PreP proteolytic activity in mitochondria caused by mutations or other factors affecting the hinge-bending opening and closing cycle of the enzyme may lead to accumulation of toxic peptides and age-dependent onset of mitochondrial dysfunction.

Acknowledgments — We greatly acknowledge the kind gift of Aβ Arctic protein from Prof. L. Lannfelt and comments on the manuscript from Dr. D. Daley.

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