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In vivo aging of rat skeletal muscle sarcoplasmic reticulum Ca-ATPase. Chemical analysis and quantitative simulation by exposure to low levels of peroxyl radicals

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Abstract

Sarcoplasmic reticulum (SR) Ca-ATPase of young adult (5 months) and aged (28 months) Fischer 344 male rat skeletal muscle was analyzed for posttranslational modifications as a result of biological aging and their potential functional consequences. The significant differences in the amino acid composition were a 6.8% lower content of sulfhydryl groups and a ca. 4% lower content of Arg residues of the Ca-ATPase from old as compared to young rats. Based on a total of 24 Cys residues the difference in protein thiols corresponds to a loss of 1.5 mol Cys/mol Ca-ATPase as a result of in vivo aging. The loss of Cys residues was not accompanied by a loss of enzyme activity though the 'aged' Ca-ATPase was more sensitive to heat inactivation, aggregation, and tryptic digestion. A comparison of the total sulfhydryl content of all SR proteins present revealed a 13% lower amount for SR vesicles isolated from aged rats. Compared to the alterations of Cys and Arg, there was only a slight and probably physiologically insignificant increase of protein carbonyls with aging, i.e. from 0.32 to 0.46 mol carbonyl groups per mol of Ca-ATPase. When SR vesicles from young rats were exposed to AAPH-derived peroxyl radicals, there was a loss of ca. 1.38×10^{-4} M total SR sulfhydryl groups per 4 mg SR protein/ml (corresponding to ca. 25%) and a loss of 9.6×10^{-5} M Ca-ATPase sulfhydryl groups (corresponding to ca. 31%) per 1.6×10^{-5} M initiating peroxyl radicals, indicating that the stoichiometry of sulfhydryl oxidation was ≥ 6 oxidized thiols per initiating AAPH-derived peroxyl radical. Besides Cys, the exposure to AAPH-derived radicals caused a slight loss of Ca-ATPase Arg, Met, and Ser residues. Most importantly, the SR Ca-ATPase exposed to this low concentration of peroxyl radicals displayed physical and functional properties quantitatively comparable to those of SR Ca-ATPase isolated from aged rats, i.e. no immediate loss of activity, increased susceptibility to heat inactivation, aggregation, and tryptic digestion. Moreover, a comparison of kinetically early tryptic fragments by HPLC-electrospray MS and N-terminal sequencing revealed that similar peptide fragments were produced from 'aged' and AAPH-oxidized Ca-ATPase which were not (or

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)hydrochloride; ATP, adenosine-5′-triphosphate; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; 2,4-DNPH, 2,4-dinitrophenylhydrazine; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; HPLC, high performance liquid chromatography; LDS, lithium dodecylsulfate; MOPS, 4-morpholinepropanesulfonic acid; NEM, *N*-ethylmaleimide; PITC, phenylisothiocyanate; PVDF, polyvinylidene difluoride; SDS, sodium dodecylsulfate; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; SR, sarcoplasmic reticulum; TFA, trifluoroacetic acid

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kinetically significantly later) generated from the 'young' Ca-ATPase, suggesting some conformational changes of the Ca-ATPase as a result of aging and AAPH-exposure. All except one of these peptides originated from locations remote from the nucleotide-binding and calcium-binding sites. The latter results suggest that aging and AAPH-exposure may target similar Cys residues, mainly at locations remote from the nucleotide-binding and calcium-binding sites, rationalizing the fact that Cys oxidation did not immediately cause inactivation of the Ca-ATPase. Our results provide a quantitative estimate of a net concentration of reactive oxygen species, here peroxyl radicals, which induces physical and chemical alterations of the SR Ca-ATPase quantitatively comparable to those induced by in vivo aging.

Keywords: Aging; Skeletal muscle; Sarcoplasmic reticulum Ca-ATPase; Oxidation; Peroxyl radical; Thiol

1. Introduction

Considerable effort has been devoted to investigate the effect of aging on muscle function which is generally characterized by an increase in contraction and half-relaxation times with age [1]. At present a molecular basis for these effects has not been clearly established but it has been suggested that modifications of proteins and/or membranes may contribute [1–3]. The sarcoplasmic reticulum (SR) Ca-ATPase serves an important function during muscle relaxation by transporting intracellular calcium into the lumen of the SR coupled to ATP hydrolysis. Specifically for homogenates from fast twitch skeletal muscle fibers, a 32% decrease of both the rate of Ca^{2+} uptake and the fractional rate of SR filling, and an 18% decrease in loading capacity of the SR were observed for old as compared to young rats [3]. Based on these findings a potential inactivation of the SR Ca-ATPase with age was hypothesized. In contrast, Gafni and Yuh [2] examined purified rat skeletal muscle SR vesicles for age-related changes of the SR Ca-ATPase and were able to demonstrate identical activities of the enzyme isolated from young and aged tissue. However, when native SR preparations were subjected to mild heating at 37°C, SR Ca-ATPase isolated from old animals exhibited slightly faster inactivation. Based upon the observation that this age-associated increased rate of inactivation was not seen upon solubilization of SR membranes with Triton-X100 it was suggested that the membrane rather than the Ca-ATPase per se had undergone age-related changes, thereby indirectly affecting the Ca-ATPase activity. More recently it has been demonstrated that rat skeletal muscle SR isolated from aged rats has a slightly different phospholipid composition as compared to that of young rats [4]. However, these compositional differences were not accompanied by changes in the physical properties of bulk or protein-associated lipids as well as in the rotational dynamics of the Ca-ATPase molecule. Thus, without a detailed analysis of SR Ca-ATPase isolated from aged muscle, chemical modifications of the protein itself as a cause of the reduced efficiency of SR Ca^{2+} uptake cannot be excluded.

Recently, we have shown that the SERCA2 (slow-twitch) isoform of the rat skeletal muscle SR Ca-ATPase isolated from aged rats contains remarkably high levels of nitrotyrosine (up to 3 mol nitrotyrosine/mol Ca-ATPase) [5]. Parallel in vitro experiments on isolated SR vesicles demonstrated that chemically the nitration of tyrosine of SERCA2 could be achieved by exposure to peroxynitrite, $ONOO^-$, a product of the reaction of nitric oxide, NO, with superoxide, $O_2^{\cdot -}$. Interestingly, in contrast to the SERCA2 (slow-twitch) isoform, the in vitro exposure of both rat [5] and rabbit [6] SERCA1 (fast-twitch) isoform to peroxynitrite resulted in no formation of nitrotyrosine even though its tyrosine content (23 Tyr residues [7]) is higher than that of the SERCA2 isoform (18 Tyr residues [8]). Other potential chemical alterations may contribute to the age-related modifications of specifically the SERCA1 which constitutes the major isoform of SR Ca-ATPase in fast-twitch skeletal muscle.

Here we report a chemical and functional analysis of the SR Ca-ATPase obtained from the hindlimb skeletal muscle of young adult (5 months old) and aged (28 months old) Fischer 344 rats. These native SR preparations are compared to protein of young adult rats which was exposed to defined low concentrations of peroxyl radicals derived from the thermolabile initiator 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH). Peroxyl radicals were selected as modifying agents since membrane proteins such as the SR Ca-ATPase are likely to be exposed to such

species in vivo resulting from the peroxidation of lipids. We will demonstrate that SR Ca-ATPase from aged rats and SR Ca-ATPase from young rats subjected to low levels of peroxyl radicals show (i) an identical profile of heat inactivation, (ii) a comparable tendency to form aggregates during exposure to elevated temperatures, (iii) a comparable higher sensitivity to tryptic cleavage with similar peptide fragments appearing, and (iv) a significant loss of protein cysteine (Cys) residues. The finding that rat skeletal muscle SR Ca-ATPase suffers oxidative modification with age, and that the effect of such modification can be simulated by exposure to low levels of peroxyl radicals, supports a role of free radicals and/or reactive oxygen species in the aging process, as initially proposed by Harman [9]. Our results will provide a quantitative estimate as to the fraction of reactive oxygen species reacting with a protein which is required to simulate age-related modifications of SR Ca-ATPase from rat skeletal muscle. Such quantitative considerations are important for a true evaluation of the potential role of reactive oxygen species in age-related modifications of biological function or etiologies of biological dysfunction.

2. Experimental

2.1. Materials

All chemicals were analytical grade products. 2,2′-Azobis(2-amidinopropane) hydrochloride (AAPH) and 2,4-dinitrophenylhydrazine (2,4-DNPH) were obtained from Eastman Kodak (Rochester, NY). Sigma Chemical (St. Louis, MO) provided adenosine 5′-triphosphate (ATP), calcium ionophore A23187, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), iodoacetic acid, sodium dodecyl sulfate (SDS), bovine pancreatic TPCK-treated trypsin, and molecular weight standards for size-exclusion chromatography. All reagents for gel electrophoresis, electroblotting, and prestained protein markers were from BIORAD (Richmond, CA). All reagents for amino acid analysis were from Pierce (Rockford, IL). HPLC grade solvents were from Fisher Scientific (Medford, MA). The potassium phosphate buffer employed in the oxidation experiments was treated with 5% (w/v) Chelex-100 (BIORAD) in order to minimize transition metal contaminations. *N*-ethyl-[^{14}C]maleimide was from NEN Life Science Products (Boston, MA).

2.2. Membrane preparations

Native SR vesicles were prepared from rat hindlimb muscles as described previously [5,10]. For each preparation, SR membranes were isolated from equal numbers (3 animals per preparation) of young (5 months) and old (28 months) adult male Fischer strain 344 rats obtained from the National Institutes of Health maintained rat colony (Harlan Sprague Dawley, Indianapolis). Prior to sacrifice, the rats were generally allowed to adapt for two weeks after arrival. SR vesicles were suspended in a medium consisting of 0.3 M sucrose and 20 mM MOPS (pH 7.0) and stored at -70°C. Protein concentration was determined by the method of Lowry [11] using bovine serum albumin as a standard.

The SR preparations were further characterized as described in detail by Ferrington et al. [12]. In brief, for SR membranes isolated from young and aged skeletal muscle, we find no age-related alterations in the mean size (ca. 151 nm diameter) or size distribution of SR vesicles, as determined by examination of electron micrographs of negatively stained vesicles. Furthermore, SR preparations from young and aged rats exhibit a similarity in both abundance of all SR proteins which copurify (as determined by gel electrophoresis), and functional properties for the Ca-ATPase protein (see Section 3). While no difference was found in the SR membrane phospholipid/protein ratio, a small but statistically significant (13%) increase in cholesterol concentration was found in SR membranes from aged skeletal muscle. However, bilayer properties (i.e., membrane viscosity and Ca-ATPase rotational motion) which directly impact Ca-ATPase function, were unaltered with aging [4,12].

Generally, in our preparations SR Ca-ATPase accounted for 40% of the total protein. There were no age-related differences in the relative contents of the fast-twitch isoform SERCA1 and the slow-twitch isoform SERCA2, respectively, as determined by Western-blot analysis [5]. The exact values were as follows: for young rats, (91.70 ± 4.29)% SERCA1 and (8.26 ± 0.96)% SERCA2, and for old rats, (90.76 ± 3.40)% SERCA1 and (9.24 ± 1.89)% SERCA2.

Subsequent purification of the Ca-ATPase for peptide mapping experiments using non-solubilizing concentrations of deoxycholate and high salt was performed by the method of Warren et al. [13]. The relative percent of Ca-ATPase protein in these SR preparations was determined as 60–70%, based on the densitometric measurement of SR proteins which were electrophoretically separated by SDS polyacrylamide gels, and stained with Coomassie blue [14].

2.3. Functional assays

Calcium-dependent ATPase activity was measured at 25°C by a colorimetric determination of inorganic phosphate [15] released from vesicles made leaky to Ca^{2+} by the addition of the ionophore A23187. ATPase activity was measured in a solution containing 0.05 mg SR protein/ml, 0.1 M KCl, 5 mM MgCl, 4 μM A23187, 25 mM MOPS (pH 7.0), 5 mM ATP, 1 mM EGTA or 0.1 mM $CaCl_2$. The activity assayed in the presence of EGTA (Ca^{2+}-independent ATPase activity) was subtracted from the activity assayed in the presence of $CaCl_2$ (total Ca-ATPase activity) in order to obtain the Ca^{2+}-dependent ATPase activity.

2.4. Determination of total free sulfhydryl groups in the SR vesicles

For the determination of sulfhydryl groups in the SR vesicles we modified the method of Ellman [16]. SR protein (100 μg) was dissolved in 10 mM phosphate buffer (pH 7.3), containing 2.5% SDS. Thereafter a final concentration of 0.2 mM DTNB was adjusted in a total volume of 1 ml. The absorbance at 412 nm was measured after 30 min of incubation at 37°C. A standard curve was obtained by reacting known concentrations of glutathione (GSH) between 10–100 μM with 0.2 mM DTNB in 10 mM phosphate buffer (pH 7.3), containing 2.5% SDS.

2.5. Determination of sulfhydryl groups of purified Ca-ATPase

SR protein was dissolved at a concentration of 1 mg/ml in 50 mM Tris–HCl (pH 6.9), containing 2% SDS, 15% sucrose, and 1 mM EDTA. The solution was then added to a three-fold molar excess of *N*-ethyl[^{14}C]maleimide (40 mCi/mmol) and incubated for 90 min at room temperature. Then the Ca-ATPase was separated from other SR protein by SDS-PAGE using a 5% separating gel according to the method of Laemmli [14]. The gels were stained with Coomassie Blue R-250, scanned on a Hewlett Packard Scan Jet IIp, sliced, and the radioactivity for the Ca-ATPase protein band was measured on a Packard Tri-Carb liquid scintillation counter (as described previously [17]). The counting efficiency was 92%. The relative amount of Ca-ATPase was determined from densitometric analysis, and the thiol content calculated from the radioactivity associated with the Ca-ATPase protein band.

2.6. Chemical oxidation

For the oxidation by AAPH-derived radicals SR vesicles were incubated with AAPH (5–20 mM) at 37°C. The incubation mixtures contained 4 mg/ml native or purified SR protein, 10 mM potassium phosphate (pH 7.3), and 100 mM NaCl. The oxidation was initiated by addition of various concentrations of AAPH (0.5 M stock solution in 10 mM phosphate buffer, pH 7.3) and terminated after different time intervals by a 10-fold dilution with phosphate buffer immediately followed by centrifugation at 100,000 $\times g$ for 30 min. The pellets containing SR proteins were used for further experiments.

2.7. Heat inactivation

Heat stability of native SR vesicles was determined by monitoring Ca-ATPase activity during the incubation of samples at 40°C for up to 4 h. The incubation medium contained 20 mM MOPS (pH 7.0), 5 mM $MgCl_2$, 0.4 mM $CaCl_2$, and 4 mg/ml SR protein. At various times, aliquots of 100 μg were withdrawn and assayed for Ca-ATPase activity, or aliquots of 1 mg (in 0.25 ml) were withdrawn for the analysis of aggregation by size-exclusion chromatography (SEC).

2.8. Size-exclusion chromatography

Aliquots of 1 mg SR protein in 0.25 ml were added to 0.3 ml containing 0.1 M Li_2SO_4, 0.05 M Li acetate, and 1% (w/v) LDS, pH 4.5. After filtration through a 0.45 mm Millipore filter, aliquots of 100 μg of SR protein were analyzed by SEC. SEC was carried out on a Shimadzu HPLC system equipped with a TSK G3000 SW column (TOSO HAAS, Montgomeryville, PA), eluted with 0.05 M lithium

acetate buffer, pH 4.5, containing 1% (w/v) lithium dodecyl sulfate (LDS) and 0.1 M $LiSO_4$ at 0.4 ml/min, essentially as described by Barrabin et al. [18]. The peaks were monitored at 280 nm, and the column was calibrated with the following molecular weight standards: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa).

2.9. Determination of protein carbonyl content

The protein carbonyl content of the SR vesicles was determined according to the general method of Levine et al. [19], modified for SR Ca-ATPase, essentially as described [6].

2.10. Preparation of samples for amino acid analysis

The SR Ca-ATPase was separated from other SR proteins by 10% tricine gel electrophoresis according to the method of Schägger and von Jagow [20]. The protein was then electrotransferred (2 h at 100 V) from gels to a PVDF membrane (BIORAD; 0.2 μm pore size) in 10 mM CAPS buffer (pH 11), containing 20% methanol. The PVDF membrane was rinsed with water, stained with 0.1% (w/v) Coomassie brilliant blue R-250 in 20% (v/v) methanol for 1 min, and subsequently destained for 5–10 min with 40% (v/v) methanol containing 10% (v/v) acetic acid, and rinsed with water. Areas of the PVDF membranes containing the band of the Ca-ATPase were excised and transferred to pyrolyzed tubes while the membrane was still moist [21].

2.11. Amino acid analysis

Amino acid analysis was done after hydrolysis of the pure Ca-ATPase (obtained by electroblotting; see above) in either 6 N HCl or 4 N methane sulfonic acid (20 or 24 h at 110°C, respectively) according to the method of Scholze [22] employing a Sperisorb ODS 2 C18-column (80 Å, 4.6×250 mm). For protein extraction from the PVDF membrane and derivatization by phenylisothiocyanate (PITC) we followed the method of Tous et al. [23] and of the operator's manual of the Pico-Tag workstation (Waters). The calculation of the amino acid composition of Ca-ATPase was based on an apparent molecular weight of 108,123 Da, not including the molecular weight of 13 Trp and 24 Cys residues. For confirmation of our analysis some protein samples were additionally analyzed by Commonwealth Biotechnologies (Richmond, VA).

2.12. Susceptibility to tryptic digestion

The susceptibility of SR proteins to tryptic digestion was assessed by incubation of 1 mg/ml SR protein with 0.02 mg trypsin at 30°C in 50 mM ammonium carbonate buffer (pH 8.5), containing 0.1 mM $CaCl_2$ and 1 mM dithiothreitol (DTT). At different time intervals between 0 and 5 min, the digestions were stopped by the addition of 10% (w/v) trichloroacetic acid (final concentration 5%, w/v), and the residual protein precipitated by centrifugation for 30 min at $6{,}000 \times g$. The content of trichloroacetic acid soluble peptides was measured using the MicroPierce BCA assay (Rockford, IL).

2.13. Tryptic hydrolysis of the protein for peptide mapping

For peptide mapping, 4 mg/ml of purified SR protein were incubated at 37°C with 0.08 mg/ml trypsin (ratio of trypsin:SR protein = 1:50) in 50 mM ammonium carbonate, pH 8.5, containing 1 mM DTT and 0.1 mM $CaCl_2$. After either 2 or 24 h, iodoacetic acid at a final concentration of 5 mM was added and the incubation continued for one additional hour. The reaction was finally quenched by a five-fold dilution with cold water, followed by centrifugation at $200{,}000 \times g$ for 90 min. The supernatant was collected, dried on a Speed-Vac tabletop centrifuge, and stored until further analysis by HPLC.

2.14. HPLC analysis of tryptic peptides

The separation of the soluble peptides from the supernatant was achieved on a Shimadzu system equipped with either a 4.6×250 mm Hypersil C_4 or C_{18} reversed-phase column (Phenomenex). For the analysis of peaks I and II (see Section 3), the dried peptide samples (see above) were redissolved in 10 mM ammonium acetate, pH 5.5 (solvent A), and injected onto the C_4 column which had been equilibrated with a 95:5 (v/v) mixture of solvent A and solvent B (10 mM ammonium acetate in 90:10 (v/v)

acetonitrile/water). After isocratic elution for 5 min with 95% solvent A, the peptides were eluted with a linear gradient of solvent B, increasing from 5 to 60% within 60 min. Peptides were monitored by UV detection at 214 nm at a flow rate of 1 ml/min. Fractions of interest were collected, lyophilized and prepared for a second step of purification on a C_{18} column by redissolving in solvent C (0.1% trifluoroacetic acid; TFA). These samples were injected onto a C_{18} column which had been equilibrated with a 99:1 (v/v) mixture of solvent C and solvent D (0.1% trifluoroacetic acid in 90:10 (v/v) acetonitrile/water), and eluted with a linear gradient of solvent D, increasing from 1 to 60% within 60 min. Fractions of interest were collected, dried and divided into two aliquots. One aliquot was used for mass spectrometric identification and the other one for characterization by N-terminal sequencing (sequencing performed by Commonwealth Biotechnologies). For the analysis of peak III (see Section 3) the dried peptide samples were dissolved in solvent A and injected onto a C_{18} column which had been equilibrated with a 99:1 (v/v) mixture of solvents A and B, and eluted with linear gradient of solvent B, increasing from 1 to 60% within 60 min.

2.15. HPLC-electrospray MS analysis of tryptic peptides

The identification of tryptic peptides was carried out by microbore HPLC on-line coupled to an electrospray mass spectrometer (Autospec-Q tandem hybrid mass spectrometer; VG Analytical, Manchester) equipped with an OPUS data system. The mass range was scanned in the positive mode from 500–2300 amu with a scan rate of 8 s/dec (resolution: 1500). The microbore HPLC instrumentation consisted of two pumps (Micro-Tech Scientific, Sunnyvale, CA), a dynamic mixing chamber with a volume of 20 μL (Micro-Tech Scientific, Sunnyvale, CA), and a Model 8125 injection valve (Rheodyne, Cotati, CA) with a 5 μL sample loop. Separations were performed on a 250×0.32 mm C18 capillary column (Vydac, C18, 5 μm, 300 Å) at 6 μL/min flow rate, and the peptides were monitored at 214 nm with a UV Detector (Model 200, Linear Instruments, Fremont, CA) equipped with a micro flow cell. The capillary-column was first equilibrated with 98% mobile phase A, consisting of a 98/2/0.1 (v/v/v) mixture of water, acetonitrile, and TFA, and 2% mobile phase B, consisting of 90/10/0.09 (v/v/v) acetonitrile, water, and TFA. The separation was performed with a linear gradient increasing the content of mobile phase B at a rate of 2%/min.

2.16. Statistics

Five groups of animals containing three rats each were sacrificed for the preparation of SR from young and old rats, respectively. The values in the figures and tables are expressed as mean ± S.E. Groups were compared by students *t*-test analysis.

3. Results

3.1. Physical characterization

Based on densitometry of Coomassie Blue stained gels, our native SR preparations from rat hindlimb

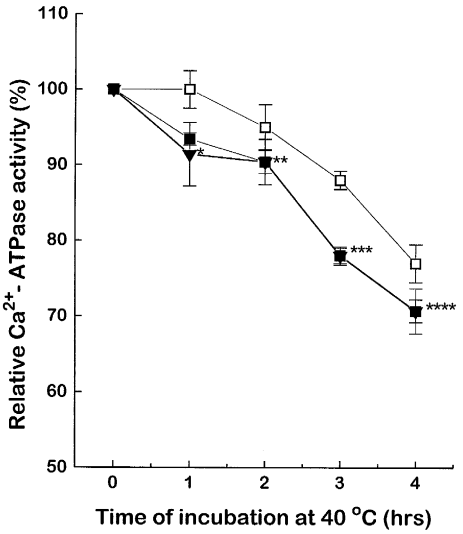

Fig. 1. Heat inactivation of Ca-ATPase in skeletal muscle SR vesicles. SR vesicles (4 mg protein/ml) from 5 months (□), 5 months, exposed to 10 mM AAPH for 10 min at 37°C (■), and 28 months old (▼) rats were incubated at 40°C in 20 mM MOPS, 5 mM $MgCl_2$, 0.4 mM $CaCl_2$ (pH 7.0). At the indicated times aliquots were withdrawn and assayed for calcium-dependent ATPase activity. Ca-ATPase activity values of 100% correspond to 3.18 ± 0.37 μmol P_i/min/mg protein for 'young' and 3.36 ± 0.16 μmol P_i/min/mg protein for 'old' SR vesicles. Samples of aged vs. corresponding young protein: * $P < 0.01$, ** $P < 0.05$, *** $P < 0.001$, **** $P < 0.02$.

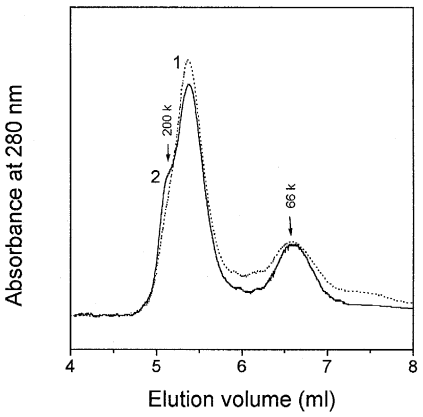

Fig. 2. Size-exclusion chromatography of SR proteins, solubilized in LDS, from 5 months (1) and 28 months (2) old rats. Samples containing 4 mg SR protein/ml were incubated for 2 h at 40°C in the medium for heat inactivation (see legend to Fig. 1). Then aliquots of 1 mg SR protein (0.25 ml) were withdrawn and added to 0.3 ml of 0.05 M lithium acetate (pH 4.5), containing 1% (w/w) LDS and 0.1 M $LiSO_4$ (elution buffer). Aliquots containing 100 μg SR protein were injected on a TSK G3000 SW column (Toso Haas), and protein peaks were monitored at 280 nm. The Ca-ATPase aggregate (most likely the dimer) elutes at the position of the 200 kDa peak, indicated by the arrow.

muscles are composed of approximately 40% Ca-ATPase protein [5]. No statistically significant age-related differences in the relative contents of the SERCA1 (91.7 ± 4.3; 90.8 ± 3.4%) and the SERCA2 (8.3 ± 1.0; 9.2 ± 1.9%) isoforms of the Ca-ATPase were detected for SR preparations of young and aged rats, respectively. While we find no initial difference in the Ca^{2+}-dependent ATPase activity, exposure to elevated temperature (40°C) caused a progressive loss of activity which was more rapid in SR vesicles isolated from old rats compared to young rats (Fig. 1), in agreement with results previously reported by Gafni and Yuh [2]. The interesting feature of Fig. 1 is that SR vesicles isolated from young rats which were exposed to 10 mM AAPH for 10 min at 37°C prior to incubation at 40°C showed a time-dependent inactivation profile identical to that of the SR vesicles from old rats. The rates of heat inactivation of the oxidized SR vesicles from young rats depended on the respective initial concentrations of AAPH used and the time of exposure to AAPH, with faster inactivation kinetics for higher AAPH concentrations and longer exposure times (data not shown). Thus, the heat inactivation kinetics observed for SR preparations exposed for 10 min to 10 mM AAPH did not represent a maximum effect but most closely mimicked the heat inactivation behavior of native SR vesicles isolated from old rats.

One of the potential mechanisms for Ca-ATPase inactivation could be the heat-induced formation of

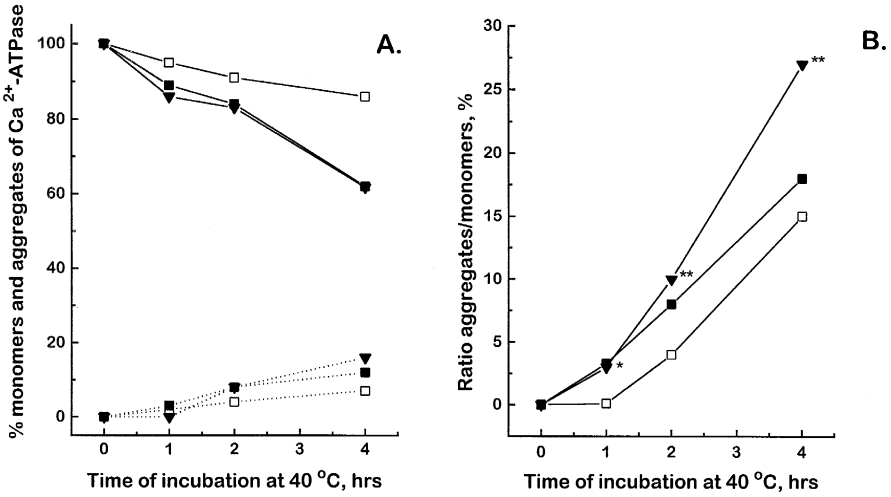

Fig. 3. Quantification of self-association of Ca-ATPase polypeptide chains as a function of incubation time at 40°C. Ca-ATPase from 5 months (□), 5 months, exposed to 10 mM AAPH for 10 min at 37°C (■), and 28 months old (▼) rats. HPLC peak areas were normalized to the peak area of the initial concentration of the Ca-ATPase monomer before the exposure to elevated temperature. (A) The content of Ca-ATPase monomers (solid lines) and aggregates (dotted lines), and (B) the time-dependent change in the ratio of Ca-ATPase aggregates to monomers. Samples of aged vs. corresponding young protein: * $P < 0.01$, ** $P < 0.001$.

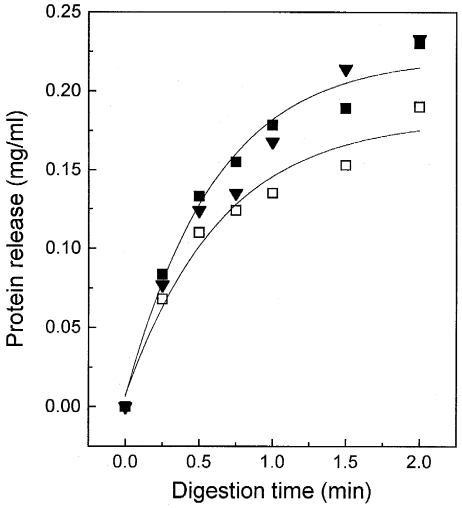

Fig. 4. Increased susceptibility of SR vesicles to tryptic hydrolysis as a result of aging or exposure to AAPH. The susceptibility of SR vesicles from 5 months (□), 5 months, exposed to 10 mM AAPH for 10 min at 37°C (■), and 28 months old (▼) rats to tryptic digestion was assessed following the incubation of 2 mg SR protein/ml with 0.04 mg trypsin (ratio 50:1, w/w) at 30°C in 50 mM ammonium carbonate (pH 7.0). The release of peptides into the supernatant was measured at various times of tryptic digestion after centrifugation of trichloroacetate precipitates, as described in Section 2.

protein aggregates. We investigated this possibility using size-exclusion chromatography to monitor the relative amounts of Ca-ATPase monomers and aggregates. Fig. 2 shows a representative size-exclusion chromatogram displaying the separation of SR Ca-ATPase monomers from higher molecular weight aggregates obtained following the exposure of SR vesicles from young and old rats to elevated temperature of 40°C for two hours. Based on the comparison with molecular weight standards and on earlier findings [18,24] we believe that the majority of the detected Ca-ATPase aggregates are actually dimers. A quantitative comparison of the content of Ca-ATPase monomers and aggregates as a function of heat exposure time is shown in Fig. 3. The Ca-ATPase molecules of SR vesicles isolated from old rats show a significantly higher tendency to aggregate as compared to those isolated from young rats. The exposure of SR vesicles isolated from young rats to 10 mM AAPH for 10 min at 37°C resulted in a preparation which behaved like one isolated from old rats on the basis of the kinetics of monomer loss, and showed similar tendencies compared to SR preparations from old rats with regard to the kinetics of aggregate formation.

It is important to note that the SR vesicles showed no age-related differences in activity and monomer/aggregate ratio prior to the incubation at 40°C. Differences only became apparent when the SR preparations were exposed to the stress of elevated temperature. In general, the greater tendency of Ca-ATPase molecules from old SR vesicles to aggregate might indicate an age-related conformational or compositional difference in the protein or membrane, resulting in a more flexible and/or open protein structure. In order to investigate age-related changes in protein conformation, we monitored the susceptibility of the protein to proteolytic digestion. Fig. 4 reveals that proteins from SR vesicles isolated from old rats exhibit faster proteolytic digestion by trypsin than SR proteins isolated from young rats, suggesting that amino acid residues recognized by trypsin (Arg and Lys) are more accessible due to a more open

Table 1
Determination of protein thiol and carbonyl groups in rat skeletal muscle SR preparations

Age	Total SH groups content (nmol/mg of SR protein [a])	*N*-ethyl-maleimide (mol/mol of Ca-ATPase)	Carbonyl groups content (mol/mol of Ca-ATPase)
5 months	138.4 ± 4.6	22.1 ± 0.07	0.32 ± 0.06
5 months + [$R_AOO^{\cdot}$]	104.2 ± 0.4	15.3 ± 0.50	n/d [b]
28 months	120.6 ± 2.6 [c]	20.6 ± 0.18 [d]	0.46 ± 0.02

[a] By the method of Ellman [16] using 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). Data represent the means (±S.E.) of four independent determinations.
[b] n/d, not determined.
[c] 28 month samples were significantly different from 6 month samples, at the $P < 0.02$ level.
[d] 28 month samples were significantly different from 5 month samples, at the $P < 0.001$ level.

protein conformation. We note that the exposure of SR vesicles from young rats to 10 mM AAPH for 10 min at 37°C resulted in a preparation which shows identical behavior as those SR vesicles isolated from old rats. In this regard it is important to realize that the SR Ca-ATPase appears to be generally more sensitive to trypsin digestion as compared to other SR proteins [25] so that the age- or oxidation-related changes in the proteolysis kinetics of the SR proteins may be directly related to changes in the conformation of the Ca-ATPase.

Thus, from a comparison of Figs. 1–4 it appears that a relatively mild oxidative stress (for quantitation, see Section 4) modifies the Ca-ATPase of SR vesicles from young rats such that it resembles that of SR vesicles isolated from old rats.

3.2. Chemical characterization

By reaction with DTNB we derived that SR vesicles from young rats showed a total content of reduced thiols of 138.4 nmol/mg SR protein (Table 1). With Ca-ATPase representing 40% of the total SR protein, a content of 24 cysteine residues per Ca-ATPase monomer [7], and $MW_{Ca\text{-}ATPase}$ = 113,679 Da (based on the sequence for rat SERCA1 [7]), we calculate that the Ca-ATPase accounted for 61% of the reduced thiols in our preparations. SR vesicles from old rats were characterized by a significantly lower (ca. 13%; $P < 0.02$) total content of DTNB-reactive sulfhydryls as compared to SR vesicles from young rats (Table 1). The exposure of SR vesicles to 10 mM AAPH for 10 min resulted in a ca. 25% loss of the total thiol content.

In order to provide an estimate for the sensitivity of thiols of specifically the Ca-ATPase to age-related modification, SR proteins were reacted with [^{14}C]-NEM and subsequently the Ca-ATPase electrophoretically separated from the other SR proteins. Quantification of [^{14}C]-NEM specifically associated with the Ca-ATPase protein reveals that the level of sulfhydryl groups decreases by approximately 6.8% with age ($P < 0.001$), corresponding to an average loss of ca. 1.5 mol sulfhydryl groups per mol of Ca-ATPase (Table 1). The exposure of SR vesicles for 10 min at 37°C to 10 mM AAPH resulted in the loss of 6.8 mol sulfhydryl groups per mol of Ca-ATPase, corresponding to a loss of ca. 31%. Thus, the SR Ca-ATPase

Table 2
Amino acid analysis of the skeletal muscle Ca^{2+}-ATPase from 5 and 28 months old Fisher strain 344 rats

Amino acid	5 months [a]	28 months [a]	5 months + AAPH [b,c]	SERCA 1a [d]
Alanine	86 ± 2	86 ± 1	85 ± 1	85
Arginine	54 ± 2	52 ± 1	51 ± 0.5	50
Aspartate + asparagine [e]	94 ± 6	99 ± 2	98 ± 6	89
Glycine	65 ± 1	66 ± 0.5	65 ± 1	65
Glutamate + glutamine	112 ± 4	113 ± 1	111 ± 3	102
Histidine	11 ± 1	10 ± 1	14 ± 1	11
Isoleucine	57 ± 1	56 ± 1	55 ± 1	67
Leucine	91 ± 3	92 ± 2	97 ± 1	94
Lysine	54 ± 3	56 ± 4	57 ± 0.5	54
Methionine	32 ± 1	33 ± 1	29 ± 1	31
Phenylalanine	35 ± 2	35 ± 1	41 ± 0.5	37
Proline	54 ± 2	52 ± 3	52 ± 1	47
Serine	56 ± 2	56 ± 1	51 ± 1	61
Threonine	56 ± 2	57 ± 2	56 ± 1	61
Tyrosine	28 ± 1	28 ± 0.5	28 ± 0.5	23
Tryptophan [b]	7 ± 1	8 ± 0.5	n/d	13
Valine	72 ± 3	73 ± 0.5	75 ± 0.5	87

[a] Data represent the means (±S.E.) of 7 independent determinations obtained with 4 groups of 3 animals each.
[b] Data represent the means (±S.E.) of 2 independent determinations obtained with 2 groups of 3 animals each.
[c] 4 mg SR protein/mL exposed for 10 min to mM AAPH at 37°C.
[d] Amino acid data from sequence of the rat SERCA 1a [7].
[e] Amino acids exhibiting significant age-related changes ($P < 0.001$) from the values of 5 month animals.

molecule accumulates a chemical modification with age which might account for the observed differences in heat inactivation, increased tendency to aggregation, and susceptibility to tryptic hydrolysis. The simulation of these age-related physical properties of SR Ca-ATPase by incubation of SR vesicles with low levels of AAPH-derived radicals is likely the result of protein Cys oxidation although the exposure to AAPH caused a significantly higher loss of Ca-ATPase sulfhydryls as compared to the net loss caused by in vivo aging (see Section 4). Table 1 further documents that the chemical modification of thiols as a result of aging or exposure to AAPH is far more significant than the accumulation of protein carbonyls on the SR Ca-ATPase. Often an increased level of protein carbonyls is taken as a measure for oxidative protein modification in particular of aged tissue [26,27], but this type of assessment would not be sufficient in the case of the SR Ca-ATPase. A detailed amino acid analysis of the Ca-ATPase protein was performed in order to define additional oxidative modifications which might occur as a result of aging or exposure to AAPH-derived peroxyl radicals (Table 2).

Besides changes in the content of protein thiols (Table 1) there was a slight loss of Arg as a result of aging and exposure of Ca-ATPase to AAPH. Oxidative N-dealkylation of Arg can lead to the formation of glutamic semialdehyde. Future experiments may show whether a sequence of further reactions such as hydrogen abstraction, net loss of CO, addition of oxygen, and disproportionation of the resulting peroxyl radicals can convert glutamic semialdehyde into aspartic semialdehyde. Aspartic semialdehyde could potentially convert to Asp in an oxidative environment, eventually rationalizing the observed increase of the combined yields of [Asp + Asn] of the Ca-ATPase as a result of aging or exposure to AAPH. No other significant differences are evident between Ca-ATPase molecules isolated from young and old rats. There was an additional slight decrease in the content of Met and Ser for AAPH-exposed SR vesicles of young rats. Since Met sulfoxide converts back to Met under conditions of acid hydrolysis this slight loss of Met observed may be the result of oxidative S-dealkylation of Met. Any oxidation of Ser likely involves hydrogen abstraction from the C_{β}–H bond. In combination with the titration of protein Cys residues presented in Table 1, these data suggest a specific role for an age-related modification of Cys and/or Arg residues of the SR Ca-ATPase. The fact that a mild oxidative stress through AAPH-derived radicals results in a modification of SR Ca-ATPase Cys and Arg residues, inducing physical properties of the protein similar to those from aged animals, suggests that the age-related modification of protein Cys and Arg residues is through oxidation. We were not able to recover the protein Cys residues through chemical reduction with DTT (data not shown) suggesting that (accessible) disulfide bridges are not a major product of Cys modifications. A detailed characterization of these products as well as their exact localization within the protein structure is currently in progress in our laboratory.

3.3. Tryptic mapping

We have shown that SR protein from old rats and from SR vesicles of young rats which were exposed to AAPH was more susceptible to tryptic digestion. It was now of interest to examine whether both species were actually proteolyzed not only at a comparable rate but also cleaved at similar positions within the protein sequence. Fig. 5A–C displays the reversed-phase chromatograms obtained on a C_4 column after a 2 h tryptic digestion of proteins from purified SR vesicles from (A) young and (C) old rats, and from (B) AAPH-exposed SR vesicles from young rats. Two areas, indicated as peaks I and II, are peaks which appear in chromatograms from old (C) and oxidant-exposed (B) preparations but are absent in the chromatogram from young SR preparations (A). These peaks represented the most obvious changes similar for in vivo aged and in vitro oxidized SR vesicles and warranted a more detailed analysis. The relative areas of peaks I and II were ca. 3–5 fold larger for in vivo aged SR vesicles, suggesting that a 10 min exposure to AAPH, though inducing comparable physical characteristics, might nevertheless lead to some chemical modifications different to those of in vivo aging, also reflected in the larger extent of thiol modification and some additional alteration of Met and Ser by AAPH (see above). Peaks I and II were collected and further purified on a C_{18} column where each peak was split into two components.

Table 3 summarizes the results of identification of these peaks by N-terminal sequencing and/or

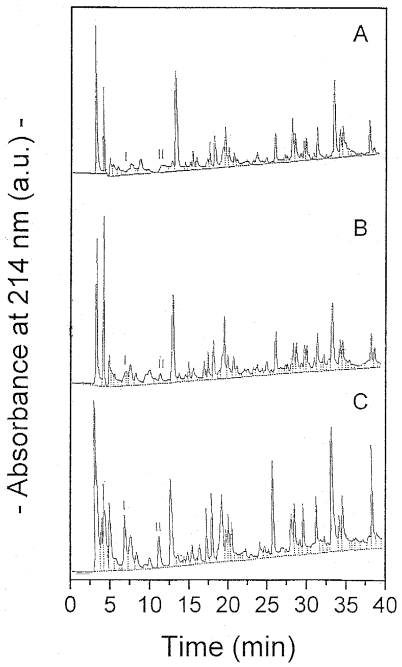

Fig. 5. Separation of peptides after 2 h of tryptic digestion of SR vesicles from 5 months (A), 5 months, exposed to 10 mM AAPH for 10 min at 37°C (B), and 28 months old (C) rats by reversed-phase HPLC. Soluble peptides derived from 1 mg purified SR vesicles, dissolved in 100 μl of the initial mobile phase, were separated onto a Phenomenex C_4 column (4.6×250 mm), as described in Section 2. Fractions I and II were collected and subjected to further purification by reversed-phase chromatography on a C_{18} column.

HPLC-electrospray mass spectrometry, together with their retention times under the different chromatographic conditions. Two components of the original peak I, assigned to the Ca-ATPase fragments $A_{199}VNQDK_{204}$ and $H_{32}LEK_{35}$, were present, though to a different extent, after 2 h of tryptic digestion only in samples from aged or AAPH-exposed SR vesicles. The fragment $A_{199}VNQDK_{204}$ constituted the main component of peak I of aged SR whereas the fragment $H_{32}LEK_{35}$ was the major component of peak I from in vitro oxidized SR. We note that the sequence $A_{199}VNQDK_{204}$ nevertheless appeared when SR vesicles isolated from young rats were reacted with trypsin for 24 h. This result is in accord with a conformational modification of the protein as a result of age or exposure to AAPH, promoting the faster proteolytic formation of the sequence $A_{199}VNQDK_{204}$.

While the first component of the original peak II, assigned to the Ca-ATPase sequence $V_{129}YR_{131}$, was present after 2 h of tryptic digestion in all samples the second component of peak II was absent from tryptic digests of young SR preparations. N-terminal sequencing of this second component gave a sequence of XGVIX′ with a molecular weight of MW = 687 amu, determined by HPLC-ESI MS. Based on the published sequence of rat SERCA1 [7] we suggest that this component represents the sequence $E_{519}GVIDR_{524}$ (MW = 687 Da), obtained via non-specific cleavage between Pro_{518} and Glu_{519}. This fragment was the major component of peak II from in vivo aged SR. When a tryptic digest of SR proteins was directly analyzed (under modified conditions) on a C_{18} column without prior separation on a C_4 column, a third peak was identified, present after 2 h of digest only from proteins of old SR vesicles, referred to as peak III in Table 3. This peak was assigned to the Ca-ATPase fragment D_{237}QMAATEQDKTPLQQK$_{252}$ which notably contains an un-

Table 3
Identification of Ca^{2+}-ATPase peptides after 2 h tryptic digestion

Ret. time on C_4	Ret. time on C_{18}	Sequence	5 months	5 months + [ROO·] (% [e])		28 months (% [e])	
6.84(I)	10.898	AVNQDK [b] (199–204)	− [d]	+	25	+	100
	13.451	HLEK [b,c] (32–35)	−	+	100	+	59
10.93(II)	16.282	VYR [c] (129–131)	+	+	100	+	14
	16.383	XGVIX [b] (519–524?)	−	+	50	+	100
n/d [a]	22.45 (III)	DQ..QK [b] (237–252)	− [d]	−	−	+	100

[a] n/d = not determined.
[b] Identified by HPLC-ESI MS.
[c] Identified by N-terminal sequencing.
[d] Found only after 24 h tryptic digestion.
[e] HPLC peak heights were normalized to the highest peak of the selected separation.

cleaved Lys residue. It did not appear in tryptic digests from AAPH-exposed SR vesicles but in SR vesicles of young rats after prolonged incubation with trypsin for 24 h. Results from these experiments identify areas of the Ca-ATPase protein in SR which may be more solvent exposed as a result of aging or exposure to AAPH, i.e., readily cleaved by trypsin, suggesting the localized alteration of protein structure. Thus, old and young AAPH-exposed SR vesicles demonstrate comparable kinetics for the overall tryptic hydrolysis and the formation of some individual (identical) peptide fragments. This result suggests that AAPH oxidation does not modify thiols in a random manner but rather chemically alters specific residues which may be identical to those altered in the SR Ca-ATPase isolated from aged rats. This result is not unexpected based on the similar physical characteristics of SR Ca-ATPase from old and young AAPH-exposed SR vesicles and suggests that a similar mechanism, i.e., oxidation of Cys and/or Arg, evokes both the age-dependent and peroxyl radical-dependent changes of SR Ca-ATPase. It should be noted that all the identified sequences originate from the SR Ca-ATPase, a result not unexpected based on the fact that we analyzed purified SR, and on the generally higher sensitivity of the Ca-ATPase to tryptic digestion as compared to other SR proteins [25].

4. Discussion

We have shown that the SR Ca-ATPase isolated from rat skeletal muscle exhibits an age-related modification of protein Cys and Arg residues. This finding provides a mechanism for the lower heat stability of the protein as well as the higher susceptibility to tryptic hydrolysis and aggregation, implying that the modification of some critical Cys and/or Arg residues results in a conformational change which may open the protein structure. This would allow for increased solvent exposure of trypsin sensitive amino acids and critical subdomains which are more hydrophobic and permit aggregation of the Ca-ATPase. However, since spectroscopic studies revealed no age-related changes of the rotational dynamics of the protein [4], such conformational opening may be restricted to certain protein subdomains not affecting the overall global structure of the protein.

Intermolecular disulfide bridges are not a major product of Cys modification, as evidenced by the absence of protein aggregates without the exposure to heat, and the lack of recovery of protein Cys residues following treatment of aged SR vesicles with DTT. A typical Cys oxidation product other than disulfide is sulfonic acid, RSO_3H. At neutral pH, sulfonic acids exists in the anionic form, RSO_3^-, whereas non-modified Cys residues are largely protonated ($pK_a \approx 8–9$ [28]; depending on the protein microenvironment). Thus, the conversion of a mostly neutral, more lipophilic, sulfhydryl group into an anionic, more hydrophilic, sulfonate may cause conformational changes of the protein responsible for the observed changes of its physical properties. Likewise, the conversion of a positively charged Arg residue into neutral or negatively charged products may induce such conformational transitions. In addition there may be steric constraints associated with the formation of a relatively bulky sulfonic acid group. We note that an age-related modification of Cys residues in skeletal muscle has also been established for other proteins such as phosphoglycerate kinase [29]. Protein carbonyl formation is not a major characteristic of the SR Ca-ATPase of aged SR, in contrast to several other proteins for which age-related carbonyl formation has been demonstrated such as glutamine synthetase [27].

An important finding is that the exposure of skeletal muscle SR vesicles from young rats to 10 mM AAPH for 10 min at 37°C causes a modification of the SR Ca-ATPase molecule which is qualitatively and quantitatively comparable to that characterized for the Ca-ATPase isolated from aged skeletal muscle. Thus, under these specific conditions we are able to quantitatively simulate the aging process and to define a concentration range of free radicals or reactive oxygen species equivalent to a fraction of reactive oxygen species to which SR vesicles must have been exposed in vivo over a specific period of time (see also below). Of course, such a comparison has to be corrected for ongoing protein synthesis and degradation in vivo, and does not account for the presence of antioxidants which may eradicate the detrimental effects of reactive oxygen species. However, it may define a lower limit of reactive oxygen species necessary to attain oxidative protein modifications such as found during aging. The quantitative simulation of

the age-related modification of the Ca-ATPase is remarkable with regard to the fact that not only the physical and functional properties of the protein from young AAPH-exposed and old SR vesicles are nearly identical but also some of the chemical features. For example, both preparations appear to be predominantly modified at Cys and less at Arg residues, and tryptic hydrolysis leads to the appearance of some identical peptide fragments at early times of tryptic digestion. This finding suggests that similar critical sulfhydryl groups and/or Arg residues of the Ca-ATPase are modified during the aging process and the exposure to AAPH. These critical residues may be located in the vicinity of the sequences from which the kinetically early tryptic fragments evolve. This would rationalize why the modification of these residues, though promoting an increased lability of the enzyme towards elevated temperature, does not cause an immediate inactivation of the Ca-ATPase. All except one (E_{519}–R_{524}) of these domains from which the kinetically early tryptic fragments originate are remote from the functionally important domains such as the Ca^{2+}- and nucleotide-binding sites [30].

4.1. Mechanisms and stoichiometry of Cys oxidation

The thermolabile azoinitiator AAPH [R_A–N=N–R_A; R_A=$C(CH_3)_2C(NH_2)$=$NH_2^+Cl^-$] yields peroxyl radicals according to Eqs. (1)–(4) where *e* denotes the fraction of initial radicals $R^{\cdot}$ escaping the solvent cage. The rate of free radical initiation is given as $R_i = 2ek_1[\text{AAPH}]$ where, based on $2ek_1 = 1.3 \times 10^{-6}$ s^{-1} at 37°C [31], a 10 min incubation of 10 mM AAPH at 37°C generates a total concentration of $[R_A^{\cdot}] = 1.6 \times 10^{-5}$ M, yielding $[R_A\text{–O–O}^{\cdot}] = 1.6 \times 10^{-5}$ M.

$$R_A\text{–N=N=}R_A \rightarrow [R_A^{\cdot} + N_2 + R_A^{\cdot}]_{\text{cage}} \quad (1)$$

$$[R_A^{\cdot} + N_2 + R_A^{\cdot}]_{\text{cage}} \xrightarrow{1-e} R_A\text{–}R_A + N_2 \quad (2)$$

$$[R_A^{\cdot} + N_2 + R_A^{\cdot}]_{\text{cage}} \xrightarrow{e} N_2 + 2R_A^{\cdot} \quad (3)$$

$$R_A^{\cdot} + O_2 \rightarrow R_A\text{–O–O}^{\cdot} \quad (4)$$

In our experiments we exposed 4 mg SR protein/ml to 10 mM AAPH. In these preparations the SR Ca-ATPase accounted for ca. 40% of the total SR protein [5]. Taking $MW_{\text{SR Ca-ATPase}}$ = 113,679 Da [7], this fraction of Ca-ATPase is equivalent to a concentration of 1.41×10^{-5} M. Based on the determination of the total content of reduced thiols in our SR vesicles (Table 1), the Ca-ATPase accounted for 61% of the available cysteine residues. Thus, our incubations with AAPH were performed under conditions of $[R_AOO^{\cdot}]$:[Ca-ATPase] = 1.13 which is sufficient to convert the SR Ca-ATPase of young SR vesicles to one with physical properties like those of old SR vesicles. More specifically, the exposure of SR vesicles to 1.6×10^{-5} M $R_AOO^{\cdot}$ resulted in the loss of 6.8 mol sulfhydryl residues per mol of Ca-ATPase molecule, corresponding to $6.8 \times 1.41 \times 10^{-5}$ M = 9.6×10^{-5} M reacted Ca-ATPase sulfhydryl groups, based on a concentration of Ca-ATPase of 1.41×10^{-5} M. Thus each AAPH-derived radical $R_AOO^{\cdot}$ could theoretically initiate the average oxidation of more than 6 Cys residues of each Ca-ATPase molecule. Considering that not necessarily all $R_AOO^{\cdot}$ will react with the thiol groups of the Ca-ATPase we have to calculate the loss of total SR thiols per $R_AOO^{\cdot}$. The total concentration of thiols of our SR vesicles was determined to be 138.4 nmol SH-groups/mg SR protein, i.e., 553.6 nmol/4 mg SR protein. We exposed 4 mg SR protein/ml, i.e., 5.53×10^{-4} M SH groups, to a concentration of $R_AOO^{\cdot}$ of 1.6×10^{-5} M (see above) which resulted in the loss of 25% of the SR thiols, corresponding to a loss of 1.38×10^{-4} M SH-groups. Thus, for total SR thiols the stoichiometry of thiol modification appears to be as large as 8.6 thiol groups per initiating radical R_A–O–$O^{\cdot}$. These product stoichiometries can easily be rationalized by a manifold of reactions which can be initiated by peroxyl and thiyl radicals, as described below.

There are several pathways established for the reactions of peroxyl radicals with sulfhydryl groups. From our mechanistic considerations we will exempt disulfide bridges (and their potential formation via thiyl radical-thiolate complexes) since those are not likely to be a major oxidation product of Cys in the aged protein (see above). The following reactions are formulated on the basis of many representative examples in the chemical literature [32] (P = protein, L = lipid).

$$R_AOO^{\cdot} + PSH \rightarrow R_AO^{\cdot} + PSOH \quad (5)$$

$$R_AO^{\cdot} \rightarrow {}^{\cdot}CH_3 + O{=}C(CH_3)C(NH_2){=}NH_2^+Cl^- \quad (6)$$

$${}^{\cdot}CH_3 + O_2 \rightarrow CH_3OO^{\cdot} \quad (7)$$

$$R_AO^{\cdot} + PSH \rightarrow R_AOH + PS^{\cdot} \quad (8)$$

$$R_AOO^{\cdot} + PSH \rightarrow R_AOOH + PS^{\cdot} \quad (9)$$

$$R_AOOH + PSH \rightarrow R_AOH + PSOH \quad (10)$$

$$PS^{\cdot} + O_2 \rightleftharpoons PSOO^{\cdot} \quad (11)$$

$$PSOO^{\cdot} + PSH \rightarrow PSO^{\cdot} + PSOH \quad (12)$$

$$PSOH \rightarrow \rightarrow PSO_3H \quad (13)$$

$$PSOO^{\cdot} \xrightarrow{\Delta} PSO_2^{\cdot} \quad (14)$$

$$PSO_2^{\cdot} + LH \rightarrow PSO_2H + L^{\cdot} \quad (15)$$

$$R_AOO^{\cdot} + LH \rightarrow R_AOOH + L^{\cdot} \quad (16)$$

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad (17)$$

$$PSO_2H \rightarrow \rightarrow PSO_3H \quad (18)$$

Considering that each peroxyl radical formed during the reaction sequence can initiate a reaction sequence analogous to that initiated by $R_AOO^{\cdot}$ we understand that the stoichiometry of modified thiol per initiating $R_AOO^{\cdot}$ is likely larger than 1:1. In support of reaction 5 we note that AAPH-derived alkoxyl radicals have been detected by means of spin-trapping experiments [33].

As a result of these quantitative considerations it may be speculated that a concentration of free radicals lower than the actual concentration of a target protein such as the SR Ca-ATPase may be responsible for the accumulation of age-related post-translational modifications such as observed. Under physiological conditions this effective concentration of free radicals available for the reaction with a specific target protein will represent only a fraction of the total pool of free radicals present of which other fractions will react competitively with biologically available antioxidants such as vitamins C and E or with other biomolecules.

We have not specifically analyzed for lipid peroxidation products, eventually formed as a consequence of a direct interaction of $R_AOO^{\cdot}$ or protein radicals with lipids (e.g., Eqs. (15) and (16)). Earlier studies had shown that in the presence of membrane proteins such as SR Ca-ATPase, the membrane proteins and not the lipids constitute the primary targets of AAPH-derived radicals [34]. Furthermore, during the exposure of SR vesicles from rabbit skeletal muscle to even larger concentrations of AAPH we found that any potential lipid peroxidation alone did not cause the inactivation of the SR Ca-ATPase [35], consistent with earlier reports on the oxidative modification of SR vesicles by a variety of oxidants [36].

4.2. Relation between the modification of the SR Ca-ATPase in vivo and in vitro

In vivo aging and the exposure to low concentrations of AAPH-derived radicals result in the modification of Cys and Arg residues of the Ca-ATPase. It has been reported that only 1–2 sulfhydryl residues of the Ca-ATPase are essential for enzyme activity [37], implying that the thiols modified during aging or AAPH-exposure under our experimental conditions are not these critical Cys residues. That the modification of Cys and Arg during aging and exposure to AAPH yields Ca-ATPase molecules of comparable physical and chemical characteristics implies, nevertheless, that their modification is not a random process but may affect similar residues. The SR Ca-ATPase has been subjected to oxidation by various oxidants where either little or significant enzyme inactivation was observed depending on the nature of the oxidant [36–39]. In one example, the oxidation of up to six sulfhydryl groups caused only less than 10% inactivation of the Ca-ATPase [39]. Based on the latter finding it does not surprise that the Ca-ATPase of in vivo aged and in vitro oxidized SR vesicles showed comparable activities and physical characteristics even though their extent of Cys modification differed significantly. An important point may be that similar thiols, critical for the observed physical and functional characteristics, are modified in both preparations. We may also have to consider that oxidative processes operative during in vivo aging initially modify more protein Cys groups and/or other amino acids of the Ca-ATPase but that the Ca-ATPase species isolated from aged rats (and characterized in this paper) only represents the protein isoform most resistant to in vivo proteolytic degradation. However, it appears that our experimental conditions provide a suitable quantitative mimic of the net extent of an age-related oxidative stress leading to the accumulation of a modified Ca-ATPase which is biologically active but shows an increased susceptibility to inactivation and aggregation by ele-

vated temperature. We note that age-related modifications are not necessarily only the result of peroxyl radicals but may involve other oxidants such as, e.g., metals, peroxides, peroxynitrite and/or singlet oxygen. Nevertheless, the AAPH-based system constitutes a valid model which may be further employed to design separation and analysis strategies for the ultimate identification of modified sites of aged Ca-ATPase.

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