In vitro oxidative inactivation of human presequence protease (hPreP)

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Abstract

The mitochondrial peptidasome called presequence protease (PreP) is responsible for the degradation of presequences and other unstructured peptides including the amyloid-β peptide, whose accumulation may have deleterious effects on mitochondrial function. Recent studies showed that PreP activity is reduced in Alzheimer disease (AD) patients and AD mouse models compared to controls, which correlated with an enhanced reactive oxygen species production in mitochondria. In this study, we have investigated the effects of a biologically relevant oxidant, hydrogen peroxide (H_2O_2), on the activity of recombinant human PreP (hPreP). H_2O_2 inhibited hPreP activity in a concentration-dependent manner, resulting in oxidation of amino acid residues (detected by carbonylation) and lowered protein stability. Substitution of the evolutionarily conserved methionine 206 for leucine resulted in increased sensitivity of hPreP to oxidation, indicating a possible protective role of M206 as internal antioxidant. The activity of hPreP oxidized at low concentrations of H_2O_2 could be restored by methionine sulfoxide reductase A (MsrA), an enzyme that localizes to the mitochondrial matrix, suggesting that hPreP constitutes a substrate for MsrA. In summary, our in vitro results suggest a possible redox control of hPreP in the mitochondrial matrix and support the protective role of the conserved methionine 206 residue as an internal antioxidant.

Keywords

Mitochondria; Presequence protease; Peptide degradation; Oxidation; Methionine; Free radicals

Mitochondria have a fundamental role in many cellular processes, including bioenergetics, metabolism of lipids and amino acids, and even coordinated cell death (apoptosis). A recent proteomic analysis estimated that mitochondria contain around 1200 different proteins [1–3], with more than 99% of them being synthesized on cytosolic ribosomes, posttranslationally targeted, and then translocated across the mitochondrial membrane

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system into one of four compartments: the outer or inner membrane, the intermembrane space, or the mitochondrial matrix (reviewed by [4]).

To ensure correct sorting and targeting after synthesis on cytosolic ribosomes, most of the mitochondrial proteins contain a targeting sequence, designated presequence. This presequence is used for recognition of the translocation machinery by binding to receptor proteins [4]. When the protein reaches its correct destination the presequence is removed by the mitochondrial processing peptidase (reviewed in [5–7]). The free presequence peptides have amphipathic characteristics and have been shown to affect mitochondrial membrane integrity, uncouple respiration, and inhibit enzyme activity [8–10].

Presequence peptides are degraded by a mitochondrial matrix resident peptidasome, the presequence protease (PreP)[1] [11–13]. This peptidase has a fundamental role in mitochondrial biogenesis, completing the last step of the protein import process, the degradation of the presequence peptide. PreP was originally identified in *Arabidopsis thaliana* [14], and homologs were found in yeast [15,16] and also in humans [17]. This important role in mitochondrial biogenesis is supported by the phenotypic analysis of PreP mutants, both in *Arabidopsis thaliana* and in *Saccharomyces cerevisiae*: double *At*PreP1/ *At*PreP2 mutants exhibit a growth phenotype, chlorosis, and uncoupling of mitochondria [18], whereas the *S. cerevisiae* Δ*mop112* strain has a severe growth defect on nonfermentative carbon sources [15].

In addition to degrading presequences, human PreP (hPreP; UniProt ID: Q5JRX3) was also shown to degrade the amyloid-β peptide (Aβ) [17], suggesting a connection between the activity of hPreP and the progression of Alzheimer disease (AD). This idea is further supported by the observation that hPreP activity is severely diminished in samples of mitochondrial matrix isolated from the temporal lobe of Alzheimer disease patients, compared to age-matched controls [19]. Additionally, this reduction in activity is recapitulated in AD transgenic mice models (overexpressing the human form of amyloid-β precursor protein) [19]. These observations, together with current evidence supporting the localization of Aβ peptide in mitochondria [20–22] and its well-established toxic effects [23–25], point toward an important role of hPreP in Aβ metabolism and in the progression of Alzheimer disease.

Interestingly, even though the activity of hPreP was reduced in AD patients and in transgenic mouse models, this was not a consequence of reduced protein levels [19]. This observation raised the hypothesis that reduced hPreP activity can be caused by oxidation of the enzyme, because an increase in the production of reactive oxygen species (ROS) was observed in AD patients [19].

In this study, we used an in vitro setup with purified hPreP to investigate the effect of oxidation by the biologically relevant oxidant hydrogen peroxide on hPreP activity and established the role of a conserved methionine residue in the process.

Materials and methods

Purification of recombinant wild-type hPreP and variants

Plasmid pGEX6p2 encoding human hPreP lacking its presequence (Δ1–28 aa) was transformed into *Escherichia coli* Rosetta 2 cells (Novagen). Cell cultures were grown in 1-L batches in Terrific Broth medium (Formedium) containing 100 μg/ml ampicillin (Sigma)

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[1]*Abbreviations used*: hPreP, human presequence protease; MsrA, methionine sulfoxide reductase A; AD, Alzheimer disease; ROS, reactive oxygen species; DTT, dithiothreitol.

and 34 μg/ml chloramphenicol (Sigma) at 37 °C to OD_{600} ~0.6 and then induced with 0.6 mM isopropyl β-ᴅ-1-thiogalactopyranoside (Formedium) for 5 h (with the temperature adjusted to 21 °C). Cells were then harvested and frozen as pellets. The cell pellets were dissolved in GSH-binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.3, 2.5 mM DTT (dithiothreitol), 10 mM $MgCl_2$, 1 μM Zn acetate) containing 1 mg/ml lysozyme (Sigma) and 10 μg/ml DNase I (Sigma) and then incubated for 1 h at 4 °C for cell lysis. The cell extract was centrifuged at 4000 g to remove unbroken cells and the supernatant further centrifuged at 100,000 g. The soluble fraction was incubated with Glutathione Sepharose (GE Healthcare) for 4 h and then washed four times with GSH-binding buffer and two times with cleavage buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM DTT). Twenty units of HRV3C protease (MoBiTec) were applied and the hPreP protein was eluted after overnight incubation at 4 °C.

hPreP variants were constructed by site-directed mutagenesis on the pGEX_hPreP vector using the QuikChange II kit (Agilent Technologies) and appropriate primers following the manufacturer's instructions and confirmed by sequencing. hPreP variants were purified using the same procedure.

Oxidation of hPreP with hydrogen peroxide (H_2O_2)

hPreP and variants (final concentration 0.26 mg ml^{-1}) were incubated with the indicated concentrations of hydrogen peroxide for 4 h at 22 °C, in 50 mM Hepes–KOH, pH 8.2. Hydrogen peroxide (Sigma) concentration was measured at 240 nm ($\varepsilon = 39.4 \pm 0.2$ M^{-1} cm^{-1}). After 4 h, the peroxide was removed by filtration using a 50 k filter (Millipore) or by dialysis against 50 mM Hepes–KOH, pH 8.2, and the hPreP samples were used for activity measurement, SDS–PAGE, and carbonyl Western blot.

hPreP activity measurements

For the analysis of $pF_1\beta$ and Aβ degradation, hPreP samples (previously incubated with peroxide as indicated) were incubated with 0.8 μg $pF_1\beta$ (purified according to [26]) or 1 μg amyloid-β (Sigma) for the indicated times, in degradation buffer (50 mM Hepes, pH 8.2, 10 mM $MgCl_2$), at 37 °C (three independent experiments). The amount of hPreP used was 0.5 μg (per reaction) in the $pF_1\beta$ assay and 1 μg in the amyloid-β assay. After incubation the reactions were stopped with SDS sample buffer and subsequently loaded on NuPAGE 4–12% Bis–Tris gels (Invitrogen) and stained with Coomassie brilliant blue (Sigma). The bands were quantified using the Multi Gauge software.

For the degradation of the C1 peptide (Promega), hPreP fractions (0.8 μg per assay) were incubated with 1 μg C1 peptide in degradation buffer and the reaction allowed to proceed for 20 min at 37 °C. The samples were chilled on ice and subsequently loaded on 0.8% agarose gel and visualized by UV light.

In the substrate V assay, hPreP fractions (0.8 μg per assay) were mixed with 1 μg substrate V (R&D Systems) in degradation buffer, and the increase in fluorescence (excitation 327 nm; emission 395 nm) was immediately recorded in a plate reader (SpectraMax Gemini) during the first 40 s. Results are shown as the substrate V degradation rate and averaged over three independent experiments.

Carbonyl detection

Carbonylation was detected using the OxyBlot kit (Milipore), according to the manufacturer's instructions.

Limited trypsin proteolysis

hPreP controls (2 μg) and samples treated for 4 h with H_2O_2 were incubated with 10 ng trypsin at 30 °C, and samples were withdrawn after 1, 10, and 30 min and subsequently analyzed using 10% Tris–glycine gels.

MsrA recovery assays

hPreP was incubated with H_2O_2 (0.5 or 5 mM) for 4 h at 22 °C. The peroxide was removed by filtration and extensive washes with reaction buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3). Then, hPreP (1.9 μM) was incubated for 3 h at 37 °C with purified recombinant mouse MsrA (as indicated), purified according to [27] in reaction buffer with 10 mM DTT and 5 mM $MgCl_2$. The activity of hPreP was then assayed using the fluorogenic substrate V.

Results

Inactivation of hPreP upon exposure to hydrogen peroxide

The main aim of this study was to investigate the connection between oxidation of hPreP and reduction in activity. To do so, we analyzed the effect of exposure to H_2O_2 on the activity of recombinant hPreP in an in vitro assay. Nevertheless, hydrogen peroxide is a biologically relevant oxidative agent being produced within the mitochondrial matrix, the cellular compartment where hPreP is localized [28].

To analyze the effect of oxidation on hPreP activity we incubated the purified enzyme with various concentrations of H_2O_2 (ranging from 0.5 to 5 mM) and assessed the peptidolytic activity of hPreP using four different peptide substrates. The peptides used were previously shown to be substrates for hPreP (or PreP homologs) [19,29–31] and reflect the broad substrate specificity of this peptidase as they vary in length and physicochemical properties. Table 1 summarizes the characteristics of the peptide substrates used: $pF_1\beta$, a typical mitochondrial presequence peptide (a 54-amino-acid peptide corresponding to the presequence of the *Nicotiana plumbaginifolia* $F_1\beta$ subunit of ATP synthase); the amyloid-β 1–40 peptide (an endogenous substrate for hPreP); and two broad-range protease substrates, the 11-amino-acid C1 peptide and the fluorogenic bradykinin-derived 9-amino-acid substrate V.

As observed in Fig. 1, exposure of hPreP to H_2O_2 resulted in a concentration-dependent inhibition of hPreP peptidolytic activity, as analyzed with all four substrates. Fig. 1A shows inhibition of C1 degradation by hPreP exposed to increased concentrations of H_2O_2. A simple qualitative analysis shows clearly that the cleavage of the C1 peptide, from the slower migrating to the faster migrating form, is reduced when hPreP is exposed to the oxidant.

To provide a more quantitative analysis of the activity inhibition by H_2O_2 we used the degradation of substrate V to assess hPreP activity. This peptide contains both the fluorescent group 7-methoxycoumarin and the quencher group 2,4-dinitrophenyl, resulting in fluorescence emission upon cleavage of a peptide bond between the two groups. In Fig. 1B we show that there is a concentration-dependent reduction in the degradation rate of substrate V by hPreP upon exposure to increasing concentrations of H_2O_2. Exposure of hPreP to 0.5 mM H_2O_2 results in a reduction of activity of about 50–60%, whereas exposure to 5 mM H_2O_2 results in a reduction of 80–90% of hPreP activity.

We then tested the influence of H_2O_2 on the hPreP ability to degrade a typical presequence peptide ($pF_1\beta$) and the endogenous substrate Aβ, using polyacrylamide gel-based assays.

Figs. 1C and 1D show time course analyses of $pF_1\beta$ and $A\beta$ degradation, respectively, by hPreP preincubated in the absence or presence of H_2O_2 (0.5 or 5 mM). In the case of both peptides, it is clear that the rate of hPreP-catalyzed degradation is reduced upon incubation with H_2O_2. This effect is particularly visible in the case of $pF_1\beta$ degradation, with the hPreP degradation activity reduced by about 70% upon exposure to 0.5 mM H_2O_2. In the case of $A\beta$ degradation, exposure of hPreP to 0.5 mM H_2O_2 results in 50% reduction in the cleavage rate.

Even though the extent of hPreP activity inhibition varies between the different assays (probably reflecting the distinct assay conditions and degradation rates), all the data shown in Fig. 1 clearly demonstrate that exposure of hPreP to the oxidative agent H_2O_2 results in a less active enzyme and consequently in a strong reduction of the degradation activity toward four different substrates.

Considering both the ease of analysis and the reproducibility of results, we selected the substrate V assay to analyze hPreP activity in the remaining experiments reported.

Molecular consequences of hPreP oxidation

It has been known for long time that incubation with H_2O_2 can result in protein degradation by chemical hydrolysis of the peptide bond [32]. We evaluated the possibility of hPreP degradation in the presence of H_2O_2 by simply monitoring hPreP by SDS–PAGE after incubation with H_2O_2. However, in the case of hPreP oxidation, no degradation was observed (Fig. 2A), indicating that the reduction in activity is rather caused by amino acid oxidation, not by protein degradation. Consistent with this idea, oxidation of hPreP by H_2O_2 resulted in protein carbonylation, a general hallmark of protein oxidation (Fig. 2B). Interestingly, oxidation probably leads to a change in hPreP conformation resulting in a less stable protein as analyzed by limited trypsin proteolysis. Oxidized hPreP is more sensitive to trypsin proteolysis than the control sample (Fig. 2C), an indicator of altered conformation, possibly reflecting an increased tendency for unfolding.

It is widely accepted that exposure of proteins to oxidizing agents such as hydrogen peroxide results in the production of oxidized forms of different amino acids [33]. Although it is certain that most amino acid residues can be targets of oxidative agents, those containing sulfur (cysteine and methionine) are among the most readily oxidizable residues. In addition, both oxidation of methionine (to methionine sulfoxide) and oxidation of cysteine (to disulfide) are processes that can be biologically reversed [34–37]. Considering the previous suggestion that hPreP oxidation would lead to formation of a disulfide bridge between C119 and C556 [17] (numbering used throughout this report refers to hPreP preprotein and corresponds to C90 and C527 in the mature portion of the protein as used by Falkevall et al. [17]), we analyzed the effect of H_2O_2 on hPreP variants in which either C119 or C556 was replaced by serine. In both variants, exposure to H_2O_2 resulted in a reduction in activity similar to that in wild-type hPreP (not shown). The reduction in activity observed in response to hydrogen peroxide is thus not (or at least not only) due to the formation of an S–S bridge between C119 and C556, as that would be prevented in these substitutions. Additionally, we did not observe any recovery of the activity of oxidized hPreP by adding DTT (see Fig. 5), further supporting the idea that hPreP inactivation by H_2O_2 is not due to the formation of a disulfide bridge.

Methionine residues are also readily oxidizable to methionine sulfoxide by reactive oxygen species, including hydrogen peroxide (further oxidation to methionine sulfone occurs only rarely under physiological conditions) [35]. hPreP contains 24 methionine residues in its mature form (residues 29–1037, corresponding to the form processed in mitochondria), with most of them being surface-exposed. However, a few methionine residues are found buried

in the structure and close to the active site of the enzyme, according to the structural model of hPreP, built based on the structure of *A. thaliana* PreP1 (Fig. 3).

An interesting feature of some methionine residues in proteins is their ability to act as internal antioxidants: being easily oxidizable residues, methionines can work as scavengers, preventing further oxidation of other amino acid residues [34,38,39].

To understand the roles of methionine residues and methionine oxidation in hPreP inactivation by H_2O_2, we analyzed on one hand the possibility of recovering hPreP activity by reduction of methionine sulfoxide and on the other hand the role of methionine residues in hPreP as antioxidants.

Substitution of methionine 206 for leucine in hPreP results in increased sensitivity to oxidation by hydrogen peroxide

To investigate the role of methionine residues located close to the enzyme active site on hPreP oxidation we produced methionine-to-leucine substitutions of residues M97, M126, M135, M206, and M504. The variants produced resulted in active proteins, with activity comparable to that of wild-type hPreP as measured with the substrate V assay, except for M97L, which showed about half of the activity (not shown). We expected that replacing methionine residues that perform a putative antioxidant function in hPreP with the nonoxidizable amino acid leucine would result in increased sensitivity to H_2O_2 exposure.

When we measured the peptidolytic activity of the hPreP variants upon exposure to various concentrations of H_2O_2 (Fig. 4A and Supplementary Fig. S1), we observed that the hPreP^M206L variant exhibited a strikingly different inhibition profile compared to wild-type hPreP (Fig. 4A) and the other variants (Supplementary Fig. S1). More specifically, a time course analysis of the oxidative inactivation of hPreP wt and hPreP^M206L showed that, upon exposure to 0.5 mM H_2O_2, the hPreP^M206L variant was inactivated three times faster than the wild type (Fig. 4B). Interestingly, in the early time points of the experiment with hPreP wt (Fig. 4B), we consistently observed a slight increase in the activity before observing the activity decrease. Although the reason for this increase is unclear, this phenomenon was observed previously in the oxidative inactivation of glutamine synthetase [40].

Experiments with hPreP variants revealed that replacing M206 with leucine results in hPreP being more vulnerable to oxidation by H_2O_2, reflected in a more pronounced effect of this oxidant on the activity, even at low H_2O_2 concentrations. Considering that in the absence of M206, hPreP activity is more sensitive to H_2O_2 exposure, it is possible that this methionine residue has a protective role, minimizing further oxidation of other residues at low concentration of the oxidant, although we cannot exclude the possibility that the substitution caused a conformational change that renders residues more susceptible to oxidation.

The importance of the M206 residue is additionally emphasized by its localization within the hPreP active site in the structural model (Fig. 3) and also by the fact that it is the only methionine residue completely conserved in PreP sequences from different organisms, including plants, parasites, yeast, and mammals (Fig. 3 and full sequence alignment in Supplementary Fig. S2). Because of its location, one can speculate that M206 may protect the active-site histidines in the zinc-binding motif (H^104ILEH^108), avoiding irreparable damage to the enzyme.

Activity of oxidized hPreP can be recovered by MsrA

Methionine oxidation to methionine sulfoxide can be enzymatically reversed by the activity of methionine sulfoxide reductases [41,42]. Msr proteins are divided into two families, MsrA and MsrB, based on the reaction stereospecificity. Oxidation of methionine to

methionine sulfoxide results in the formation of two stereoisomers at the sulfur atom (Met-S-SO and Met-R-SO), with MsrA reducing the S isomer and MsrB reducing the R isomer. In mammals, MsrA is dually localized to the cytosol and to the mitochondrial matrix because of alternative translation initiation sites [27,43,44]. In vivo, the regeneration of active MsrA, as part of its catalytic cycle, requires the sequential action of thioredoxin and thioredoxin reductase, whereas in vitro DTT can replace the thioredoxin system as a source of reductant [41].

To evaluate the possibility of recovering hPreP activity upon oxidation we performed incubation with recombinant mouse MsrA [27]. The incubation with MsrA was performed using as substrate hPreP oxidized under either low (0.5 mM H_2O_2) or high oxidant conditions (5mM H_2O_2). Fig. 5B shows that when hPreP was oxidized in the presence of 5 mM H_2O_2, the incubation with MsrA did not increase the activity of hPreP. However, when hPreP was oxidized in the presence of 0.5 mM H_2O_2 (Fig. 5A) the incubation with MsrA resulted in a significant increase in hPreP activity, whereas incubation with DTT alone had essentially no effect.

Incubation with MsrA resulted in close to the maximal recovery theoretically possible, considering the stereospecificity of MsrA (only one stereoisomer of methionine sulfoxide is reduced and therefore the recovery of activity is about 50% of the activity "lost" because of oxidation).

Our results in an in vitro system with purified proteins suggest that hPreP may be a substrate for mitochondrial MsrA based on the recovery of oxidized hPreP activity by MsrA and also on the known intracellular localization of both hPreP and MsrA in the mitochondrial matrix [17,27,43,44].

Discussion

In this report we show that exposure of purified hPreP to an oxidizing agent, hydrogen peroxide, results in decreased peptidolytic activity. Additionally, we suggest that the evolutionarily conserved methionine 206 plays a protective role against hPreP oxidation, as when this residue is substituted by leucine, hPreP shows increased sensitivity to inactivation by hydrogen peroxide. Our observations show that hPreP is inactivated by H_2O_2 in a concentration-dependent manner and that oxidation with low concentrations of H_2O_2 can be reversed by mitochondrial MsrA. Thus, at low concentrations of H_2O_2, methionine residues in hPreP can be oxidized, resulting in a reduction in activity of about 60%, which can be reversed by the action of MsrA. However, exposure to higher concentrations of H_2O_2 leads to a reduction in activity of 90–100%, which is not reversed by MsrA. The exposure to 5 mM H_2O_2 may result in oxidation of residues other than methionines (an increase in protein carbonylation is clearly observed in Fig. 2A, demonstrating further oxidation of other amino acids), reactions that cannot be biologically reversed. Mapping of oxidation sites has been successfully achieved for a number of proteins, especially utilizing HPLC coupled to mass spectrometry [45,46]. However, the large size of hPreP (> 1000 amino acids) and the existence of 24 methionine residues in the mature form make the precise mapping of each methionine oxidation a challenging task. Nevertheless, our current efforts are aimed at tackling this problem.

The present results substantiate the critical role of methionine residues in proteins for protection against oxidative inactivation, in cases in which the proteins are exposed to low concentrations of oxidants. This protective role of methionine residues has been demonstrated in several previous reports [34–36,38,39,47]. For example, it was shown thatmethionine residues in α_2-antitrypsin work as internal antioxidants protecting the critical

tryptophan residue fromirreversible oxidation and inactivation [47]. Additionally, a recent report showed that partial substitution of methionine residues for the nonoxidizable amino acid nor-leucine in *E. coli* cells results in increased sensitivity to H_2O_2 oxidation and in increased protein carbonylation [39]. Our present study further supports the proposal that methionine residues play an important role in protecting proteins from oxidative inactivation by scavenging oxidizing agents and limiting the damage inflicted on catalytically essential residues.

Additionally, the possibility of controlling hPreP activity by reversible methionine oxidation may constitute a novel regulatory mechanism. The activity of hPreP could potentially be controlled within the mitochondrial matrix by localized production of hydrogen peroxide and possibly also by other reactive oxygen species. Although the biological significance of this phenomenon remains to be elucidated, it may provide a new strategy to regulate hPreP activity. Future experiments in human cell lines knocked down for MsrA may provide clues to understanding hPreP regulation.

Although the regulation of protein function by reversible methionine oxidation is still poorly understood, there have been some reports of such a regulatory strategy [48]. One such example is the regulation of Ca^{2+}–calmodulin protein kinase II (CaMKII) [49]. Oxidation of two methionine residues in CaMKII results in activation of the kinase. In CaMKII, a helical segment containing M281/282 is exposed upon binding of Ca^{2+} and calmodulin, and methionine oxidation to sulfoxide results in retained kinase activity even in the absence of Ca^{2+}–calmodulin. This oxidation can be reversed by MsrA and constitutes an additional regulatory mechanism for CaMKII.

Conclusions

In summary, this report highlights the inhibitory effect of hydrogen peroxide on hPreP activity, describes the role of methionine 206 as an internal antioxidant, and suggests the possibility of hPreP activity being regulated by MsrA. Inactivation by reactive oxygen species was also observed for other amyloid-β-degrading peptidases, such as insulin-degrading enzyme and neprilysin, using in vitro assays [50]. Considering that hPreP activity is reduced in Alzheimer disease patients, our future efforts will be focused on assessing the role of hPreP oxidation in the progression of this pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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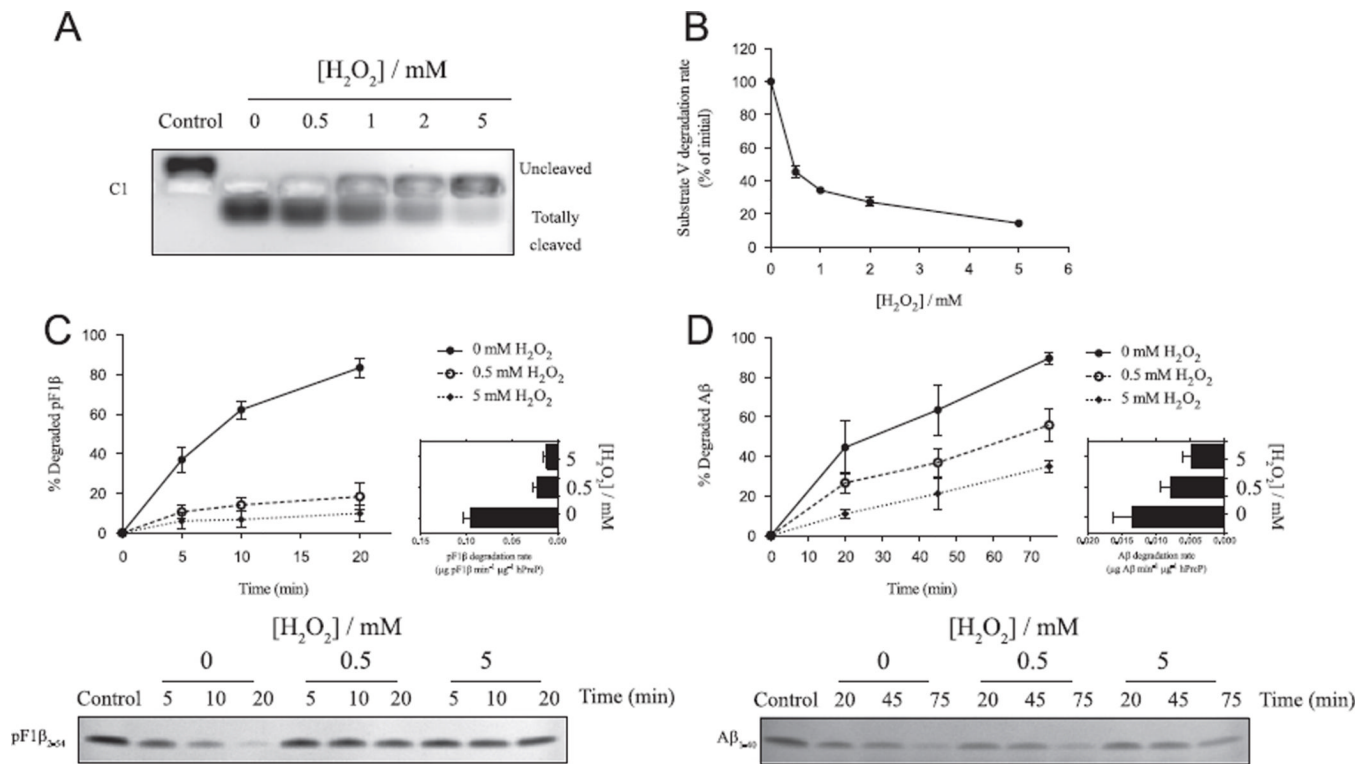
Fig. 1.
Effect of exposure to hydrogen peroxide on hPreP wild-type activity. After 4 h incubation with H_2O_2, the hydrogen peroxide was removed and hPreP activity assayed as the cleavage of four substrates. (A) Representative gel showing cleavage of C1 by hPreP, resulting in a change in migration on agarose gel due to the charge profile of the peptide. (B) Effect of oxidation on the rate of substrate V degradation by hPreP (average of three experiments). Time course analysis of (C) $pF_1\beta$ and (D) $A\beta$ degradation by hPreP incubated in the absence or presence (0.5 or 5 mM) of H_2O_2. Shown are representative gels (corresponding to one of the three experiments) and an estimation of the degradation rate (in the first 10 min for $pF_1\beta$ and in the first 45 min for $A\beta$).
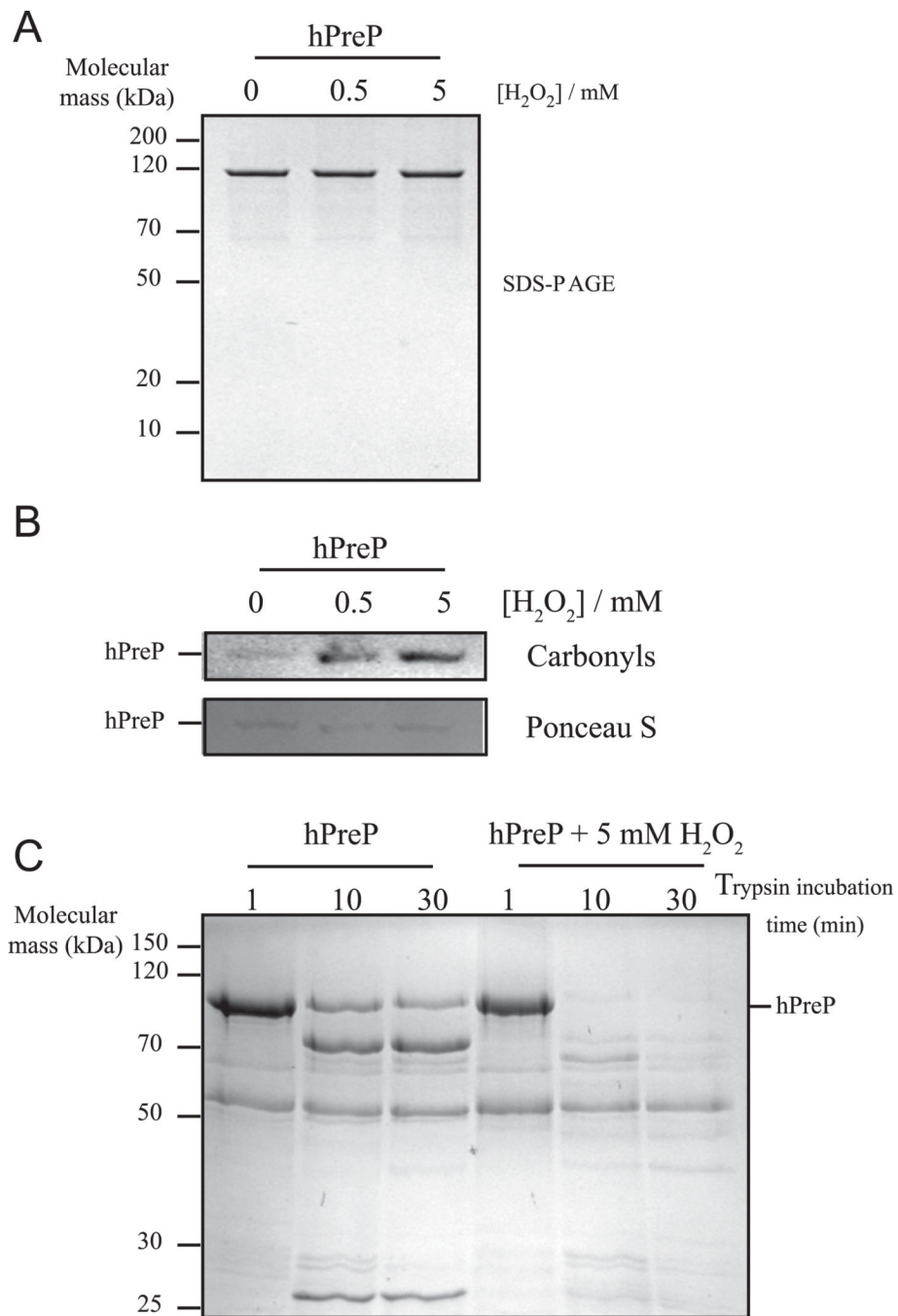
Fig. 2.
Consequences of hPreP wild-type oxidation. Upon incubation with H_2O_2 for 4 h, hPreP was analyzed by (A) SDS–PAGE (0.7 µg per lane) and (B) carbonyl immunoblot (4 µg per lane), as described under Materials and methods. Ponceau S staining is shown as a loading control. (C) hPreP samples were incubated in the presence or absence of 5 mM H_2O_2 for 4 h; subsequently treated with 10 ng trypsin for 1, 10, and 30 min; and then analyzed by SDS–PAGE (2 µg per lane).
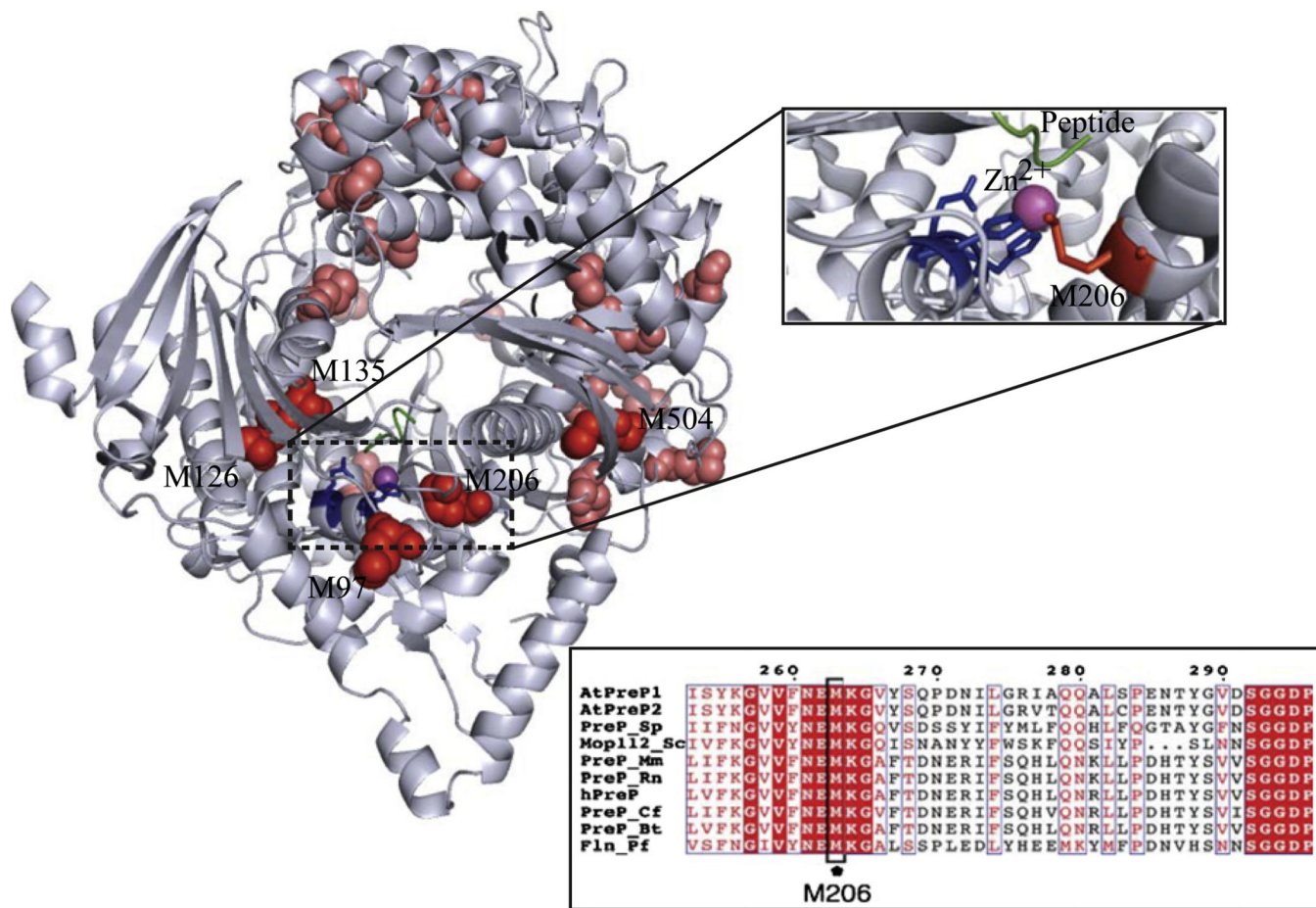
Fig. 3.
Localization of the analyzed methionine residues in the hPreP structural model, constructed based on the structure of *At*PreP1 (PDB ID: 2FGE) [51]. Methionines are shown in red, the active-site histidines are blue, the substrate peptide is green, and the zinc ion is magenta. The detailed location of M206 within the hPreP active site is highlighted. Inset shows an alignment of PreP sequences from *A. thaliana* (AtPreP1 and AtPreP2), *Plasmodium falciparum* (falcilysin, Fln), *S. cerevisiae* (Mop112), *Bos taurus* (PreP_Bt), *Canis familiaris* (PreP_Cf), *Mus musculus* (PreP_Mm), *Rattus norvergicus* (PreP_Rn), *Schizosaccharomyces pombe* (PreP_Sp), and *Homo sapiens* (hPreP). The alignment is restricted to the region around M206 (full alignment is shown in Supplementary Fig. S2).
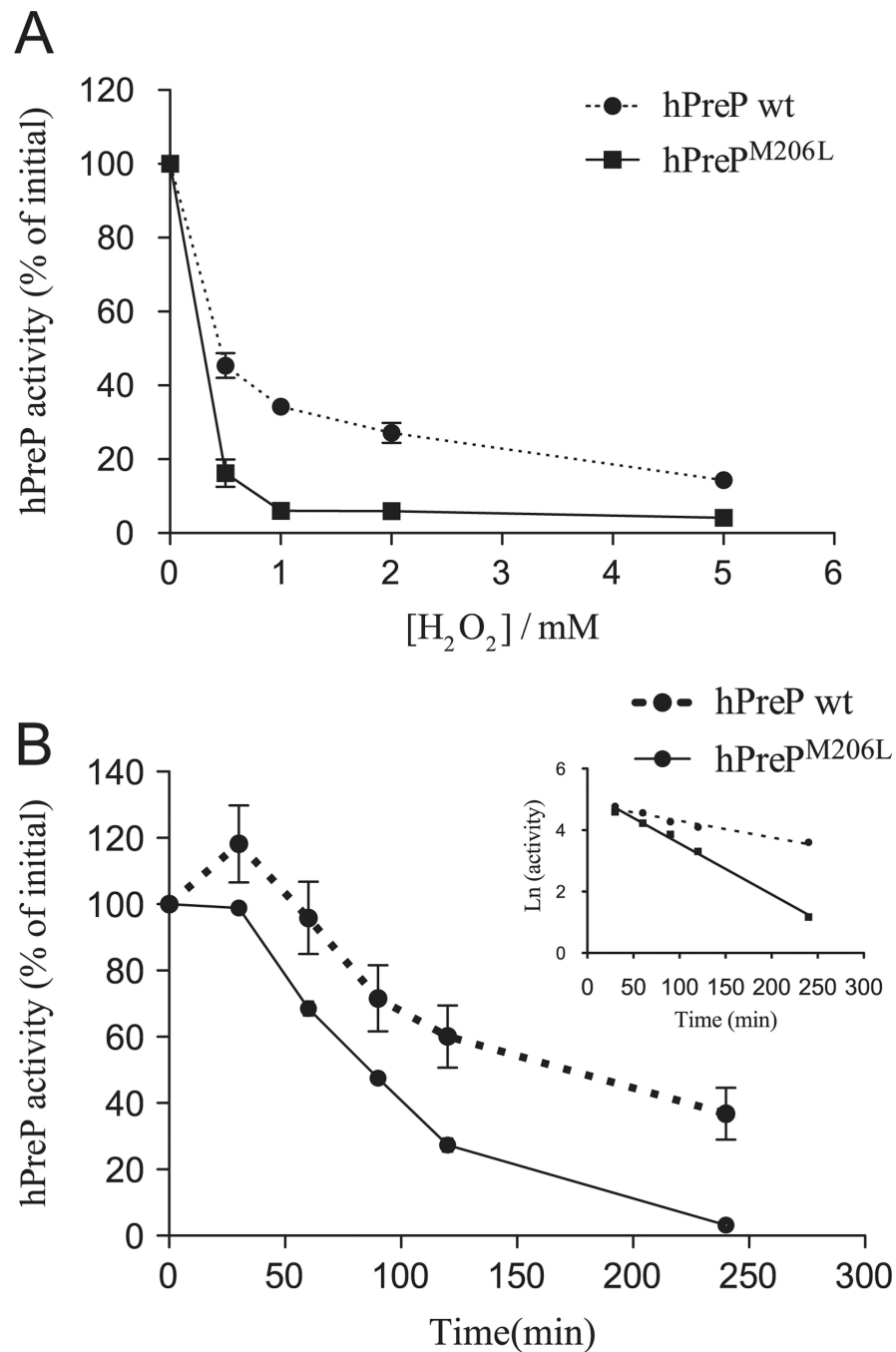
Fig. 4.
(A) Effect of H_2O_2 exposure (4 h) on the activity of hPreP[M206L] compared to hPreP wild type (wt), as assayed by the degradation of the fluorogenic substrate V. (B) Time course inactivation of hPreP wt and hPreP[M206L] upon exposure to 0.5 mM H_2O_2 analyzed by the substrate V assay (results shown are averages of three experiments). The regression lines (inset) were fit for a first-order reaction for time points from 30 to 240 min and gave R^2 of 0.96 and 0.99, for the hPreP wt and hPreP[M206L] data sets, respectively. The ratio between the inactivation rates of hPreP[M206L] and hPreP wt showed a threefold difference. 100% activity corresponds to a specific activity of 491.6 ± 54.2 ng substrate V degraded min^{-1} μg

protein^{-1} for wt hPreP and 656.2 ± 32.7 ng substrate V degraded min^{-1} µg protein^{-1} for hPreP^{M206L}.
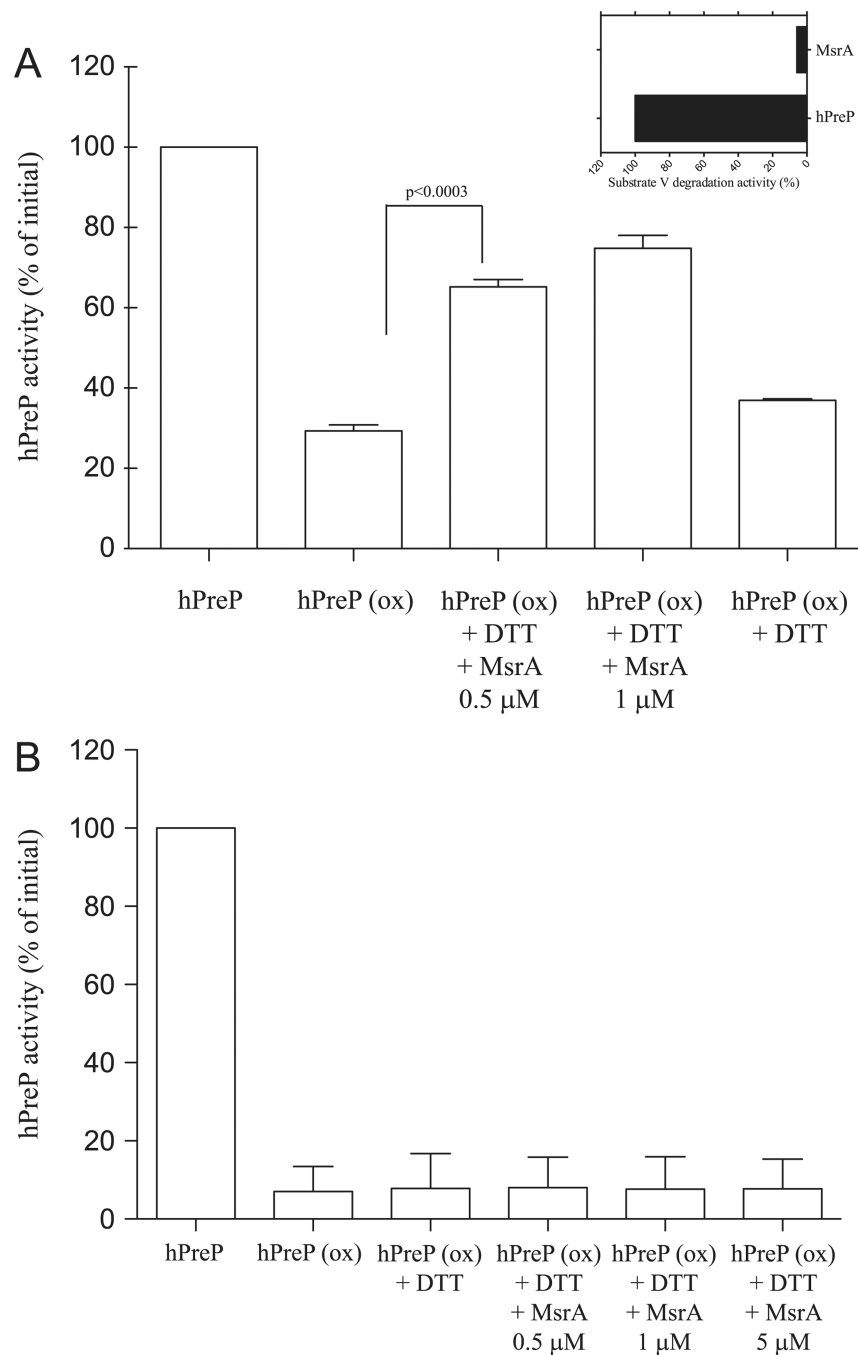
Fig. 5.
Recovery of hPreP wt activity by MsrA. hPreP was exposed to H_2O_2 for 4 h at the concentrations of 0.5 or 5 mM. The H_2O_2 was then removed by filtration and hPreP further incubated for 2 h at 37 °C with DTT and MsrA as indicated. After this incubation the hPreP activity was assayed using substrate V. (A) MsrA effect on hPreP oxidized with 0.5 mM H_2O_2. (B) MsrA effect on hPreP oxidized with 5 mM H_2O_2. Statistical analysis was made using Student's *t* test. Inset in (A) shows that MsrA itself has no degradation activity against substrate V.

Table 1

Properties of the peptide substrates used to measure hPreP activity.

Peptide	No. amino acids	Sequence
Substrate V	9	(7-Methoxycoumarin-4-yl)acetyl–RPPGFSAFK–(2,4-dinitrophenyl)
C1	11	PLSRTLSVAAK
Amyloid-β	40	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV
pF₁β	53	ASRRLLASLLRQSAQRGGGLISRSLGNSPKSASRASSRASPKGFLLNRAVQYM