Rat peptide methionine sulphoxide reductase: cloning of the cDNA, and down-regulation of gene expression and enzyme activity during aging

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Peptide methionine sulphoxide reductase (PMSR, EC 1.8.4.6), the *msrA* or *pmsR* gene product, is a ubiquitous enzyme catalysing the reduction of methionine sulphoxide to methionine in proteins. Decreased expression and/or activity of the PMSR with age could explain, at least in part, the accumulation of oxidized protein observed upon aging. To test this hypothesis, the rat *pmsR* cDNA was cloned and sequenced. The recombinant protein was expressed, its catalytic activity checked with a synthetic substrate and polyclonal antibodies were raised against recombinant PMSR. The expression of the *pmsR* gene and protein as well as its catalytic activity were then analysed as a function of age in the rat brain and in two organs that express the most PMSR, liver and kidney. It appears that *pmsR* gene expression decreases with age in liver and kidney as early as 18 months, whereas protein level and protein activity are reduced in the three organs at the very end of the life of the rat (26 months). These results suggest that the down-regulation of PMSR can contribute to the accumulation of oxidized protein that has been associated with the aging process.

Key words: post-translational modification, protein oxidation, protein repair.

INTRODUCTION

Accumulation of oxidized proteins is a hallmark of cellular aging. Indeed, proteins are amongst the cellular macromolecules that can be modified by free radicals and reactive oxygen species and these modifications are strongly believed to be deleterious for cellular functions and to contribute to the aging process [1,2]. In the cell, the oxidized proteins are rapidly degraded by the proteasome [3–5], although some oxidative modifications to protein may be repaired by specific enzymes [6,7]. In proteins, methionine residues are especially susceptible to oxidation, leading to the formation of methionine sulphoxide [8]. Oxidation of methionine is generally associated with a loss of biological activity in a wide range of biological peptides and proteins and it was suggested that this modification may be implicated in aging and in several pathologies including cataract formation [9], pulmonary emphysema [10] and more recently in Alzheimer's disease [11]. In contrast to other oxidation products of amino acids, methionine sulphoxide in proteins can be enzymically reduced back to methionine by a ubiquitous enzyme, peptide methionine sulphoxide reductase (PMSR) [6]. This enzyme is present in the majority of living organisms and has been described as one of the 300 genes encoding the minimal set of proteins sufficient for cellular life [12]. The mammalian protein is detected in all tissues investigated, although with differing expression levels; kidney, brain and liver express the most PMSR enzyme [13,14]. It has been postulated that PMSR could have a potential protective role against oxidative stress [15]. Indeed, null mutants of *pmsR* in *Escherichia coli* and *Saccharomyces cerevisiae* show a decreased resistance toward oxidative-stress conditions [15,16]. Moreover, overexpression of the enzyme in *S. cerevisiae* and human T-cells results in an increased resistance of the cells to oxidative stress [17]. Furthermore, it has been proposed that methionine residues constitute an important antioxidant defence mechanism since a variety of oxidants react readily with methionine and the reduction back by PMSR would allow the antioxidant system to function catalytically [18,19].

The accumulation of oxidized methionine in proteins during aging suggests that the activity of PMSR might be not sufficient to maintain proteins in a reduced and functional state. This could be explained by either decreased expression of the *pmsR* or impaired activity of the enzyme during aging. To test these hypotheses, we have cloned and sequenced the rat *pmsR* gene. The recombinant protein has been expressed, purified and its catalytic activity assayed by using the synthetic substrate 4-dimethylaminoazobenzene-4′-sulphonyl-methionine sulphoxide [dabsyl-Met(O)] [20]. Then, expression of the *pmsR* gene and protein as well as its catalytic activity were analysed in different organs (brain, liver and kidney) of Fisher 344 rats as a function of age.

EXPERIMENTAL

Cloning and sequencing of the rat *pmsR*

Based on the mammalian *pmsR* published sequences [14,21], degenerate PCR primers (5′-CTCGGATCCATGGGNTGYTTCTGGGGNG-3′, 5′-CAGGAATTCAGYTARTGCTGGTGGT-3′) were used to isolate a partial cDNA sequence of 422 bp from a rat liver 5′-STRECH PLUS cDNA library (Clontech). The resulting PCR fragment was cloned in the pBluescript II

Abbreviations used: PMSR, peptide methionine sulphoxide reductase; dabsyl-Met, 4-dimethylaminoazobenzene-4′-sulphonyl-methionine; dabsyl-Met(O), 4-dimethylaminoazobenzene-4′-sulphonyl-methionine sulphoxide; DTT, dithiothreitol.

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SK(+) vector, fully sequenced and ^{32}P-labelled by using the Rediprime labelling kit (Amersham Pharmacia Biotech) to make a probe. The probe was used to identify the complete *pmsR* gene by colony hybridization using standard protocols. DNA from the positive phage was purified by using the Qiagen Lambda System according to the manufacturer's recommendations, cloned into the *Eco*RI restriction site of the pBluescript II SK(+) vector and was sequenced in both directions.

Overexpression and purification of the rat recombinant PMSR protein

The complete rat *pmsR* coding region was ligated into the *Nde*I and *Bam*HI restriction sites of the prokaryotic expression vector pET-15b (Novagen). BL21 cells (Novagen) were transformed with the recombinant plasmid and grown in LB medium containing 50 μg/ml ampicillin at 37 °C. When cells reached an absorbance at 600 nm of 0.9, isopropyl β-D-thiogalactoside was added to a final concentration of 0.5 mM and the culture was continued for an additional 4 h. The cells were centrifuged at 5000 ***g*** for 10 min at 4 °C and the pellet was suspended in buffer A (20 mM Tris/HCl, pH 7.9/500 mM NaCl/0.5 mM imidazole) and sonicated four times for 30 s. The lysate was centrifuged at 39000 ***g*** for 20 min at 4 °C. Supernatant was applied to a 4 ml His-Bind resin column (Novagen) previously equilibrated with the same buffer A. Column was washed with buffer A containing 60 mM imidazole and PMSR was then eluted with buffer A containing 1 M imidazole. Fractions of 5 ml were collected and protein concentrations were determined using the Bradford method (Bio-Rad Protein Assay). The purity of the PMSR was verified by SDS/PAGE stained with Coomassie Brilliant Blue. The protein solution was dialysed overnight against 50 mM potassium phosphate buffer, pH 7, containing 100 mM KCl. Dithiothreitol (DTT) was then added to a final concentration of 5 mM and the protein was concentrated in a 10000 Da molecular-mass cut-off Centricon concentrator (Amicon). The N-terminal His-tag sequence of the recombinant protein was removed using the Thrombin kit (Novagen). Protein (500 μg) was digested for 18 h at 20 °C with 1 unit of thrombin. The efficiency of the cleavage was checked by SDS/PAGE and MS.

Analytical ultracentrifugation

Centrifugation of recombinant PMSR was performed in a Beckman Optima-XLA analytical centrifuge, using standard double-sector cells with aluminum centrepieces of 1.2 mm thickness. The temperature was 4 °C. The cells were scanned at 280 nm. After reaching 16500 ***g***, radial scans were recorded at 2 h intervals for 18 h to check that equilibrium was reached. When equilibrium was achieved, 10 successive scans were recorded and averaged to improve the signal-to-noise ratio of the recordings. The centrifuge was then accelerated to 200000 ***g*** to clear the meniscus from any residual protein and determine the absorbance baseline. The protein distribution at equilibrium was analysed with the Origin-based Optima XL-A data analysis software (Beckman). The different fitting models (Ideal 1, Ideal 2 and Assoc 4) for single data sets (XLA-Single program) were tested systematically, and the best fitting was retained on the basis of both the χ^2 value and the lack of systematic deviation of the residuals. In all cases, the baseline was first fixed at the value observed after meniscus clearing and allowed to float only as an ultimate fitting refinement, which was accepted only if the resulting baseline differed by less than 0.02 absorbance units from that determined experimentally. The value of the partial specific volume used for molecular-mass calculations was 0.726 ml/g, as determined according to Cohn and Edsall [22] from the amino acid composition of the recombinant PMSR. The buffer density was 1.009 g/ml.

RNA isolation and Northern-blot analysis

Male Fisher 344 rats of different ages (9, 18 and 24 months old) were obtained from Iffa Credo (L'Arbresle, France). The rats were killed, and the organs were excised and frozen immediately in liquid nitrogen and stored at −70 °C. The organs were thawed at 4 °C, cut into small pieces and homogenized with a polytron in TRIzol reagent (Life Technologies). Total RNAs were isolated from kidney, liver and brain using the single-step guanidinium thiocyanate/phenol method. RNA (35 μg) was separated by 1.2% (w/v) agarose/6.6% (v/v) formaldehyde denaturing gel electrophoresis and capillary-transferred on to nylon membrane (GeneScreen, NEN Life Science Products). The blots were hybridized with a [α-^{32}P]dCTP-labelled PCR fragment that included nucleotides 207–629 of the rat *pmsR* coding sequence. The gels were autoradiographed, the films were scanned and submitted to densitometric analysis by using Image Master 1D quantification software (Amersham Pharmacia Biotech). The amount of specific RNA was normalized by hybridization with the 18 S ribosomal RNA probe.

Preparation of tissue extract and enzymic assays

Kidney, liver and brain samples from 9, 18, 24 and 26 month-old Fisher 344 rats were thawed at 4 °C, suspended and homogenized in 25 mM Tris/HCl, pH 7.4, containing 200 mM sucrose, 12.5 mM $MgCl_2$, 5 mM EDTA and 1 mM DTT, with a Potter homogenizer. The homogenates were centrifuged at 12000 ***g*** for 2 h at 4 °C. The supernatant was recovered and protein concentrations were determined by using the Bradford method (Bio-Rad Protein Assay). The activity of PMSR in the different crude extracts was determined by monitoring the reduction of the synthetic substrate dabsyl-Met(O). The reaction mixture containing 400 μg of total proteins (or 8 μg of purified recombinant protein) in 15 mM Hepes, pH 7.4/10 mM $MgCl_2$/30 mM KCl/20 mM dithioerythritol/0.5 mM dabsyl-Met(O) in a final volume of 100 μl was incubated for 1 h at 37 °C [20]. The reaction was stopped by addition of diamide to a final concentration of 30 mM. After centrifugation at 12000 ***g*** for 30 min at 4 °C, 20 μl of the resulting supernatant were loaded on a C_{18} reversed-phase column (Beckman) using the Beckman Gold HPLC system. The elution was monitored at 472 nm. Using a linear gradient from 20 to 66.7% of acetonitrile in 29 mM ammonium acetate, pH 4.1, over 10 min, 4-dimethylamino-azobenzene-4′-sulphonyl-methionine (dabsyl-Met) and dabsyl-Met(O) were differentially eluted. The peaks corresponding to dabsyl-Met were integrated and the activities in the different organs of young and old rats were determined in pmol of dabsyl-Met/min per mg of protein.

Western-blotting experiments

Rabbit antibodies were raised against the recombinant rat PMSR and were purified on a Protein A–Sepharose column (Amersham Pharmacia Biotech). Total protein extract (20 μg) of liver, brain and kidney from 9, 18, 24 and 26 month-old Fisher 344 rats were separated on SDS/PAGE (12%, w/v, gel) and then electro-transferred on to nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech). Membranes were incubated with anti-PMSR polyclonal antibodies at a dilution of 1:100000. Blots were developed using ECL detection reagents (Amersham

Pharmacia Biotech). Films were scanned and the amount of PMSR was quantified by densitometric analyses using Image Master 1D quantification software.

Statistical analysis

Data are expressed as means ± S.E.M. Results were compared by ANOVA followed by the Student's t test for unpaired data. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Cloning and sequencing of the rat PMSR

The complete cDNA encoding the PMSR was isolated from a 5′-STRECH PLUS cDNA library as described in the Experimental section. The cDNA insert of 1268 nucleotides contains an open reading frame which encodes a protein of 233 amino acids with a calculated molecular mass of 25851 Da. Nevertheless, the first ATG codon is flanked by the sequence 5′-TCC**ATG**C-3′, while the ATG codon at position 21 is flanked by the sequence 5′-ATG**ATG**G-3′, which is a better consensus sequence for translational initiation (the most important conserved nucleotides are underlined) [23], suggesting that a shorter protein could be translated from this codon. The open reading frame is followed by a 522-nucleotide 3′-untranslated region which does not present the polyadenylation signal. The consensus motif ^{71}GCFWG^{75}, which is in the active site, and in particular the conserved Cys-72, as well as the two cysteines at positions 218 and 227 that may be also involved in the catalytic activity of the PMSR [24–28], are also present in the rat sequence (Figure 1). Alignment of the rat PMSR protein and the two other known mammalian enzymes reveals 82% identity between rat and bovine PMSR and 80% between rat and human PMSR (Figure 1).

Overexpression, purification and biochemical characterization of the recombinant PMSR protein

The complete open reading frame was cloned into the pET-15b vector and PMSR was expressed as a fusion protein with an N-terminal His tag in *E. coli*. Recombinant PMSR was purified by Ni^{2+}-chelation chromatography as shown in Figure 2 (lanes A and B). On SDS/PAGE, recombinant PMSR exhibits an ap-

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                10        20        30        40        50        60
                 |         |         |         |         |         |
Human    MLSATRRACQLLLLHSLFPVPRMGNSASNIVSPQEALPGRKEQTPVAAKHHVNGNRTVEP
Bovine   MLSVTRRA--LQLFHSLFPIPRMGDSAAKIVSPQEALPGRKEPLVVAAKHHVNGNRTVEP
Rat      MLSASRRT--LQLLSSSIPVRMMGDSSSKVISAEEALPGRTESIPVAAKHHVSGNRTVEP
         ***  **   * *  *  *   ** *     *  ****** *   ******* *******

                70        80        90       100       110       120
                 |         |         |         |         |         |
Human    FPEGTQMAVFGMGCFWGAERKFWVLKGVYSTQVGFAGGYTSNPTYKEVCSEKTGHAEVVR
Bovine   FPEGTQMAVFGMGCFWGAERKFWTLKGVYSTQVGFAGGYTPNPTYKEVCSGKTGHAEVVR
Rat      FPEGTQMAVFGMGCFWGAERKFWLLKGVYSTQVGFAGGYTRNPTYKEVCSEKTGHAEVVR
         ********************** ********** ********* *********

               130       140       150       160       170       180
                 |         |         |         |         |         |
Human    VVYQPEHMSFEELLKVFWENHDPTQGMRQGNDHGTQYRSAIYPTSAKQMEAALSSKENYQ
Bovine   VVFQPEHISFEELLKVFWENHDPTQGMRQGNDHGSQYRSAIYPTSAEHVGAALKSKEDYQ
Rat      VVYRPEHVSFEELLKVFWENHDPTQGMRQGNDCGTQYRSAVYPTSAVQMEAALKSKEEYQ
         **  *** ************************ * ***** *****     *** *** **

                190       200       210       220       230
                 |         |         |         |         |
Human    KVLSEHGFGPITTDIREGQTFYYAEDYHQQYLSKNPNGYCGLGGTGVSCPLGIKK
Bovine   KVLSEHGFGLITTDIREGQTFYYAEDYHQQYLSKDPDGYCGLGGTGVSCPLGIKK
Rat      KVLSKHGFGPITTDIREGQVFYYAEDYHQQYLSKNPDGYCGLGGTGVSCPTAIKK
         **** **** ********* ************** * *************  ***
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Figure 1 Sequence alignment of human, bovine and rat PMSR

The amino acid sequence of rat PMSR deduced from the longest open reading frame was aligned with the sequences of human and bovine PMSR using the CLUSTAL W algorithm (Institut de Biologie et Chimie des Proteines, Lyon, France). The amino acid residues conserved between the three proteins are highlighted with asterisks.

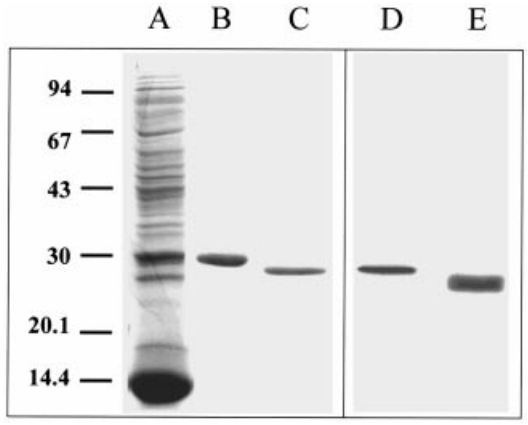

Figure 2 SDS/PAGE and Western-blotting analysis of recombinant and native PMSR

Lane A, crude protein extract of *E. coli* cells transformed with the pET-15b vector harbouring the rat *pmsR* gene and induced with 0.5 mM isopropyl β-D-thiogalactoside. Lanes B and C, affinity-purified recombinant PMSR before or after thrombin cleavage, respectively. Lanes D and E, recombinant PMSR without His tag and protein extract from rat liver, respectively, were subjected to SDS/PAGE (12% acrylamide gel), electrotransferred on to a nitrocellulose membrane and immunoblotted with anti-PMSR polyclonal antibodies.

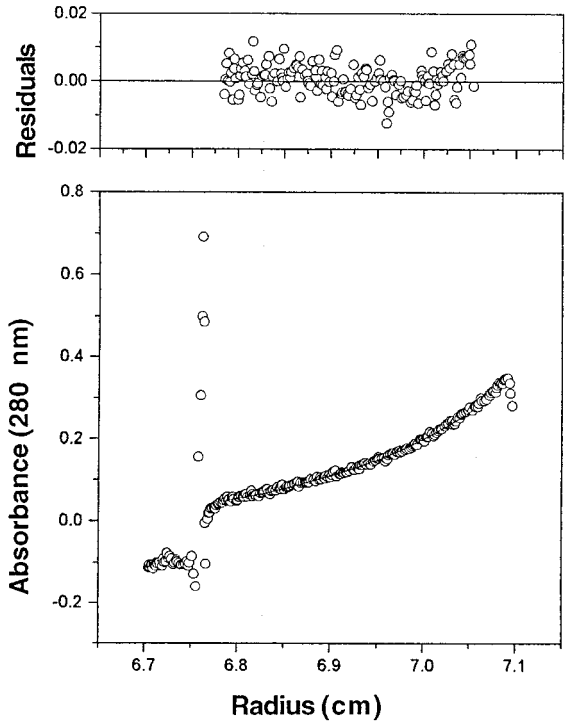

Figure 3 Sedimentation equilibrium analysis of recombinant rat PMSR

Recombinant rat PMSR (0.35 mg/ml) in potassium phosphate buffer (50 mM, pH 7.0) with 50 mM KCl and 2 mM DTT was subjected to centrifugation at 16500 ***g*** and 4 °C as described in the Experimental section. When equilibrium was reached, 10 successive scans at 280 nm were recorded and averaged. The resulting data and the corresponding fitted curve are shown in the main panel, and the distribution of residuals is shown in the top panel.

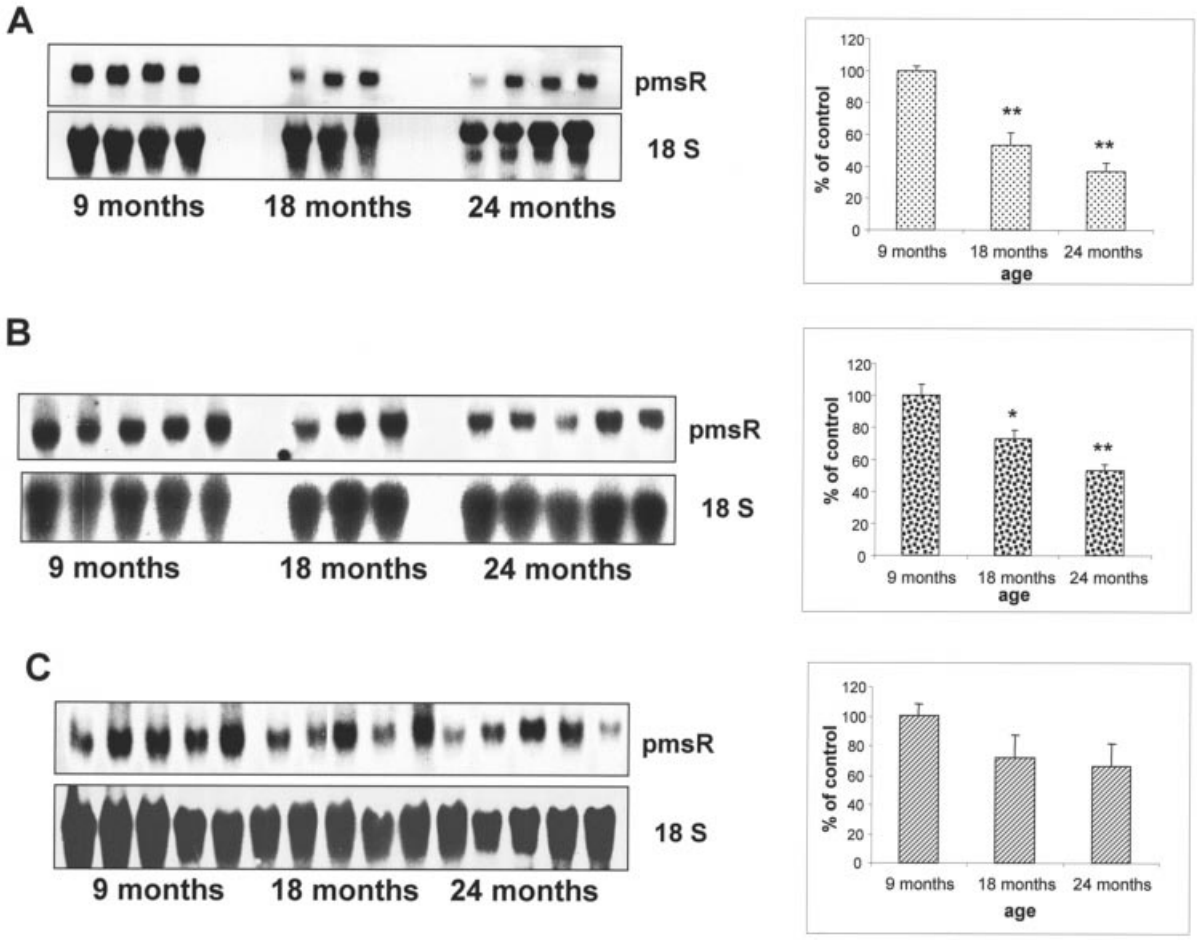

Figure 4 Northern-blot analysis of the *pmsR* gene in rat tissues during aging

(**A**) Total RNAs (35 μg) extracted from kidney of rats of different ages (9 months old, $n = 4$; 18 months old, $n = 3$; 24 months old, $n = 4$) were separated by 1.2% (w/v) agarose denaturing formaldehyde gel electrophoresis and transferred on to a nylon membrane. The blots were probed with a [α-^{32}P]dCTP-labelled PCR fragment, which included nucleotides 207–629 of the rat *pmsR* coding sequence (upper panel), or with the 18 S ribosomal RNA probe (lower panel). In the densitometric analysis (right-hand panel), the amounts of RNA were normalized by hybridization with the 18 S ribosomal RNA probe. The measurement corresponding to that obtained from 9-month-old rats was taken as 100%; $^{**}P < 0.01$ compared with 9-month-old rats. (**B**) As described for (**A**), expression of the *pmsR* gene in liver from rats of different ages was analysed by Northern blotting followed by densitometric analyses (9 months old, $n = 5$; 18 months old, $n = 3$; 24 months old, $n = 5$); $^{*}P < 0.05$, $^{**}P < 0.01$. (**C**) As described for (**A**), expression of the *pmsR* gene in brain from rats of different ages was analysed by Northern blotting followed by densitometric analyses (9 months old, $n = 5$; 18 months old, $n = 5$; 24 months old, $n = 5$).

parent molecular mass of approx. 30 kDa. The N-terminal His tag of the protein was removed by thrombin cleavage (Figure 2, lane C). The molecular masses of the recombinant PMSR with and without the His tag were determined by MS to be 27883 Da and 26132 Da respectively, which is in agreement with the cDNA sequence. Polyclonal antibodies were used in Western-blot experiments to probe the cellular rat PMSR, as described in the Experimental section. A major band is detected at a molecular mass slightly lower than that of the recombinant PMSR (Figure 2, lanes D and E).

A sample of PMSR dialysed against 50 mM potassium phosphate buffer, pH 7.0, containing 50 mM KCl/2 mM DTT, and adjusted at 0.35 mg/ml with the dialysed buffer, was submitted to analytical centrifugation to determine the quaternary structure of the recombinant enzyme in solution (Figure 3). A good fit of the data was obtained assuming a unique species with ideal behaviour ($\chi^2 = 2.1 \times 10^{-5}$, random distribution of the residuals), with an estimated molecular mass of 28115 ± 346 Da. Thus under these experimental conditions PMSR appears to be monomeric in solution.

To check the enzymic activity of the recombinant PMSR, with or without the His tag, the reduction of the synthetic substrate dabsyl-Met(O) to dabsyl-Met was monitored by reversed-phase HPLC. The two proteins exhibited the same specific activity (10 μmol of dabsyl-Met/min per mg of protein).

Age-related decrease of mRNA expression in several rat tissues

mRNA levels were determined in kidney, liver and brain, organs that are known to express the most the PMSR enzyme [14,21]. Total mRNA from young (9 month-old), middle-aged (18 month-old) and old (24 month-old) rats were analysed by probing tissue blots with the ^{32}P-labelled PCR product (see the Experimental section). The probe hybridized with a unique mRNA species of about 1.4 kb in the three tissues tested (Figure 4). In all organs, the expression of the enzyme appeared down-regulated with age,

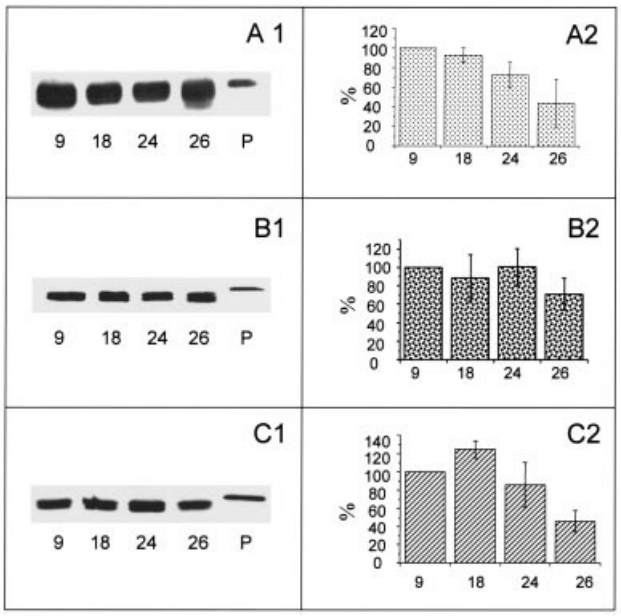

Figure 5 Quantification of PMSR protein in rat tissues during aging

Protein extract (20 μg) from kidney (**A1**), liver (**B1**) and brain (**C1**) of rats of different ages (9, 18, 24 and 26 months) were subjected to SDS/PAGE (12% gel), electrotransferred and Western blotted as described in the Experimental section. The blots were processed with anti-PMSR antibodies at a dilution of 1:100000. Typical blots from three independent experiments are shown. For densitometric analysis of kidney (**A2**), liver (**B2**) and brain (**C2**), the measurement corresponding to that obtained from 9-month-old rats was taken as 100%.

with a decline in expression seen from as early as 18 months old, and to a greater extent at 24 months old. In kidney and liver, expression of the *pmsR* gene in middle-aged rats was 53% and 73% of the levels observed in the 9-month-old rats, respectively. The decline was more significant in old rats in which the expression levels dropped to 37% and 53%, respectively, compared with the young rats ($P < 0.01$). Furthermore, decreased expression was also observed in the brain but the differences did not reach statistical significance due to variability between individuals within each sample age group (Figure 4C).

Quantification of PMSR in rat tissues during aging

The amount of PMSR in rat liver, kidney and brain was determined by Western blotting using polyclonal antibodies raised against the recombinant protein. For each organ, the quantities of PMSR detected at 18, 24 and 26 months were quantified relative to that detected at 9 months. In contrast with the results observed previously concerning the mRNA levels, the quantity of PMSR appears stable from 9 to 24 months in the three organs analysed, with the differences observed not being statistically significant due to the variability within the individual data (Figure 5). In addition, the level of PMSR in the brain from 18-month-old rats reached more than 120% of that observed in the brain from 9-month-old rats. To determine whether PMSR activity is maintained throughout lifespan, we examined the PMSR content in 26-month-old rats, at the very end of their life. For this oldest age, the amount of PMSR decreased significantly in the three organs. In brain and kidney it was about 50% of the amount of PMSR present in 9-month-old rats and 70% in the liver.

Table 1 The effect of aging on PMSR activity

The ability of PMSR to reduce protein-bound methionine sulphoxide was assayed by using dabsyl-Met(O) as substrate in the presence of 400 μg of total protein from kidney, liver and brain from rats of different ages. After elution on a C_{18} reversed-phase column (as described in the Experimental section), the peak corresponding to dabsyl-Met was integrated and the specific activities were determined. Data are expressed as the mean percentage variability ($\pm$S.E.M.) from at least three independent experiments.

Tissue	Age (months)	PMSR activity (pmol of dabsyl-Met/min per mg)
Kidney	9	296.4 $\pm$ 13.6
	18	250.2 $\pm$ 32.1
	24	269.7 $\pm$ 14
	26	157.7 $\pm$ 35.8 ($P < 0.01$)
Liver	9	180.8 $\pm$ 17
	18	214.2 $\pm$ 18
	24	206.2 $\pm$ 3.8
	26	85.5 $\pm$ 13.1 ($P < 0.01$)
Brain	9	166 $\pm$ 15.5
	18	186.75 $\pm$ 16.82
	24	185.53 $\pm$ 3.7
	26	145.4 $\pm$ 12.06

Determination of PMSR activity in rat tissues during aging

In order to determine the PMSR protein's activity in the different tissues, in correlation with the modulation of protein expression, the specific activity of PMSR in crude extracts of the different organs was measured by monitoring the reduction of the synthetic substrate dabsyl-Met(O) to dabsyl-Met. The PMSR activities observed in the different organs were in agreement with the expression levels of the enzyme, as reported previously, i.e. highest in kidney, followed by liver and brain [14,21]. As shown in Table 1, the catalytic activity of PMSR in the different organs is maintained until 24 months since the differences observed between the rats of different ages are not significant. In the 26-month-old rats there was approx. 50% less activity in the liver and kidney compared with that exhibited by the 9-month-old rats. On the other hand, the 20% decline observed in the brain was not statistically significant.

DISCUSSION

We have identified rat PMSR, a novel member of the mammalian PMSR family. Only the bovine and the human *pmsR* genes [14,21] have been cloned so far. Analysis of the rat PMSR sequence confirms the very high sequence homology between members of the family, particularly the residues involved in the catalytic mechanism [24–28] and emphasises the crucial role of this protein in the defence against oxidative stress in all living species. From the sequence analysis, it is interesting to note that although there is an open reading frame encoding a protein of 233 amino acids, there is also an ATG codon encoding Met-21. This methionine could well be the initial methionine for translation since it is flanked with a better consensus translation-initiation motif than is Met-1. Western-blot analysis after SDS/PAGE separation of both the recombinant and cellular PMSRs indicates that the cellular PMSR may actually be a little bit shorter than the recombinant enzyme (Figure 2, lanes C and D), arguing for Met-21 as the initial methionine. However, only MS analysis or N-terminal sequencing of PMSR purified from rat organs will give a clear answer to this question.

As the PMSR has been described as an oxidized protein-repair enzyme that may constitute an important antioxidant-defence system [29], we have focused our studies on the gene and protein expression and activity in relation to the aging process. Meth-

ionine is one amino acid that is oxidized easily by various oxidants and this modification has been associated with many physiological and pathological conditions [30]. In many cases, methionine oxidation in proteins has been associated with a loss of activity and a disruption of structural conformation, but another possible role for this post-translational modification is to participate in the regulation of protein function [31]. Intracellular oxidized protein content has been shown to increase with age and, besides degradation by the proteasome [32], reversion of methionine sulphoxide back to methionine in protein can be achieved by the PMSR enzyme as a way to eliminate oxidized proteins [7]. Consequently, impaired expression and/or activity of PMSR with age may well explain the accumulation of methionine sulphoxide within the proteins and therefore contribute to the age-related accumulation of oxidized protein. By analysing *pmsR* gene expression, protein levels and specific enzymic activities in different organs as a function of age, we have shown that gene expression is indeed notably down-regulated with age in kidney and liver (Figures 4A and 4B), two organs often considered to have house-keeping roles. In contrast, in the brain, the differences that we have determined in gene expression are not statistically significant (Figure 4C), but both the enzymic activity and protein level are reduced in 26-month-old rats (Table 1 and Figure 5). It has been reported previously [33] that some proteins like calmodulin, isolated from the brain of senescent Fisher 344 rats, exhibited increased amounts of methionine sulphoxide, which can be reversed back to methionine by PMSR *in vitro*, and it has been postulated [33] that in aging brain the specific activity of the PMSR enzyme should be insufficient to maintain calmodulin in a fully functional state. Our findings give experimental evidence that PMSR function may be impaired in the aging brain, although at very old age. In this study, we have examined extracts from the whole brain but other investigations have demonstrated that the protein is not present at the same level in all regions of the brain [11,13,14] and may be not affected in the same way by aging. Future studies will be directed at PMSR expression and activity status during aging in the different regions of the brain. This will be of particular interest in such regions as the superior and middle temporal gyri, inferior parietal lobule and the hippocampus, as it has been observed in Alzheimer's disease that PMSR activity is decreased while mRNA levels are not affected [11].

In addition, there has recently been a great deal of interest in gene-expression patterns in relation to aging. This question has been addressed using DNA-biochip technology in different systems taken as models for aging; skeletal muscle and brain from mice [34,35] and human dermal fibroblasts [36]. Highly different patterns were found depending on the post-mitotic or mitotic status of the cells. In fact, less than 2% of the 6347 genes whose expression was monitored were affected with age. Although expression of the *pmsR* gene was not analysed in these studies, our results indicate that *pmsR* is also an important gene whose expression is affected with age. As suggested by Lee et al. [35], oxidative stress may be the underlying cause of the aging process of post-mitotic tissues, and increased protein turnover and decreased macromolecular damage may explain the anti-aging effect of caloric restriction. Therefore, repair of oxidized protein by PMSR is also likely to be of importance in aging. It remains to be known, however, whether the age-related decrease of *pmsR* gene expression is reversed with age-matched calorie-restricted animals.

Finally, the PMSR activity we measured was assayed in crude extracts in the presence of saturating amounts of reductant dithioerythritol and synthetic substrate dabsyl-Met(O). Although a similar trend for a decrease of both protein level and enzymic activity was observed, there is a possibility that the PMSR can be itself oxidatively damaged, which will probably affect its activity. Indeed Cys-72, which is crucial for PMSR activity, as well as Cys-218 and Cys-227, appear as potential targets for enzyme inactivation under oxidative conditions. Such a mechanism, in addition to the age-related decrease in gene and protein expression we report in this study, may also explain the accumulation of methionine sulphoxide within proteins and consequently contribute to the alteration of cellular functions.

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