Peroxynitrite Modification of Protein Thiols: Oxidation, Nitrosylation, and S-Glutathiolation of Functionally Important Cysteine Residue(s) in the Sarcoplasmic Reticulum Ca-ATPase†

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ABSTRACT: Skeletal muscle contraction and relaxation is efficiently modulated through the reaction of reactive oxygen−nitrogen species with sarcoplasmic reticulum protein thiols in vivo. However, the exact locations of functionally important modifications are at present unknown. Here, we determine by HPLC−MS that the modification of one (out of 24) Cys residue of the sarcoplasmic reticulum (SR) Ca-ATPase isoform SERCA1, Cys_{349}, by peroxynitrite is sufficient for the modulation of enzyme activity. Despite the size and nature of the SR Ca-ATPase, a 110 kDa membrane protein, identification and quantitation of Cys modification was achieved through labeling with 4-(dimethylamino)phenylazophenyl-4′-maleimide (DABMI) and/or *N*-(2-iodoethyl)trifluoroacetamide (IE-TFA) followed by an exhaustive tryptic digestion and on-line HPLC−UV−electrospray MS analysis. The reaction with IE-TFA generates aminoethylcysteine, a new trypsin cleavage site, which allows the production of specific peptide fragments that are diagnostic for IE-TFA labeling, conveniently identified by mass spectrometry. Exposure of the SR Ca-ATPase to low concentrations (0.1 mM) of peroxynitrite resulted in the fully reversible chemical modification of Cys at positions 344, 349, 471, 498, 525, and 614 (nitrosylation of Cys_{344} and Cys_{349} was seen), whereas higher concentrations of peroxynitrite (0.45 mM) additionally affected Cys residues at positions 636, 670, and 674. When the SR Ca-ATPase was exposed to 0.45 mM peroxynitrite in the presence of 5.0 mM glutathione (GSH), thiol modification became partially reversible and S-glutathiolation was detected for Cys residues at positions 344, 349, 364, 498, 525, and 614. The extent of enzyme inactivation (determined previously) quantitatively correlated with the loss of labeling efficiency (i) of a single Cys residue and (ii) of the tryptic fragment containing both Cys_{344} and Cys_{349}. Earlier results had shown that the independent selective modification of Cys_{344} is functionally insignificant [Kawakita, M., and Yamashita, T. (1987) *J. Biochem. (Tokyo) 102*, 103−109]. Thus, we conclude that modification of only Cys_{349} is responsible for the modulation of the SR Ca-ATPase activity by peroxynitrite.

Nitric oxide (NO) is an important modulator of muscle contraction through the formation of cGMP and its interaction with reactive oxygen species which modify regulatory thiols in the sarcoplasmic reticulum (SR)[1] (*1*). For example, the calcium release channel (ryanodine receptor) is efficiently modulated by exposure to reactive oxygen species (*2*), NO, and NO donors (*3*−*5*), and S-nitrosylation of the calcium release channel has been documented in vitro and in vivo (*5*). The simultaneous production of NO (*1*) and superoxide ($O_2^{\bullet -}$) (*6*) in skeletal muscle can promote the formation of peroxynitrite ($ONOO^-$), a strong but selective oxidant which efficiently modifies protein thiols (*7*).

Muscle relaxation requires the transport of cytosolic calcium into the lumen of the SR, primarily achieved by the SR Ca-ATPase, a 110 kDa membrane protein (*9*, *10*). Specifically, the fast-twitch skeletal muscle isoform of the SR Ca-ATPase, SERCA1, contains 24 Cys residues (*11*−*13*) [two or three disulfide bonds (*13*)], of which only one or two appear to be essential for the activity of the enzyme (*12*−*15*). Through the presence of such critically important Cys residues, the SR Ca-ATPase, like the calcium release channel, represents a suitable target for modulation of muscle function by reactive oxygen species (*16*). Skeletal muscle expresses two highly similar (84%) SR Ca-ATPase isoforms, the SERCA1, located predominantly in fast-twitch muscle, and the SERCA2a, located predominantly in slow-twitch muscle (*9*). In vivo, both isoforms exhibit a significant loss of reduced Cys residues as a consequence of biological aging (*17*, *18*), indicating that protein oxidation occurs in both muscle types. The fact that especially the SERCA2a isoform also accumulates 3-nitrotyrosine (*18*, *19*) parallel to the loss of Cys indicates the involvement of reactive nitrogen intermediates in the modification of the SR Ca-ATPase in vivo and supports the hypothesis that this protein is

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[1] Abbreviations: [^{14}C]NEM, ^{14}C-labeled *N*-ethylmaleimide; DABMI, 4-(dimethylamino)phenylazophenyl-4′-maleimide; GSH, glutathione; IE-TFA, *N*-(2-iodoethyl)trifluoroacetamide; SIN-1, 3-morpholinosydnonimine-*N*-ethylcarbamide; SR, sarcoplasmic reticulum; TCP, tris(2-cyanoethyl)phosphine.

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modulated by NO- and/or peroxynitrite-dependent redox modification.

Recent experiments revealed that low levels of peroxynitrite inactivated the SR Ca-ATPase specifically through Cys modification, fully reversible through subsequent reduction of the enzyme by a disulfide reducing agent (*20*). In addition, an initially irreversible inactivation of the SR Ca-ATPase by higher concentrations of peroxynitrite became partially reversible (ca. 50%) when the enzyme was exposed to peroxynitrite in the presence of added glutathione (GSH), suggesting the intermediate S-glutathiolation of the protein (*20*).

Three important structural domains of the SR Ca-ATPase are the nucleotide-binding domain, the phosphorylation site (Asp_{351}), and the calcium-binding and -translocating domain (*9*). A correlation of a reversible functional inactivation through peroxynitrite with thiol modification requires the identification of the modified cysteine residue(s) critically important for enzyme activity. Here, we have identified by HPLC−MS the location and the functional importance of oxidized, nitrosylated, and/or S-thiolated cysteine residues within the SERCA1 isoform of the SR Ca-ATPase, exposed to peroxynitrite. Data were obtained for two representative oxidizing conditions which produced either fully (0.1 mM peroxynitrite) or partially (0.45 mM peroxynitrite/5.0 mM GSH) reversible Cys modification (*20*).

MATERIALS AND METHODS

Materials. The following chemicals were obtained from the respective sources: amyloid P component (FTLCFR-NH_2) from Advanced Chem Tech, trypsin (sequencing grade) from Promega, 4-(dimethylamino)phenylazophenyl-4′-maleimide (DABMI), *N*-(2-iodoethyl)trifluoroacetamide (IE-TFA), and tris(2-cyanoethyl)phosphine (TCP) from Molecular Probes, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and glutathione (GSH) from Sigma, and 3-morpholinosydnonimine-*N*-ethylcarbamide (SIN-1) from Alexis Biochemicals (San Diego, CA). All reagents for gel electrophoresis and prestained protein markers were from Bio-Rad (Richmond, CA). Peroxynitrite was synthesized according to the method of Pryor et al. (*21*). The potassium phosphate buffer employed in the oxidation experiments was treated with 5% (w/w) Chelex-100 (Bio-Rad) to minimize transition metal contaminations.

Membrane Preparations. Native SR vesicles (longitudinal "light" fraction) were prepared from rabbit skeletal white (fast-twitch) muscle, essentially as described previously (*22*). Electron microscopy studies of our preparations indicated that we obtained highly purified, right-oriented, intact SR vesicles (*20*). Protein concentrations were determined according to method of Lowry et al. (*23*) using bovine serum albumin (BSA) as a standard, and/or by absorbance, using an E_{280} of 1.05 for 1 mg/mL SR protein in 1% SDS (*13*). Generally, the Ca-ATPase accounted for ca. 72 ± 4% of the total mass of SR protein as quantified by gel electrophoresis and densitometry, corresponding to ca. 6.5 nmol of Ca-ATPase/mg of protein.

Determination of Sulfhydryl Groups. The total free sulfhydryl content of the SR vesicles was determined according to the general method of Ellman (*24*), modified as described previously (*17*).

Oxidation Conditions. The oxidation reactions were carried out using intact SR vesicles containing 10 mg/mL SR protein, 10 mM phosphate buffer, and 100 mM NaCl at 25 °C in a total volume of 250 μL. The SR vesicles were incubated either for 1 h with 400 μM SIN-1 or with bolus amounts of different concentrations of peroxynitrite. The starting pH was adjusted to 7.4 and shifted less than 0.2 unit through the addition of the peroxynitrite stock solution. In control experiments, peroxynitrite was added to the buffer in the absence of protein and allowed to decompose; SR vesicles were added after 20 min (reverse-order-of-addition control). In some experiments, a final concentration of 5 mM GSH was added before the initiation of oxidation by the addition of the peroxynitrite stock solution.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was performed using a 5% Laemmli gel with a 3% stacking gel (*25*). The samples were dissolved in a solution of 2% SDS, 50 mM Tris-HCl (pH 6.8), 15% sucrose, and 0.05% bromphenol blue in the absence or presence of 2.5% β-mercaptoethanol. After gel electrophoresis, gels were stained for protein with Coomassie blue R-250. The relative amount of monomeric and aggregated SR Ca-ATPase was determined from computerized densitometric measurements of the protein bands.

Labeling of the Protein. After centrifugation for 30 min at 100000*g*, native and peroxynitrite-exposed SR vesicles were dissolved in 2% SDS, 50 mM Tris-HCl (pH 7.0), and 0.1 mM EDTA to a final protein concentration of 4 mg of SR protein/mL. A freshly prepared solution of 10 mM DABMI in dimethylformamide (DMF) was added to the sample (an approximately 400-fold molar excess of DABMI over the Ca-ATPase), and the reaction mixture was stirred for 2 h in the dark at room temperature. Such labeling conditions were sufficient to quantitatively monitor all peroxynitrite-sensitive Cys residues (see Results). Excess reagent not bound to the protein, SDS, salts, and lipids were removed by precipitation with acidified acetone (*26*). The protein precipitate was washed repeatedly with DMF, and finally with water, and dried. The amount of DABMI covalently bound to SR protein was quantified as described previously (*27*). Aliquots of the samples labeled with DABMI were then dissolved to a final protein concentration of 2 mg/mL in 6 M guanidine hydrochloride and 100 mM Tris-HCl (pH 8.6) and incubated with 5 mM tris(2-cyanoethyl)phosphine (TCP) in DMF for 1 h at 50 °C to reduce potential disulfide bonds. After cooling, a final concentration of 5 mM IE-TFA (in DMF stock solution) was added, and the sample stirred for 2 h at room temperature in the dark (*28*). Excess reagent was removed as described for DABMI.

Trypsin Digestion. An aliquot of 1 mg of labeled protein was reacted with 0.05−0.1 mg of trypsin for 16−24 h at 37 °C in 50 mM NH_4HCO_3 (pH 8.2) containing 1 M urea. The resulting peptides were analyzed by HPLC−MS immediately, or stored at 4 °C for not more than 24 h.

Preparation of Standards for Mass Spectrometry. A cysteine-containing peptide (FTLCFR-NH_2, amyloid P component) was used as a standard to confirm the fidelity of the labeling chemistry and to search for ions characteristic of the label in the MS/MS experiments which could be used for the identification of labeled Ca-ATPase peptides. FTLCFR-NH_2 at a final concentration of 1 mM was dissolved in 100 mM ammonium acetate buffer at pH 7.0 for labeling

with DABMI or at pH 8.6 for labeling with IE-TFA, and incubated with 20 mM label for 2 h at room temperature in the dark. The labeled peptide was separated from excess labeling reagent by reversed-phase HPLC. As indicated by mass spectrometry, a byproduct (MW of 530 amu) of the DABMI reaction coeluted with FTLC(DABMI)FR-NH_2 and had to be removed from the collected and/or concentrated HPLC peak via toluene extraction.

FAB MS/MS. FAB experiments were performed with the VG flow FAB probe using a solvent system of H_2O/MeOH/glycerol (70/25/5, v/v/v) flowing at a rate of 5 μL/min into the source which was heated to 55 °C. Ionization was achieved with a cesium gun operated at 25 kV and 2 μA emission. The instrument was operated at 8 kV, and linked scans at a constant B/E were performed without adding gas to the first collision cell; the flow FAB solvent elevated the source pressure sufficiently for collision activation. Collision-induced decomposition (CID) spectra were acquired from activation in the collision octopole with precursor ions attenuated 50% with xenon and at 45 eV. The analyzer quadrupole was tuned to 1.5 amu full width at half-height, and the precursor ion was transmitted with MS1 tuned to a resolution of 1500. The scan rate was 1 s/100 amu, and 20 scans were integrated.

HPLC−ESI-MS. The identification of labeled standard and tryptic peptides was performed by microbore HPLC on-line coupled to an electrospray mass spectrometer (Autospec-Q tandem hybrid mass spectrometer; VG Analytical, Manchester, U.K.) equipped with an OPUS data system. The mass range was scanned in the positive mode from 500 to 2500 amu with a scan rate of 8 s/decade (s/dec) (resolution of 1500). The microbore HPLC instrumentation consisted of two pumps (Micro-Tech Scientific, Sunnyvale, CA), a dynamic mixing chamber with a volume of 20 μL, and a model 8125 injection valve (Rheodyne, Cotati, CA) with a 50 μL sample loop. Separations were performed on a 150 mm × 1.0 mm C_{18} column (Zorbax, 5 μm, 300 Å) at a flow rate of 50 μL/min where postcolumn flow splitting diverted 8 μL/min into the electrospray interface, and 42 μL/min for fraction collection. The peptides were monitored at either 310 or 500 nm with a UV/Vis detector (model 200, Linear Instruments, Fremont, CA) equipped with a micro flow cell. The column was first equilibrated with 95% (v/v) mobile phase A, consisting of a 98/2/0.1 (v/v/v) mixture of water, acetonitrile, and trifluoroacetic acid, respectively, and 5% (v/v) mobile phase B, consisting of a 10/90/0.1 (v/v/v) mixture of the same solvents. The peptides were eluted with the following gradient program: a linear gradient from 5 to 20% mobile phase B between 0 and 10 min, a linear gradient increasing mobile phase B to 64% between 10 and 80 min, and a linear gradient increasing mobile phase B to 100% between 80 and 90 min, followed by a 10 min isocratic elution with 100% mobile phase B. Collected fractions of interest were concentrated and stored at −70 °C.

RESULTS

HPLC−MS Analysis of a Standard Peptide Labeled with IE-TFA or DABMI

Before the analysis of labeled Ca-ATPase peptides, we established the HPLC−electrospray MS, FAB-MS, and MS/MS characteristics of a Cys-containing standard peptide, FTLCFR-NH_2 (amyloid P component), labeled by either IE-TFA or DABMI. The reaction of FTLCFR-NH_2 with IE-TFA and DABMI yielded the expected modified peptides, FTLC(aminoethyl)FR-NH_2 (MW of 827.4 amu) and FTLC-(DABMI)FR-NH_2 (MW of 1104.5 amu), when ionized in the FAB or electrospray mode. The starting peptide and both labeled products were subjected to MS/MS analysis by either linked scan at a constant B/E or CID in the collision octopole, or both. The masses of the observed ions are listed in Table 1 along with an interpretation of the structures of the respective ions, labeled according to the standard nomenclature for peptide fragmentation (*29*).

Table 1: Fragment Ions Obtained by FAB MS/MS for Native and Labeled Amyloid P Component FTLCFR-NH_2

	FTLC(X)FR-NH_2[a]		
fragment[b]	X = H	X = $CH_2CH_2NH_2$	X = DABMI
A_1 (or F immonium)	120	120	120
$(Y_5B_3)_2'$	215	215	215
$(Y_4B_4)_2'$	217	260	537
A_2	221	221	221
B_2-H_2O	231	231	231
B_2	249	249	249
$(Y_2B_6)_2'$	304		
$(Y_5B_4)_3'$	318	361	
Y_2''	321		321
B_3-H_2O	344		
B_3	362		
Y_3''-XSH	390	390	
$(Y_3B_6)_3'$	407		727
Y_3''	424	467	744
A_4	437		
B_4-H_2O	447	490	
B_4	465	508	785
Y_4''-XSH	503	503	
$(Y_4B_6)_4'$	520	563	840
Y_4''	537	580	857
$(Y_5B_6)_5'$	621	664	941
B_6-H_2O	750		

[a] C = HN-CH(CH_2S)CO. [b] Nomenclature from ref *29*.

The addition of a DABMI group to the cysteine side chain resulted in the CID spectra being dominated by the DABMI group. The most abundant product ion was DABMI(SH)$^+$ (*m*/*z* 353) followed by the DABMI-Cys immonium ion (*m*/*z* 396). This shift in product ion distribution is illustrated in Figure 1. In an HPLC−electrospray MS experiment in which the ESI source lenses were set to enhance cone fragmentation, the *m*/*z* 353 ion was also quite abundant. Thus, the DABMI(SH)$^+$ daughter ion can be used as a marker ion to screen for DABMI-labeled peptides. The CID spectrum of the aminoethyl-labeled cysteine was not dramatically different from that of the unmodified peptide; usually, the aminoethyl side chain was lost. Similarly, no significant yields of aminoethyl-Cys immonium ions could be detected in the linked scan at a constant B/E.

Reaction with low concentrations of peroxynitrite

Quantification of SR Thiols and Labeling. The titration of nonmodified SR vesicles with DTNB yielded 21.8 ± 1.0 mol of reduced Cys residues/mol of Ca-ATPase in good agreement with our earlier measurements (*20*) and data reported by Murphy (*12*) and Thorley-Lawson and Green (*13*) (see Table 2). Determination of free sulfhydryls per mole

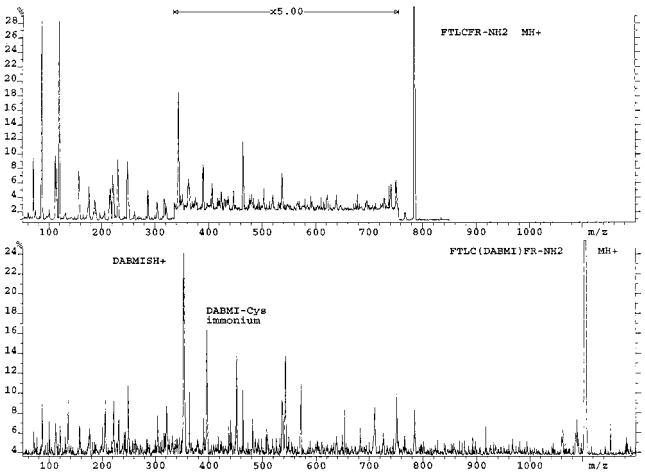

FIGURE 1: FAB MS/MS spectra of native (upper spectrum) and DABMI-labeled (lower spectrum) amyloid P component FTLCFR-NH_2. Clearly, the major fragment ions of FTLC(DABMI)FR-NH_2 are DABMI$(SH)^+$ and the DABMI-Cys immonium ion.

Table 2: Identification of Protein Thiol Groups[a]

sample	mol of SH/mol of Ca-ATPase[b]	mol of DABMI/mol of Ca-ATPase
control	21.8 ± 1.0	9.8 ± 0.7
0.10 mM $ONOO^-$	20.46 ± 0.36	8.40 ± 0.35
0.45 mM $ONOO^-$	10.28 ± 0.37	2.21 ± 0.15
0.45 mM $ONOO^-$ and 5 mM GSH	n/d[c]	7.41 ± 0.13

[a] Data represent the mean (±SD) of three determinations. [b] Data obtained with DTNB according to the method of ref *20*. Determination of the number of free thiol groups per mole of Ca-ATPase is based on the facts that (i) each milligram of SR contains 72 ± 4% Ca-ATPase (SDS−PAGE) and (ii) 95% of all thiol grops present in SR proteins reside on the Ca-ATPase (*12*). [c] Not determined.

of Ca-ATPase was based on the fact that each milligram of "light" SR vesicles contains 72 ± 4% Ca-ATPase and that >95% of all Cys residues of the SR proteins belong to the SR Ca-ATPase (*12*). By UV spectroscopy, we quantified that the reaction with DABMI led to the incorporation of 9.8 ± 0.7 mol of DABMI/mol of native SR Ca-ATPase, corresponding to a labeling efficiency of ca. 63% for the DABMI-reactive Cys residues (see Table 2).

The exposure of the SR vesicles (10 mg of SR protein/mL; corresponding to 65 μM SR Ca-ATPase) to 100 μM peroxynitrite resulted in the loss of ca. 1.3 mol of Cys residues/mol of SR Ca-ATPase, as quantified by DTNB (Table 2). The quantification of DABMI labeling showed that after reaction with 100 μM peroxynitrite the SR Ca-ATPase incorporated 1.4 mol of DABMI/mol of Ca-ATPase less compared to the control, well in agreement with the loss of thiols quantified by DTNB. A similar level of thiol modification was achieved when the SR vesicles were incubated for 1 h with 400 μM SIN-1, a compound which simultaneously releases NO and superoxide (*30*). Thus, low levels of peroxynitrite exclusively target Cys residues which also react with DABMI. Therefore, HPLC−MS experiments with DABMI-labeled peptide fragments will give a quantitative measure of the peroxynitrite-sensitive Cys residues.

HPLC−MS Analysis of Labeled Nonoxidized SR Ca-ATPase. Native SR Ca-ATPase in intact SR vesicles was manipulated in the following order: (1) labeling with DABMI, (2) reduction by TCP, (3) labeling with IE-TFA,

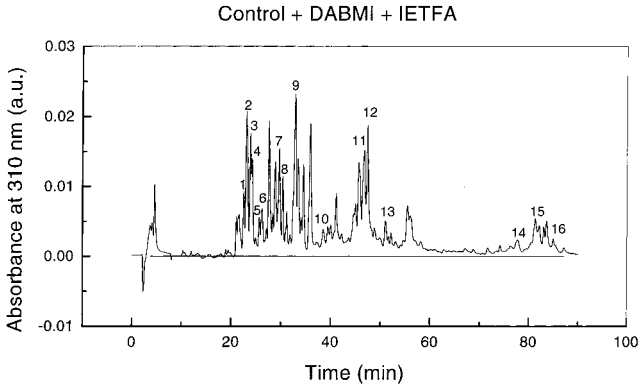

FIGURE 2: Analysis of an exhaustive tryptic digest of DABMI- and IE-TFA-labeled SR Ca-ATPase by HPLC−UV−electrospray MS. For conditions, see Materials and Methods.

(4) exhaustive digestion by trypsin, and (5) analysis by HPLC on-line connected to a UV/VIS detector (set at either 310 or 500 nm) and electrospray MS in series. Labeling by DABMI led to the incorporation of a chromophore and to a theoretical mass increase of an original Cys residue by 320.35 amu. Labeling by IE-TFA yielded 2-aminoethylcysteine through simultaneous nucleophilic substitution at the carbon−iodide bond and cleavage of the amide bond (*28*), associated with a theoretical mass increase of the original Cys residue by 43 amu. We note that trypsin cleaves C-terminal to 2-aminoethylcysteine; i.e., labeling by IE-TFA incorporates an additional proteolytic cleavage site. DABMI-labeled fragments were monitored by spectroscopic detection at either 310 or 500 nm and identified by mass spectrometry, whereas the 2-aminoethylcysteine-containing fragments were monitored by mass spectrometry only.[2] A representative chromatogram is displayed in Figure 2, and a cartoon indicating the locations of the respective Cys residues in the SERCA1 sequence is shown in Figure 3. Overall, 19 Cys residues were labeled comprising all cytosolic and two transmembrane Cys residues of the Ca-ATPase (Cys_{268} and Cys_{938}) (Table 3). Three Cys residues were exclusively labeled by IE-TFA (Cys_{12}, Cys_{377}, and Cys_{420}). All other identified Cys residues reacted with DABMI. However, for five of the DABMI-labeled Cys residues, Cys_{498}, Cys_{561}, Cys_{614}, Cys_{670}, and Cys_{675}, we identified additional labeling with IE-TFA after TCP exposure, indicating an incomplete labeling of these specific residues by DABMI [incomplete labeling has also been reported for other maleimide- and iodoacetamide-based labels (*27*)].

HPLC−MS Analysis of Peroxynitrite-Modified SR Ca-ATPase. After the exposure of 10 mg/mL SR protein (corresponding to a total of 65 μM SR Ca-ATPase) to 100 μM peroxynitrite, we identified five Cys-containing fragments by HPLC-MS (and parallel UV detection) which incorporated less DABMI compared to the control (Figure 4A,B), namely, peak 1 (−16%, Cys_{614}), peak 2 (−27%, Cys_{498}), peak 3 (−14%, Cys_{525}), peak 7 (−20%, Cys_{471}), and peak 9 (−22%, Cys_{344} and Cys_{349}). A similar pattern was observed when SR vesicles were exposed for 1 h to 400 μM SIN-1. Quantification of the total integrated peak area lost

[2] It should be noted that the MS scans exhibit the masses of theoretically 103 tryptic fragments (and even more as a result of some incomplete digestion). Parallel UV−vis detection allows us to search for the regions of DABMI-labeled fragments in the chromatograms.

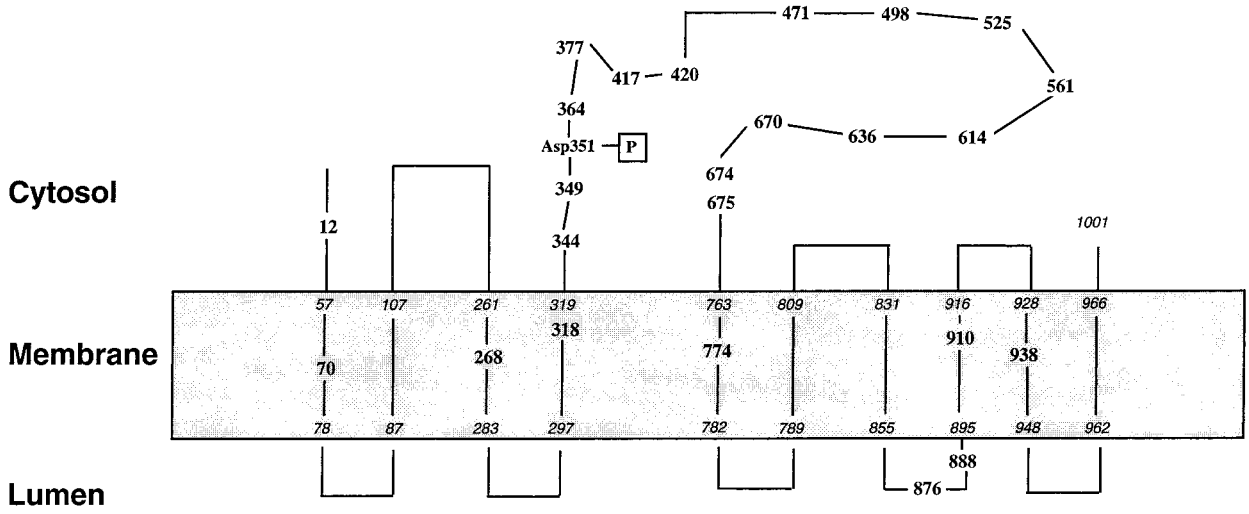

FIGURE 3: Cartoon of the SERCA1 indicating the location of the Cys residues. Cys residues are numbered in bold. Also shown is the phosphorylation site at Asp_{351} (location of Cys residues from ref *11*).

Table 3: Identification of Cysteine-Containing Tryptic Peptides of the SR Ca-ATPase by HPLC−ESI-MS

peak[a]	tryptic fragment	Cys positions	theoretical MW[b]	measured MW[c]	number and type of label[d]
12	Ser_8−Lys_{30}	12	2473.14	2516.25	1 IE-TFA
n/f[e]	Ile_{64}−Arg_{110}	70	5257.87		
15	Val_{263}−Arg_{334}	268, 318	7713.26[f]	8354.99	2 DABMI
9	Ser_{335}−Lys_{352}	344, 349	1837.09[f]	2478.48	2 DABMI
11	Thr_{353}−Lys_{365}	364	1381.65	1701.22	1 DABMI
6	Thr_{353}−Cys_{377}	377	2764.26	2807.82[g]	1 IE-TFA
13	Ser_{404}−Lys_{431}	417, 420	2987.35	3307.76[h]	1 DABMI
14	Met_{366}−Cys_{420}	377, 417, 420	5969.07[f]	6057.24[i]	2 IE-TFA
7	Ala_{468}−Arg_{476}	471	945.46	1265.48	1 DABMI
2	Ser_{493}−Lys_{502}	498	1070.47	1390.65	1 DABMI
3	Cys_{525}−Arg_{529}	525	652.29	973.48	1 DABMI
5	Cys_{561}−Arg_{567}	561	745.40	1066.6	1 DABMI
1	Glu_{606}−Arg_{615}	614	1133.54	1452.08	1 DABMI
4	Gly_{630}−Arg_{637}	636	802.42	1123.61	1 DABMI
8	Glu_{668}−Arg_{671}	670	476.26	796.96	1 DABMI
10	Ala_{673}−Arg_{678}	674, 675	667.26	1309.21	2 DABMI
n/f	Tyr_{763}−Arg_{822}	774	6425.43		
n/f	Tyr_{837}−Arg_{924}	876, 888, 910	9740.38		
16	Met_{925}−Lys_{958}	938	3987.95[f]	4309.44	1 DABMI

[a] Peak numbers are assigned in the order of retention times as shown in Figure 2. [b] Theoretical values of MW are calculated on the basis of the most abundant monoisotopic species of the original peptide. [c] These experimental values are reported as $[(m/z \times n) - n]$, where n is the charge. [d] The mass increase per added equivalent of DABMI is 320.35 amu and that of aminoethyl 43 amu. [e] Not found. [f] These MW values are calculated as average masses. [g] Cys_{364} in this peptide was not modified. [h] Cys_{420} in this peptide was not modified. [i] Incomplete digestion after aminoethylcysteine at either position 377 or 417.

after peroxynitrite exposure revealed a loss of 1.2 mol of DABMI/mol of Ca-ATPase, well in accord with the data derived by DTNB titration (−1.3 mol/mol) and spectroscopic quantification of DABMI incorporation (−1.4 mol/mol) (Table 2). Thus, an average loss of 1.3 ± 0.1 mol of Cys residues/mol of Ca-ATPase can be calculated. Except for Cys_{498} (−27%), the quantified loss of peptide labeling for each of the other affected peptides (average loss of −18 ± 4%) is comparable to the loss of activity of the enzyme under these conditions measured earlier [−17% (*20*)]. This result suggests that only one of the affected Cys residues at positions 344, 349, 471, 525, or 614 is functionally important for activity.

The enzyme initially labeled by DABMI was reduced by TCP and subsequently reacted with IE-TFA. This doubly

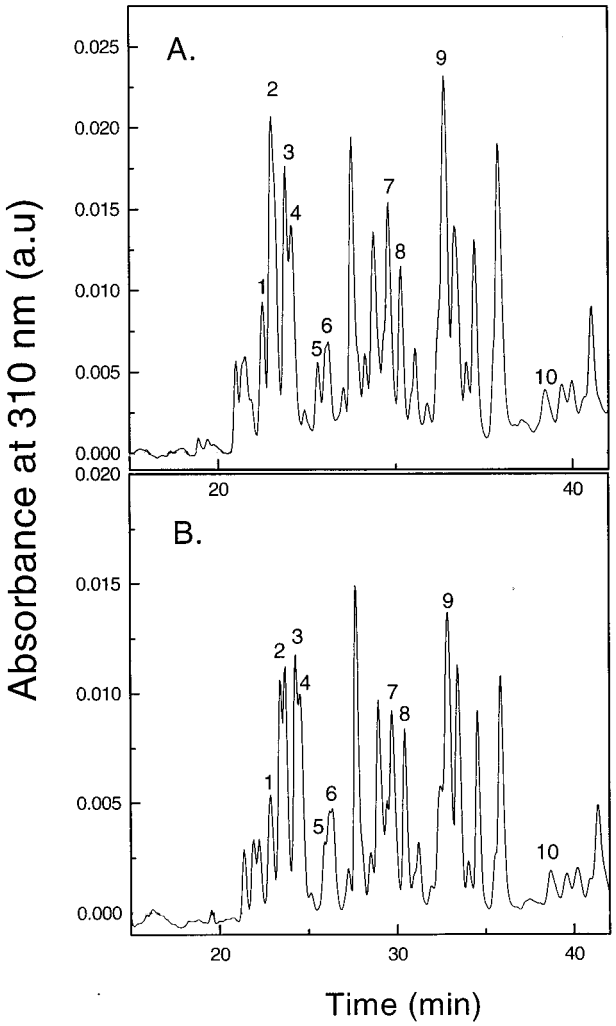

FIGURE 4: HPLC−UV−electrospray MS analysis of native (A) and peroxynitrite-exposed (0.1 mM) SR Ca-ATPase (B) after labeling with DABMI and IE-TFA and exhaustive tryptic digestion. The total amount of injected protein was 25% less for Figure 3B. For conditions, see Materials and Methods.

labeled protein revealed the formation of aminoethyl-Cys residues for all previously modified Cys residues, i.e., at positions 344, 349, 471, 498, 525, and 614 (note that aminoethyl labeling results in tryptic cleavage C-terminal

to aminoethyl-Cys_{344} and aminoethyl-Cys_{349}, generating two new fragments from the sequence of $Ser_{335}-Lys_{352}$). The reversible modification of the individually identified Cys residues is in accord with the earlier observed reversibility of inactivation and loss of total Cys (*20*), and the hypothesis that one of the identified Cys residues is functionally important for activity.

Products of Cys Modification. On the basis of the reversibility of Cys modification, we anticipate the reaction products to be either disulfides or nitrosothiols (RSNO) but not higher oxidation products such as, for example, sulfinic or sulfonic acid (*7*, *30*). We analyzed an exhaustive tryptic digest of peroxynitrite-exposed SR Ca-ATPase which was labeled with DABMI, but not reduced with TCP, by HPLC−MS specifically for the presence of nitrosothiols, i.e., for fragments with molecular weights 29 amu higher than those theoretically expected for nonmodified Cys-containing peptides. Screening over all MS scans, we located (a) the doubly (*m*/*z* 1288.86) and triply (*m*/*z* 859.41) charged ions corresponding to a peptide X with a MW_X of 2575.41 ± 0.2, eluting with a t_R of 27 min, and (b) the doubly (*m*/*z* 1274.6) and triply (*m*/*z* 849.6) charged ions corresponding to a peptide Y with a MW_Y of 2546.5 ± 0.7, eluting with a t_R of 34 min. Peptide X nearly coelutes with the nonderivatized tryptic SR Ca-ATPase fragment of $Lys_{329}-Lys_{352}$ with a MW of 2517.31 (t_R = 26.5 min) which contains Cys_{344} and Cys_{349} (this peptide originates from an incomplete proteolytic cleavage of nonlabeled SR Ca-ATPase between Lys_{328} and Lys_{329} and contains intact Arg_{334}). Peptide X exhibits a molecular weight 58 amu higher than that of $Lys_{329}-Lys_{352}$, consistent with an assignment to the bis-nitrosylated sequence [$Lys_{329}-Lys_{352}$ (Cys_{344}-NO and Cys_{349}-NO)] which would have a theoretical MW of 2575.31. Peptide Y is 29 amu heavier than $Lys_{329}-Lys_{352}$ and can be assigned to the mononitrosylated sequence [$Lys_{329}-Lys_{352}$(NO)] which would exhibit a theoretical MW of 2546.31. Due to the small quantities of peptides X and Y, we were unsuccessful at obtaining satisfactory MS/MS data. No other Cys-nitrosylated peptides were detected.

High Concentrations of Peroxynitrite

Absence of GSH. Following the reaction of 10 mg/mL SR protein with 450 μM peroxynitrite, DABMI labeled only 2.2 mol of Cys residues/mol of Ca-ATPase, i.e., 78% less than in the control samples (Table 2). HPLC−MS analysis of DABMI-labeled protein revealed the modification of Cys_{636}, Cys_{670}, and Cys_{674} in addition to the residues lost at low levels of peroxynitrite (see above). Furthermore, no IE-TFA labeling could be identified anymore for Cys_{12} (even after reduction with TCP), suggesting the modification of Cys_{12} to higher oxidation products different from disulfides or nitrosothiols, e.g., sulfinic or sulfonic acid.

Presence of GSH. After the exposure of the SR vesicles to 450 μM peroxynitrite in the additional presence of 5.0 mM GSH (GSH added before peroxynitrite), DABMI labeled 7.4 mol of Cys residues/mol of Ca-ATPase (Table 2), i.e., 2.4 mol/mol of protein less compared to control samples. Quantitative HPLC−UV−MS analysis of an exhaustive tryptic digest revealed a net loss of the following DABMI-labeled peaks: peak 1 (−17%, Cys_{614}), peak 2 (−11%, Cys_{498}), peak 3 (−20%, Cys_{525}), peak 5 (−10%, Cys_{561}), peak 9 (−39%, Cys_{344} and Cys_{349}), peak 10 (−53%, Cys_{674} and Cys_{675}), and peak 11 (−60%, Cys_{364}). In total, this net loss of DABMI-labeled peaks amounts to −3.0 mol of DABMI /mol of protein compared to a control sample, in reasonable agreement with the net loss of −2.4 mol of DABMI/mol of protein determined by UV−vis spectroscopy. Thus, on the basis of both measurements, we calculate an average net loss of −2.7 ± 0.3 mol of DABMI-reactive Cys residues/mol of Ca-ATPase. This loss of Cys is associated with a 40% loss of Ca-ATPase activity determined earlier, of which at most half can be reversed by subsequent incubation with dithiothreitol (*20*).

A direct search for S-glutathiolated Cys-containing peptides present in the mass spectrometric scans revealed S-thiolation for the following Cys residues: Cys_{344}, Cys_{349}, Cys_{364}, Cys_{498}, Cys_{525}, Cys_{561}, and Cys_{614}. S-Glutathiolation of a Cys-containing peptide leads to a theoretical mass increase of 305.3 amu.

DISCUSSION

Location of the Functionally Important Cys Residue. It has been suggested that only one or two Cys residues of the SR Ca-ATPase are critically important for enzyme function (*12*−*15*). The following considerations suggest that peroxynitrite modification of specifically Cys_{349} causes the loss of Ca-ATPase activity. At low peroxynitrite concentrations, we observed a nearly stoichiometric (1:1) correlation of the loss of the following DABMI-reactive Cys residues, Cys_{344} and Cys_{349} (22%, both residues in one tryptic peptide), Cys_{471} (20%), Cys_{525} (14%), and Cys_{614} (16%), with the loss of enzyme activity (−17%), determined previously (*20*). As expected, free sulfhydryl groups, available for a second labeling with IE-TFA, are obtained for all five Cys residues (in their respective peptides) after reduction of the modified Ca-ATPase by TCP. This result is consistent with the complete reversibility of enzyme inactivation and the loss of total Cys under these conditions (*20*). The nearly 1:1 correlation of the loss of enzyme activity with the loss of each of the five DABMI-reactive Cys residues is well in accord with the hypothesis that the peroxynitrite modification of only one individual Cys residue out of those five is functionally important. When SR vesicles are exposed to 450 μM peroxynitrite in the presence of GSH, only the loss of DABMI labeling for the peptide containing Cys_{344} and Cys_{349} (39%) correlates with the loss of activity (−40%) under such conditions, determined earlier (*20*). All other affected peptides exhibit significantly lower (10−20%) or higher (53−60%) extents of Cys modifications. On the basis of the partial reversibility of enzyme inactivation by DTT, we suggested the formation of S-glutathiolated SR Ca-ATPase (*20*). In support of this suggestion, we have now identified S-glutathiolation by HPLC−MS for both Cys residues, Cys_{344} and Cys_{349}. Thus, at this point we conclude that peroxynitrite modification of either Cys_{344} or Cys_{349} has functional consequences for the Ca-ATPase.

Earlier, Kawakita and Yamashita (*15*) reported that labeling of the Ca-ATPase with *N*-ethylmaleimide (NEM) to an extent of 1.2 mol of label incorporated per mole of protein did not significantly affect both the phosphorylation at Asp_{351} (formation of the E−P intermediate) and Ca^{2+} transport activity of the enzyme. Under these conditions, the NEM

label was equally distributed between Cys_{344} and Cys_{364}. This was unambiguously demonstrated by labeling with [^{14}C]-NEM, thermolysin digestion, and HPLC purification and characterization of two [^{14}C]NEM-containing peptides, VC_{364}-(NEM)KM and LGC_{344}(NEM)TS. The finding that labeling of Cys_{364} does not result in enzyme deactivation is consistent with our conclusions that peroxynitrite modification of Cys_{364} does not contribute to the loss of Ca-ATPase activity. However, the fact that independent labeling of Cys_{344} by NEM is functionally insignificant has important implications for our experiments; it suggests that, in a first approximation, peroxynitrite modification of Cys_{349} represents the enzyme deactivating process. We need to caution, however, that labeling with NEM and peroxynitrite modification result in the formation of different reaction products. On the basis of the reversibility of the Cys modification, we expect that especially low concentrations of peroxynitrite convert Cys_{344} and Cys_{349} into either disulfides or nitrosothiols. Gel electrophoretic analysis of the modified SR Ca-ATPase showed the presence of some higher-molecular weight aggregates only under nonreducing conditions (data not shown), indicating the formation of intermolecular disulfide bonds. Evidence for the nitrosylation of Cys_{344} and Cys_{349} has been obtained by HPLC−MS. Whether these nitroso-Cys residues are formed through the direct interaction of peroxynitrite with the respective Cys residues or through transnitrosylation after the initial modification of other Cys residues cannot be specified. At higher peroxynitrite concentrations, irreversible inactivation was observed (*20*), suggesting the formation of higher oxidation products of Cys such as sulfinic or sulfonic acids. However, at present we have not succeeded in identifying these products by HPLC−MS.

Both Cys residues, Cys_{344} and Cys_{349}, are located in the immediate vicinity of the phosphorylation site of the enzyme, Asp_{351}, within the sequence $GCTSVICSD_{351}KT$. Phosphorylation of Asp_{351} is very sensitive to changes in the spatial arrangement of the phosphorylation domain. For example, switching of the subsequence Asp-Lys to Lys-Asp resulted in a loss of the ATP-dependent phosphorylation of the enzyme (*32*). By analogy to the crystal structure of an enzyme with a catalytic domain similar to that of the P-type ATPase, haloacid dehalogenase (*33*), a short distance between Asp_{351} and Lys_{684} in the SR Ca-ATPase is essential for the phosphorylation of the enzyme. In fact, site-directed mutagenisis of Lys_{684} resulted in a nonphosphorylatable SR Ca-ATPase (*34*). The short distance between Asp_{351} and Lys_{684} may be impaired especially through disulfide formation between Cys_{349} and any other Cys residue, either intra- or intermolecularly. Nitrosylation of Cys_{349} would introduce a sterically bulkier group and eventually perturb any hydrogen-bond framework involving Cys_{349}.

Various mechanisms for the peroxynitrite-mediated modification of thiols have been described involving nucleophilic and free radical pathways, directly yielding nitrosothiols (*31*), nitrothiols (*35*), sulfenic acid (*7*, *36*), or thiyl radicals (*36*). Our experimental results do not permit any conclusion about which mechanism is ultimately responsible for the observed modifications of the SR Ca-ATPase, in particular as any of the potentially formed intermediates has its own complex chemistry. An additional pathway of thiol modification may involve N_2O_3 (*37*). Peroxynitrite exists in equilibrium with its conjugate acid, peroxynitrous acid [ONOOH, equilibrium 1; $pK_{a,1} = 6.5-6.8$ (*38*, *39*)]. Reactions 2 and 3 represent two homolytic pathways, contributing to the pH-dependent decomposition of peroxynitrite (*40*). Subsequently, NO and $^{\bullet}NO_2$ can combine to give N_2O_3, an electrophilic thiol modifying reagent (*37*).

$$ONOO^- + H^+ \rightleftharpoons ONOOH \quad (1)$$

$$ONOO^- \rightleftharpoons NO + O_2^{\bullet -} \quad (2)$$

$$ONOOH \rightleftharpoons HO^{\bullet} + {}^{\bullet}NO_2 \quad (3)$$

The peroxynitrite-induced modification of the SERCA1 is quite selective with respect to Cys (*20*), in accordance with the measured rate constants for the reaction of peroxynitrite with various amino acids where Cys represents the most reactive target (*41*). Such selectivity would also be in accordance with N_2O_3 being the actual modifying agent. Also, the incubation of the SERCA1 with H_2O_2 (*42*), Fenton reaction-derived hydroxyl radicals (or their metal-bound equivalents) (*14*), or phenoxyl radicals (*43*) led to enzyme inactivation through Cys modification. In contrast, peroxyl radicals modified up to 5 mol of Cys/mol of SERCA1 without affecting enzyme activity (*42*), a result comparable to simple air oxidation where the modification of up to six Cys residues had little effect on activity (*13*). Besides modifying Cys, peroxyl radicals also targeted Tyr residues, yielding bityrosine (*42*), a modification not observed for the peroxynitrite incubation of the SERCA1 (*20*). In vivo, biological aging of SR containing predominantly SERCA1 (90%) resulted in the modification of 1.5 mol of Cys/mol of SR Ca-ATPase without altering the initial activity of the protein (*17*). This modification was not reversible. Thus, most oxidizing conditions target the Cys residues of the SR Ca-ATPase. However, the actual level of inactivation will depend on the location of the modified Cys residue and the reaction product.

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REFERENCES

1. Kobzik, L., Reid, M. B., Bredt, D. S., and Stamler, J. S. (1994) *Nature 372*, 546−548.
2. Stoyanovsky, D. A., Salama, G., and Kagan, V. E. (1994) *Arch. Biochem. Biophys. 308*, 214−221.
3. Mézáros, L., Minarovic, I., and Zahradnikova, A. (1996) *FEBS Lett. 380*, 49−52.
4. Stoyanovsky, D., Murphy, T., Anno, P. R., Kim, Y.-M., and Salama, G. (1997) *Cell Calcium 21*, 19−29.
5. Xu, L., Eu, J. P., Meissner, G., and Stamler, J. S. (1998) *Science 279*, 234−237.
6. Reid, M. B., Haack, K. E., Franchek, K. M., Valberg, P. A., Kobzik, L., and West, M. S. (1992) *J. Appl. Physiol. 73*, 1797−1804.
7. Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) *J. Biol. Chem. 266*, 4244−4250.
8. Viner, R. I., Ferrington, D. A., Hühmer, A. F. R., Bigelow, D. J., and Schöneich, Ch. (1996) *FEBS Lett. 379*, 286−290.
9. Møller, J. V., Juul, B., and le Maire, M. (1996) *Biochim. Biophys. Acta 1286*, 1−51.
10. MacLennan, D. H., Rice, W. J., and Green, N. M. (1997) *J. Biol. Chem. 273*, 5885−5891.

11. Brandl, C. J., Green, N. M., Korczak, B., and MacLennan, D. H. (1986) *Cell 44*, 597−607.
12. Murphy, A. J. (1976) *Biochemistry 15*, 4492−4496.
13. Thorley-Lawson, D. A., and Green, N. M. (1977) *Biochem. J. 167*, 739−748.
14. Scherer, N. M., and Deamer, D. W. (1986) *Arch. Biochem. Biophys. 246*, 589−601.
15. Kawakita, M., and Yamashita, T. (1987) *J. Biochem. (Tokyo) 102*, 103−109.
16. Kukreja, R. C., Okabe, E., Schrier, G. M., and Hess, M. L. (1988) *Arch. Biochem. Biophys. 261*, 447−457.
17. Viner, R. I., Ferrington, D. A., Aced, G. I., Miller-Schlyer, M., Bigelow, D. J., and Schöneich, Ch. (1997) *Biochim. Biophys. Acta 1329*, 321−335.
18. Viner, R. I., Ferrington, D. A., Williams, T. D., Bigelow, D. J., and Schöneich, Ch. (1999) *Biochem. J. 340*, 657−669.
19. Viner, R. I., Ferrington, D. A., Hühmer, A. F. R., Bigelow, D. J., and Schöneich, Ch. (1996) *FEBS Lett. 379*, 286−290.
20. Viner, R. I., Hühmer, A. F. R., Bigelow, D. J., and Schöneich, Ch. (1996) *Free Radical Res. 24*, 243−259.
21. Pryor, W. A., Cueto, R., Jin, X., Koppenol, W. H., Ngu-Schwemlein, M., Squadrito, G. L., Uppu, P. L., and Uppu, R. M. (1995) *Free Radical Biol. Med. 18*, 75−83.
22. Saito, A., Seiler, S., Chu, A., and Fleischer, S. (1984) *J. Cell Biol. 99*, 875−885.
23. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
24. Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70−77.
25. Laemmli, U. K. (1970) *Nature 227*, 680−685.
26. Ozols, J. (1990) *Methods Enzymol. 182*, 581−601.
27. Wawrzynów, A., and Collins, J. H. (1993) *Biochim. Biophys. Acta 1203*, 60−70.
28. Schwartz, W. E., Smith, P. K., and Royer, G. P. (1980) *Anal. Biochem. 106*, 43−48.
29. Roepstorff, P., and Fohlmann, J. (1984) *Biomed. Mass Spectrom. 11*, 601−609.
30. Lomonosova, E. E., Kirsch, M., Rauen, U., and de Groot, H. (1998) *Free Radical Biol. Med. 24*, 522−528.
31. Van der Vliet, A., Hoen, P. A. C., Wong, P. S.-Y., Bast, A., and Cross, C. E. (1998) *J. Biol. Chem. 273*, 30255−30262.
32. Maruyama, K., and MacLennan, D. H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3314−3318.
33. Aravind, L., Galperin, M. Y., and Koonin, E. V. (1998) *Trends Biochem. Sci. 23*, 127−129.
34. Vilsen, B., Andersen, J. P., and MacLennan, D. H. (1991) *J. Biol. Chem. 266*, 16157−16164.
35. Balazy, M., Kaminski, P. M., Mao, K., Tan, J., and Wolin, M. S. (1998) *J. Biol. Chem. 273*, 32009−32015.
36. Quijano, C., Alvarez, B., Gatti, R. M., Augusto, O., and Radi, R. (1997) *Biochem. J. 322*, 167−173.
37. Goldstein, S., and Czapski, G. (1996) *J. Am. Chem. Soc. 118*, 3419−3425.
38. Pryor, W. A., and Squadrito, G. L. (1995) *Am. J. Physiol. 268*, L699−L722.
39. Kissner, R., Nauser, T., Bugnon, P., Lye, P. G., and Koppenol, W. H. (1997*) Chem. Res. Toxicol. 10*, 1285−1292.
40. Goldstein, S., Czapski, G., Lind, J., and Merényi, G. (1999) *Chem. Res. Toxicol. 12*, 132−136.
41. Alvarez, B., Ferrer-Sueta, G., Freeman, B., and Radi, R. (1999) *J. Biol. Chem. 274*, 842−848.
42. Viner, R. I., Krainev, A. G., Williams, T. D., Schöneich, Ch., and Bigelow, D. J. (1997) *Biochemistry 36*, 7706−7716.
43. Ritov, V. B., Goldman, R., Stoyanovski, D. A., Menshikova, E. V., and Kagan, V. E. (1995) *Arch. Biochem. Biophys. 321*, 140−152.

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