Potential Role of Methionine Sulfoxide in the Inactivation of the Chaperone GroEL by Hypochlorous Acid (HOCl) and Peroxynitrite (ONOO\textsuperscript{−})\textsuperscript{*}

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GroEL is an Escherichia coli molecular chaperone that functions in vivo to fold newly synthesized polypeptides as well as to bind and refold denatured proteins during stress. This protein is a suitable model for its eukaryotic homolog, heat shock protein 60 (Hsp60), due to the high number of conserved amino acid sequences and similar function. Here, we will provide evidence that GroEL is rather insensitive to oxidants produced endogenously during metabolism, such as nitric oxide (NO) or hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), but is efficiently modified and inactivated by reactive species generated by phagocytes, such as peroxynitrite (ONOO\textsuperscript{−}) and hypochlorous acid (HOCl). For the exposure of 17.5 μM GroEL to 100–250 μM HOCl, the major pathway of inactivation was through the oxidation of methionine to methionine sulfoxide, established through mass spectrometric detection of methionine sulfoxide and the reactivation of a significant fraction of inactivated GroEL by the enzyme methionine sulfoxide reductase B/A (MsrB/A). In addition to the oxidation of methionine, HOCl caused the conversion of cysteine to cysteic acid and this product may account for the remainder of inactivated GroEL not recoverable through MsrB/A. In contrast, HOCl produced only negligible yields of 3-chlorotyrosine. A remarkable finding was the conversion of Met\textsuperscript{111} and Met\textsuperscript{114} to Met sulfone, which suggests a rather low reduction potential of these 2 residues in GroEL. The high sensitivity of GroEL toward HOCl and ONOO\textsuperscript{−} suggests that this protein may be a target for bacterial killing by phagocytes.

GroEL and its eukaryotic analog, heat shock protein 60 (Hsp60),\textsuperscript{1} are highly sequence-related members of the Group I subclass of chaperonin 60 (Cpn60) (1). These proteins assist the folding of newly synthesized polypeptides (GroEL) or translocated preproteins (mitochondrial Hsp60). The functional unit of GroEL (and of most Cpn60 proteins) is a sandwich of two heptameric rings, which are stacked end to end. Depending on the protein substrate, different ligands such as K\textsuperscript{+}, Mg\textsuperscript{2+}, ATP, and the cofactor GroES (or Hsp10) may be required for proper folding. Following the trapping of an unfolded or misfolded protein substrate in the hydrophobic interior of GroEL, the binding of ATP and GroES causes a conformational transition, which changes the interior surface properties from hydrophobic to hydrophilic, thus triggering protein folding (1, 2). Mammalian Hsp60 differs from GroEL in that it forms stable and functional heptameric rings in the absence of ATP and its cofactor Hsp10 (3). Our rationale for investigating the oxidative inactivation of GroEL is 2-fold: (i) its potential involvement in bacterial killing by phagocytes and (ii) a potential role for its analog, Hsp60, in an inflammatory and proapoptotic response during cardiovascular disease, as described below.

Hsp60 proteins play an important role in the cellular protection against oxidative stress (4, 5). Studies with mutant strains of Saccharomyces cerevisiae exposed to various oxidants show that a decrease in Hsp60 expression results in an increased sensitivity toward oxidative stress (reduced cell viability) and increased levels of oxidized mitochondrial proteins, including Hsp60 itself (5). An important question, which has not been addressed, is whether the oxidation of Hsp60 leads to its inactivation. If so, Hsp60 oxidation would further decrease the levels of active Hsp60 eventually leading to a more pronounced sensitivity of cells toward oxidative stress. Here, we will provide evidence that the bacterial analog of Hsp60, GroEL, is fairly insensitive to oxidants produced endogenously during metabolism, such as nitric oxide (NO) or hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). In contrast, GroEL is efficiently modified and inactivated through reactive species generated by phagocytes, such as peroxynitrite (ONOO\textsuperscript{−}) and hypochlorous acid (HOCl). Such sensitivity toward phagocyte-derived oxidants suggests that GroEL oxidation may represent an effective mechanism for bacterial killing by neutrophils and macrophages. Peroxynitrite, the product of NO and superoxide (O\textsubscript{2}\textsuperscript{−}), is highly toxic toward bacteria (6, 7). Hypochlorous acid is generated from chloride ions (Cl\textsuperscript{−}) and H\textsubscript{2}O\textsubscript{2} by the heme enzyme myeloperoxidase (8–10). A prominent role for myeloperoxidase in bacterial killing has been proposed (11–16) and confirmed in vivo involving a mouse model of polymicrobial sepsis (17). Aberrant Hsp60 may also play a role during inflammation of human tissue. Inflammatory processes contribute to the pathogenesis of some cardiovascular diseases such as atherosclerosis (18–21). Mechanical shear stress, such as observed in atherosclerotic aorta, triggers the expression of Hsp60, which stimulates the expression of E-selectin, intercellular adhesion molecule-1.
(ICAM-1), and interleukin-6 (22), thus promoting an inflammatory response (19). Interestingly, stressed aortic endothelial cells present Hsp60 on the cell surface (23). A similar observation was made for oxidatively stressed myocytes (hypoxia/reoxygenation), where translocation of cytosolic Hsp60 to the cell surface induced apoptosis (24). In the cytosol, Hsp60 complexes the propaptotic protein bax, and upon translocation of Hsp60 to the cell surface, the Hsp60/bax complex dissociates and bax relocates into the mitochondria (24). The molecular reason for Hsp60 translocation is not known. Coronary artery disease correlates with increased levels of myeloperoxidase (25) and myeloperoxidase-dependent markers for protein oxidation have been detected in low density lipoprotein isolated from human atherosclerotic intima (26). Based on the sensitivity of GroEL to HOCl demonstrated in this paper, it is possible that myeloperoxidase-derived reactive species target human Hsp60 in the aorta and that oxidative modification and inactivation of Hsp60 contribute to the pathogenesis of atherosclerosis.

MATERIALS AND METHODS

GroEL and GroES

The GroE molecular chaperons of Escherichia coli were isolated from the lysate of cells containing the appropriate overexpression plasmid (gifts from Dr. Edward Eisenstein (27) and Dr. Lorimer (28), respectively) as described by Vosjan and Fisher (29). Because GroEL and GroES do not contain tryptophan residues, as assayed by second derivative analysis of the absorption spectra and tryptophan fluorescence, was used as a criterion for purity of the chaperonin preparations in addition to size separation of the components resulting in the following concentrations: 1 μM (oligomer) native or oxidant-treated GroEL, 0.5 μM MDH, 2 μM GroES, and 5 mM ATP. The refolding reaction was carried out for 3 h at 37 °C before the enzymatic activity of MDH was determined using 1 mM ATP. The rate of oxidation of NADH at 340 nm was followed for 3 min using a U3210 Hitachi (Tokyo, Japan) spectrophotometer.

Quantitation of Cys with Thioglo-1

Native or oxidant-treated GroEL was denatured with 6 M guanidine HCl in 18.75 mM sodium phosphate buffer, pH 7.5. Stock solutions of Thioglo-1 were prepared in dimethyl formamide and added to denatured GroEL samples to give a final molar ratio of 5:1 dye to free thiol (Cys). Calibration curves in the same concentration range were generated with N-acetylt-cysteine as a standard. Fluorescence yields were determined after 30-min incubation at room temperature using a BioTek (Winooski, VT) FL600 Microplate Spectrophotometer.

Methanesulfonic Acid Hydrolysis and Amino Acid Analysis

Hydrolysis was performed according to the method of Simpson et al. (38). Briefly, GroEL samples were hydrolyzed in vacuo with 4 N methanesulfonic acid at 115 °C for 22 h and neutralized with 3.5 N sodium hydroxide. Methionine sulfoxide was separated from other amino acids by HPLC after precolum o-phthalaldehyde derivatization as described previously (39). The o-phthalaldehyde derivatization reagent was prepared by mixing equal volumes of stock (25 mg of o-phthalaldehyde, 0.625 ml of methanol, 25 ml of 2-mercaptoethanol, 11.2 ml of 0.4 M K₂HPO₄, pH 9.5) with 1% Brij solution. The sample (10 μl) was mixed with 10 μl of the derivatization reagent for 1 min before adding 0.4 μl of 2 N KH₂PO₄. After another min, 100 μl of the sample was injected onto a Hypersil ODS column (4.6 × 250 mm; ThermoHypersil-Keystone, Bellefonte, PA). Methionine sulfoxide and Met were separated from the other amino acids at 35 °C at a flow rate of 1 ml/min using a linear gradient of 33–100% solvent B within 15 min, where solvents A and B were 95:5 (v/v) water/acetonitrile and 95:5 (v/v) methanol/tetrahydrofuran, respectively. Detection was by fluorescence (Shimadzu RF-10AAS; Shimadzu, Columbia, MD) with excitation at 330 nm and emission at 450 nm. The same separation conditions were also sufficient to resolve the o-phthalaldehyde-derivatized 3-chlorotyrosine from the other amino acids.

Incubation with Methionine Sulfoxide Reductase (MsrBA)

Oxidant-treated GroEL samples were incubated with 1 μM MsrBA and 15 mM DTT for 2 h at 37 °C. The final GroEL concentration was 15.8 μM in buffer B.

Tryptic Digestion

Prior to tryptic digestion, native and oxidant-treated GroEL (starting concentration: 1 mg/ml) were reduced with 1 mM DTT in 6 M guanidine HCl for 30 min at 37 °C and alkylated with 3 mM sodium iodoacetate for 30 min at 37 °C. Subsequently, dialysis into 50 mM sodium phosphate buffer, pH 8.0, was carried out using Pierce Mini Dialysis Units, with the final buffer exchange containing 10% acetonitrile. Tryptsin was initially added at a 1:20 molar ratio to GroEL. After 1 h at 37 °C, another aliquot was added to adjust the final trypsin to GroEL molar ratio to 1:10 (total trypsin to GroEL). At this stage, samples contained ~0.4 mg/ml GroEL. After 18 h, the digestion was stopped by cooling to ~20 °C.

Analysis of Tryptic Digests

On-line HPLC-MS/MS Analysis—For on-line HPLC-MS/MS analysis we used a ThermoElectron LCQ-Duo mass spectrometer (ThermoElectron Corp., San Jose, CA) coupled to a gradient HPLC consisting of two Micro-Tech Ultra Plus II gradient pumps. Samples (10 μl of ~0.4 mg/ml peptides) were injected into a Vydac 218SM530 C18 column (300-A pore diameter, 50 × 0.32 mm) equilibrated with 0.1% mobile phase A (99.9% ultrapure water/0.1% formic acid) and 10% mobile phase B (99.9% acetonitrile/0.1% formic acid). Gradient separation was achieved by a linear increase of the mobile phase ratio to 100% solvent B within 60 min and holding at this ratio for 5 min before returning to starting conditions.
conditions. The following instrumental conditions were used for mass spectrometric analysis: number of microscans = 3, length of microscans = 200 ms, capillary temperature = 200 °C, spray voltage = 4.5 kV, capillary voltage = 14 V, tube lens offset = −17 V. Data acquisition was performed in the data-dependent fashion, i.e. a MS scan followed by MS/MS measurement with the normalized collision energy for MS/MS set at 35% and the isolation width of 2.0 m/z. A minimal signal for MS/MS acquisition was set to 2 × 10^5. Additionally, the dynamic exclusion option was enabled and set with the following parameters: repeat count = 3, repeat duration = 5 min, exclusion list size = 25, exclusion duration = 5, and exclusion mass width = 3. The sequence of native protein was matched using the TurboSEQUEST search option in the Bioworks Browser 3.1 software (ThermoElectron Corp.). Oxidatively modified sequences were matched manually by searching the data using the Xcalibur™ software package (ThermoElectron Corp.). The criteria for positive identification of a tryptic fragment were 1) matching of the observed m/z to the theoretical m/z and 2) matching of the collision-induced dissociation (CID) pattern to at least 3 consecutive fragment ions. Furthermore, confirmation of the location of modification in a peptide required the absence of the native residue.

**Off-line HPLC and MS/MS Analysis**—For off-line mass spectrometric analysis, 50–100-μg aliquots of a tryptic digest were separated using a 5-μm 250 × 4.6 mm-Hypersil ODS column (ThermoHypersil-Keystone). The mobile phases used were 3% acetonitrile/97% water containing 0.1% trifluoroacetic acid (solvent A) and 60% acetonitrile/40% water containing 0.1% trifluoroacetic acid (solvent B). A gradient of 0–100% solvent B over 120 min was generated using two Shimadzu LC-10A pumps. The flow rate was 1 ml/min with detection at 215 nm using a Shimadzu SPD 10AV UV/VIS detector. Peaks were collected, lyophilized, and stored at −20 °C.

Dry samples were solubilized in 90% methanol, 0.5% formic acid and introduced into an electrospray ionization source from a 20 μl injector loop at 10 μl/min. Spectra were acquired on a Q-ToF-2™ hybrid mass spectrometer (Micromass Ltd., Manchester, UK). The instrument was operated for maximum resolution with all lenses optimized on the [M+2H]2+ ion from the cyclic peptide Gramicidin S. The cone voltage was 55 eV, argon was admitted to the collision cell at a pressure that attenuates the beam to about 20%, and the cell was operated at 8 eV (maximum transmission). Spectra were acquired at 11,364-Hz pusher frequency covering the mass range 100 to 3,000 atomic mass units and accumulating data for 5 s per cycle. Time-to-mass calibration was made with C13 cluster ions acquired under the same conditions. CID spectra were acquired by setting the MS1 quadrupole to transmit a precursor ion from the cyclic peptide Gramicidin S. The cone voltage for CID was 30 eV, argon was admitted to the collision cell at a pressure that attenuates the beam to about 20%, and the cell was operated at 8 eV (maximum transmission). Spectra were acquired at 11,364-Hz pusher frequency covering the mass range 100 to 3,000 atomic mass units and accumulating data for 5 s per cycle.

**Size Exclusion Chromatography**

Chromatographic analysis of native and oxidant-treated GroEL was performed on a BIOSEP-SEC-S4000 300 × 7.8-mm column (Phenomenex, Torrence, CA) connected to a Shimadzu LC-10A pump. The run buffer was 50 mM sodium phosphate, pH 7.5, 10 mM magnesium chloride, 50 mM potassium phosphate (buffer B), at 1 ml/min flow rate. Detection was by a Shimadzu SPD 10AV UV/VIS detector at 260 nm. The injection volume was 50 μl of 1 mg/ml GroEL sample.

**Native Gel Electrophoresis**

Samples were run on 4–15% Phast Gels (non-reducing conditions) using a Pharmacia (Peapack, NJ) LKB Phast System. Protein was monitored by Coomassie Blue staining.

**RESULTS**

**Activity and Cysteine Content of Oxidant-treated GroEL**

The plots displayed in Fig. 1A reflect the activity of GroEL at refolding denatured MDH following treatment with various concentrations of oxidant. MDH is a class III type substrate (2), which requires the complete chaperonin system (GroEL and GroES) to refold. Any oxidation-dependent defects in the chaperonin system are more likely to show up with such a stringent substrate because of the multiple reactions that are necessary for a fully functioning chaperonin reaction (ATPase, GroES binding, GroES release, GroEL conformational changes, complex formation, polypeptide release). No significant inactivation of GroEL was observed when treated with 1 mM DEA/NO for 30 min. Even treatment with 10 mM H2O2 for 3 h only caused 40% inactivation (data point not shown but was used to extrapolate data for 1 mM H2O2 in Fig. 1A). On the other hand, more than half of the activity of GroEL was lost when treated with either 0.5 mM ONOO− or 0.25 mM HOCI and ≥80% inactivation occurred with 1 mM ONOO− and 0.5 mM HOCI, respectively. The data demonstrate an efficient inactivation of GroEL by HOCI and ONOO− but negligible effects of NO and H2O2.

We examined the potential reversibility of inactivation representatively for the exposure to HOCI (Fig. 1B). The inactivation of GroEL treated with 0.1–1.0 mM HOCI could not be reversed by reaction with 15 mM DTT alone (data not shown). In contrast, especially for GroEL treated with 0.1–0.25 mM HOCI, some of the lost activity could be recovered through exposure to 1 μM MsrB/A in the presence of 15 mM DTT. DTT is a suitable substitute for the physiological electron donor of methionine sulfoxide reductase, thioredoxin (40). The incubation with MsrB/A recovered ~70%, 85, and 60% of the activity lost after treatment with 0.1, 0.175, and 0.25 mM HOCI, respectively. For HOCI concentrations ≥ 0.5 mM, the combination of MsrB/A with 15 mM DTT did not restore the activity. The activity of MsrB/A was independently confirmed through amino acid analysis of methionine sulfoxide after methanesul-
Oxidation of GroEL by HOCl and ONOO⁻

M. A. K. O. A. V. K. F. N. 1
M. A. K. O. A. V. K. F. N. 2
M. A. K. O. A. V. K. F. N. 3
M. A. K. O. A. V. K. F. N. 4

HOCl did not result in a significant loss of free thiols. In function of oxidant treatment. Clearly, the exposure to /H18528 not shown). Cysteic acid was only detected at Cys138 and Cys458 for 0.1 and through reaction with 1

Cys even in highly oxidized samples (1.0 mM HOCl). No cysteic acid was present in native GroEL and GroEL exposed to peroxynitrite (0.5 and 1.0 mM). A representative CID spectrum for the tryptic fragment T57, containing Cys458, is displayed in Fig. 3. The presence of the fragments y12 (1229.3 Da) effectively localizes the addition of 3 oxygen atoms (+48 Da) to the Cys458 residue. Evidence for carboxymethyl-cysteine (Cys(Cm), molecular mass of Cys + 58 Da) as a result of iodoacetic acid derivatization was obtained for all 3 Cys residues in native and oxidant-treated samples, indicating at least some traces of residual free Cys even in highly oxidized samples (1.0 mM HOCl). Of the 23 Met residues (this includes the N-terminal Met) in GroEL, 12 were found oxidized by 1.0 mM HOCl, 10 by 0.25 mM HOCl, 10 by 0.1 mM HOCl, 7 by 1.0 mM ONOO⁻, and 8 by 0.5 mM ONOO⁻. The apparently lower number of oxidized Met residue after exposure to higher levels of ONOO⁻ is likely due to secondary reactions of oxidized peptide fragments. Tyr nitration at Tyr203 was observed for GroEL treated with 0.5 and 1.0 mM ONOO⁻. An unambiguous location of Tyr nitration in the ONOO⁻-treated GroEL peptide T57 +45 could not be made; here the MS/MS fragmentation pattern excluded the possibility of nitration at Tyr203 but could not discriminate the exact location of nitration between the two remaining tyrosines, Tyr476 and Tyr478. No mass spectrometric evidence for 3-chlorotyrosine was obtained for GroEL treated with 0.1 mM HOCl. By comparison with an authentic standard of 3-chlorotyrosine, amino acid analysis (after methanesulfonic acid hydrolysis) also yielded negligible amounts of this product (<0.1 mol of 3-chlorotyrosine/mol of GroEL for all applied concentrations of HOCl between 0.1 and 1.0 mM). The apparently lower number of oxidized Met residues for T16, which originally contains 2 Met residues, Met111 and Met114. Fragment ions y6 (1095.6 Da), y9 (1024.5 Da), y8 (953.5 Da), and their corresponding neutral losses of 64 Da (HSOCH₃) from the b or y

 Mass Spectrometric Identification of Oxidation Products

Analysis by On-line HPLC-MS/MS—Tryptic digests of native and oxidized GroEL (0.1, 0.25, and 1.0 mM HOCl; 0.5 mM and 1.0 mM ONOO⁻) were analyzed for oxidative modifications. For native GroEL, we obtained a sequence coverage >85% matched to the primary sequence of GroEL (NCBI accession number NP_313151), as shown in Fig. 2. Table I summarizes all the detected modifications for the respective oxidizing conditions. We note that the employed mass spectrometric conditions did not allow for the quantitation of the protein modifications.

Cysteic acid (cysteine sulfonic acid, molecular mass of Cys 48 Da) was observed for all 3 cysteine residues (Cys138, Cys458 and Cys519) for GroEL treated with 1.0 mM HOCl. In contrast, cysteic acid was only detected at Cys138 and Cys458 for 0.1 and 0.25 mM HOCl. No cysteic acid was present in native GroEL and GroEL exposed to peroxynitrite (0.5 and 1.0 mM). A representative CID spectrum for the tryptic fragment T57, containing Cys458, is displayed in Fig. 3. The presence of the fragments y12 (1229.3 Da) and y11 (1289.3 Da) effectively localizes the addition of 3 oxygen atoms (+48 Da) to the Cys458 residue. Evidence for carboxymethyl-cysteine (Cys(Cm), molecular mass of Cys + 58 Da) as a result of iodoacetic acid derivatization was obtained for all 3 Cys residues in native and oxidant-treated samples, indicating at least some traces of residual free Cys even in highly oxidized samples (1.0 mM HOCl). Of the 23 Met residues (this includes the N-terminal Met) in GroEL, 12 were found oxidized by 1.0 mM HOCl, 10 by 0.25 mM HOCl, 10 by 0.1 mM HOCl, 7 by 1.0 mM ONOO⁻, and 8 by 0.5 mM ONOO⁻. The apparently lower number of oxidized Met residue after exposure to higher levels of ONOO⁻ is likely due to secondary reactions of oxidized peptide fragments. Tyr nitration at Tyr203 was observed for GroEL treated with 0.5 and 1.0 mM ONOO⁻. An unambiguous location of Tyr nitration in the ONOO⁻-treated GroEL peptide T57 +45 could not be made; here the MS/MS fragmentation pattern excluded the possibility of nitration at Tyr203 but could not discriminate the exact location of nitration between the two remaining tyrosines, Tyr476 and Tyr478. No mass spectrometric evidence for 3-chlorotyrosine was obtained for GroEL treated with 0.1 mM HOCl. By comparison with an authentic standard of 3-chlorotyrosine, amino acid analysis (after methanesulfonic acid hydrolysis) also yielded negligible amounts of this product (<0.1 mol of 3-chlorotyrosine/mol of GroEL for all applied concentrations of HOCl between 0.1 and 1.0 mM). The apparently lower number of oxidized Met residues for T16, which originally contains 2 Met residues, Met111 and Met114. Fragment ions y6 (1095.6 Da), y9 (1024.5 Da), y8 (953.5 Da), and their corresponding neutral losses of 64 Da (HSOCH₃) from the b or y

Analysis by Off-line HPLC Separation and MS/MS—Off-line tandem MS analysis of 1 mM HOCl-treated GroEL identified two tryptic fragments that contained Met sulfoxide. One characteristic of fragments containing Met sulfoxide is the neutral loss of 64 Da (H₂SO₃) from the b or y ion (this was observed in almost every spectrum). Fig. 4 displays representative data for T16, which originally contains 2 Met residues, Met111 and Met114. Fragment ions y10 (1095.6 Da), y9 (1024.5 Da), y8 (953.5 Da), and their corresponding neutral losses of 64 Da (1031.6, 960.5, and 889.5 Da) are labeled. Fragment ions y6 and y5 are labeled twice in the spectra. The 733.4-Da fragment containing a sulfone at Met114. This is also the case for y5, where the first and second designated m/z are 16 Da apart, indicating that Met114 is present in two forms, as a sulfoxide and a sulfone. Since the parent masses of these MS/MS spectra were identical, we conclude that 3 oxygen atoms were distributed such to yield either the combination of sulfoxide at Met111/sulfone at Met114 or sulfone at Met113/sulfide at Met114.

Fig. 2. Sequence coverage and location of modifications as determined by online LC-MS and MS/MS of whole tryptic digests. More than 85% sequence coverage was achieved (underlined sequences were not found). All Met, Cys, and Tyr residues are represented in bold, and residues found modified as a consequence of oxidant exposure are indicated by their respective oxygen and nitrogen additions.
Structural Changes as Detected by Native Gel Electrophoresis and Size Exclusion Chromatography

The results from native gel electrophoresis demonstrate structural changes due to the oxidation of GroEL (Fig. 5). The right lane of each gel represents a control of native GroEL, which establishes a standard band for intact oligomeric GroEL. Based on this reference, we see that all samples contain oligomeric GroEL except samples in which GroEL was treated with high concentrations (1.0 mM) of HOCl and ONOO⁻. Large smears extending to lower molecular weight regions are observed in the lanes for 0.5 mM and 1.0 mM HOCl and ONOO⁻.

Similar results are observed with size exclusion chromatography. In Fig. 6, the peaks representing oligomeric and monomeric GroEL were established using thyroglobulin (669 kDa) and bovine serum albumin (60 kDa) as reference data (not shown). The peak eluting with $t_R = 13$ min represents small molecular weight components of the reaction mixtures. A gradual loss of the GroEL oligomer occurs for treatment with increasing concentrations of HOCl, paralleled by the formation of GroEL monomer. Notably, the exposure of GroEL to 0.1 and

| Fragment + modification | Sequence $^a$ | ms HOCl 0.25 | ms HOCl 0.10 | ms HOCl 0.0 | ms HOCl 1.0 | ms ONOO⁻ 0.5 | ms ONOO⁻ 0.0 |
|------------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| T12 13+32              | EIELEDKFENM$^{338}$GAQM$^{380}$OYK | x x x          | x x x          | x x           | x x          | x x          | x x          |
| T16 13+32              | AVAAGFM$^{124}$ONFM$^{144}$OYDK    | x x x          | x x x          | x x          | x x          | x x          | x x          |
| T20 13+58              | ALSVM$^{338}$Cys(Cm)SDSK             | x x x          | x x x          | x x          | x x          | x x          | x x          |
| T20+48                 | ALSVM$^{143}$O$_2$SDSK               | x x x          | x x x          | x x          | x x          | x x          | x x          |
| T22+16                 | LIAEM$^{148}$OYDK                    | x x x          | x x x          | x x          | x x          | x x          | x x          |
| T24+16                 | EGTVGFDGTLQDLEGEM$^{338}$OEQFD        | x x x          | x x x          | x x x        | x x          | x x          | x x          |
| T26+45                 | GY$^{143}$LSPY$^{203}$OJFINKPGEAVELEPSFILLADK | x x          | x x          | x x          | x x          | x x          | x x          |

$^a$ x indicates peptide identified by at least 3 consecutive fragment ions (MS/MS). ? indicates matching m/z but lack of unambiguous MS/MS. (O) indicates an oxygen addition at this position, characterized by an increase of the $m/z$ of the native peptide by +16 Da. (O$_2$) indicates the addition of three oxygens, characterized by an increase of the $m/z$ of the native peptide by +48 Da. (NO$_2$) indicates tyrosine nitration, characterized by an increase of the $m/z$ of the native peptide by +45 Da. (Cys(Cm)) indicates carboxymethylcysteine, characterized by an increase of the $m/z$ of the native peptide by +58 Da.

**Structural Changes as Detected by Native Gel Electrophoresis and Size Exclusion Chromatography**

The results from native gel electrophoresis demonstrate structural changes due to the oxidation of GroEL (Fig. 5). The right lane of each gel represents a control of native GroEL, which establishes a standard band for intact oligomeric GroEL. Based on this reference, we see that all samples contain oligomeric GroEL except samples in which GroEL was treated with high concentrations (1.0 mM) of HOCl and ONOO⁻. Large smears extending to lower molecular weight regions are observed in the lanes for 0.5 mM and 1.0 mM HOCl and ONOO⁻. Similar results are observed with size exclusion chromatography. In Fig. 6, the peaks representing oligomeric and monomeric GroEL were established using thyroglobulin (669 kDa) and bovine serum albumin (60 kDa) as reference data (not shown). The peak eluting with $t_R = 13$ min represents small molecular weight components of the reaction mixtures. A gradual loss of the GroEL oligomer occurs for treatment with increasing concentrations of HOCl, paralleled by the formation of GroEL monomer. Notably, the exposure of GroEL to 0.1 and
0.25 mM HOCl resulted in an ~15 and 60% loss of oligomer (Fig. 6A), which is paralleled by the levels of inactivation, ~18 and 60%, respectively, displayed in Fig. 1A. In contrast, the exposure of GroEL to 0.25 mM ONOO− did not cause any loss of oligomer but ~30% loss of activity (Fig. 6B). Hence, some of the ONOO−-treated inactive protein is still present in the oligomeric state. Consistent with the gel electrophoresis measurements, GroEL treated with either 1 mM DEA/NO or 10 mM H2O2 showed no conformational changes (Fig. 6C).

DISCUSSION

As an intracellular protein-folding machine, the Cpn60 class interacts with a wide range of substrates and is an essential component of the cell. Thus, the accumulation of aberrant Cpn60 may have many deleterious consequences. First, Cpn60 is involved in folding a wide variety of proteins, particularly those whose folding pathway includes aggregation prone intermediates or metastable states and those that are susceptible to thermal denaturation (1). Cpn60 function is crucial, and a deletion of this protein is thought to be lethal in virtually every organism. Second, in the case of the mitochondrial Cpn60 (mt-Cpn60), the accumulation of damaged or malfunctional Cpn60 inside the mitochondria will have deleterious effects on general protein folding in the entire organelle. Here, mt-Cpn60 is not only responsible for folding important oxygen scavenging enzymes (such as manganese-superoxide dismutase) (41) but also required for the folding of newly imported Cpn60 (42). Thus, a decrease in the amount of available chaperonin, compounded with the fact that replenishment of native mt-Cpn60 requires pre-existing mt-Cpn60 to fold, is predicted to severely compromise the overall function of the mitochondria. In fact, a genetic disease state that results in a simple decrease in the amount of native chaperonin in the mitochondria appears to be lethal (43). In the third instance, Cpn60 also serves indirectly as an extracellular signaling protein. These less understood effects include its proinflammatory cytokine-like actions, its immunological cross-reactivity between bacterial and eukaryotic homologs that could lead to age-related inflammatory diseases (44), and the role of Hsp60 in the mechanism of apoptosis that is a pathogenic factor in cardiovascular disease (45). The high sensitivity of GroEL toward oxidation by HOCl suggests that GroEL oxidation may represent an effective pathway of bacterial killing by phagocytes. Moreover, HOCl-dependent oxidation of Hsp60 may accompany inflammatory processes during human pathologies. For the incubation of 17.5 μM GroEL (based on protein monomer) with up to 250 μM...
HOCl, the oxidation of Met to Met sulfoxide constituted the predominant mechanism of inactivation, indicated by ≥60% recovery of the lost activity through reaction with MsrB/A. GroEL Cys residues suffer oxidation during HOCl treatment. However, neither the formation of disulfides nor of stable sulfenic acids (CysSOH) contribute to protein inactivation, based on the inability of DTT alone to recover protein activity. Our mass spectrometric analysis of Cys oxidation products reveals that HOCl exposure generates predominantly cystic acid, and part of the loss of activity that is not recoverable through incubation with MsrB/A may be associated with this product. The reaction of HOCl with thiol can yield sulfenyl chloride (RSO2Cl), all these chlorides will eventually hydrolyze to the respective acids, sulfenic, sulfonic, or sulfonyl acid, where both sulfenic and sulfonic acid will ultimately oxidize to sulfonic acid (46). On the other hand, the reaction with nearby Lys residues could yield sulfenyl, sulfinyl, or sulfonyl amides, respectively (46). The latter cannot be reduced by disulfide-reducing agents. In contrast to HOCl, the exposure of GroEL to ONOO− generated no measurable yields of cystic acid. This difference in the oxidation products from Cys may have consequences for the oligomeric state of GroEL (see below). While some previous studies have indicated that the modification of single thiols in GroEL, particularly Cys138, can result in aberrant GroEL function (47), other studies report that thiols in GroEL are not absolutely critical for function (57, 58). In contrast, GroEL treated with 10 mM H2O2 retained significant activity (60%) in our study and retained full activity in studies of Hsp21, another stress-induced chaperone, converts 6 of its 8 Met residues to sulfoxides when subjected to high concentrations of H2O2 (7 mM for 2 h at 37 °C) (57). Even treatment with 1.5 mM H2O2 was sufficient to abolish activity (57, 58). In contrast, GroEL treated with 10 mM H2O2 retained significant activity (60%) in our study and retained full activity in studies by Wang et al. (59).

Of mechanistical interest is the oxidation of Met to Met sulfoxide by HOCl, which, to our knowledge, has not been documented elsewhere. While sulfoxide formation is often quoted as a theoretical possibility, few actual examples have been reported. For example, a peroxide-resistant mutant catalase of Proteus Mirabilis and its wild type both contain a Met sulfoxide close to the equivalent Cys positions in GroEL are replaced with serine (46). Of mechanistical interest is the oxidation of Met to Met sulfoxide by HOCl, which, to our knowledge, has not been documented elsewhere. While sulfoxide formation is often quoted as a theoretical possibility, few actual examples have been reported. For example, a peroxide-resistant mutant catalase of Proteus Mirabilis and its wild type both contain a Met sulfoxide close to

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**Fig. 6.** A–C, size exclusion chromatography indicating the effect of oxidation on the oligomeric state of GroEL. Oxidation reactions were carried out in buffer B containing 1 mg/ml GroEL (17.5 μM monomer) and various concentrations of oxidant. All reactions were carried out at room temperature for 30 min except for the incubation with H2O2, which was run for 3 h, after which catalase (final concentration: ~5 mM) was used to quench residual H2O2. For HOCl and ONOO−, a final concentration of 5 mM Met was used to terminate the reactions. Oxidant concentrations are as labeled. Size exclusion chromatography was run at a flow rate of 1 ml/min in 50 mM sodium phosphate, pH 7.5, 50 mM potassium chloride, 10 mM magnesium chloride, using a BIOSEP S-4000 column and detection at 280 nm.

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Brockeri and Karlin (49)). Amino acid analysis revealed that HOCl-treatment results in negligible yields of 3-chlorotyrosine (<0.1 mol/mol of GroEL). This may be rationalized by the kinetic preference of HOCl for Met (k = 3.8 × 107 M−1 s−1) over Tyr (k = 44 M−1 s−1) (50) and the higher abundance of Met (23 Met versus 7 Tyr residues in native GroEL). In addition, out of a total of 7 Tyr residues only one (Tyr328) is located within a Tyr-XXX-Lys-X motif (X = unreactive amino acid), where intermedialy chlorination of Lys (kHOCl-Lys = 5 × 103 M−1 s−1; Ref. 50) could promote the formation of 3-chlorotyrosine (51).

The functional importance of intact Met residues (here, for chaperonin activity) requires a repair system, which reduces Met sulfoxide to Met in vivo (52). In this paper, we used a bacterial enzyme (MsrB/A), which contains two separate domains specific for the reduction of Met-(S)-SO and Met-(R)-SO, respectively (31). Human tissue expresses two separate proteins, MsrA (40), specific for Met-(S)-SO (39, 53), and hCBS1, specific for Met-(R)-SO (54) (analogous to the bacterial MsrB (55)). Importantly, MsrA is targeted to the mitochondria consistent with an important role for the maintenance of reduced Met in this organelle (56). There are additional examples for chaperone-inactivation through Met oxidation. For example, Hsp21, another stress-induced chaperone, converts 6 of its 8 Met residues to sulfoxides when subjected to high concentrations of H2O2 (7 mM for 2 h at 37 °C) (57). Even treatment with 1.5 mM H2O2 was sufficient to abolish activity (57, 58). In contrast, GroEL treated with 10 mM H2O2 retained significant activity (60%) in our study and retained full activity in studies by Wang et al. (59).

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**Fig. 6.** A–C, size exclusion chromatography indicating the effect of oxidation on the oligomeric state of GroEL. Oxidation reactions were carried out in buffer B containing 1 mg/ml GroEL (17.5 μM monomer) and various concentrations of oxidant. All reactions were carried out at room temperature for 30 min except for the incubation with H2O2, which was run for 3 h, after which catalase (final concentration: ~5 mM) was used to quench residual H2O2. For HOCl and ONOO−, a final concentration of 5 mM Met was used to terminate the reactions. Oxidant concentrations are as labeled. Size exclusion chromatography was run at a flow rate of 1 ml/min in 50 mM sodium phosphate, pH 7.5, 50 mM potassium chloride, 10 mM magnesium chloride, using a BIOSEP S-4000 column and detection at 280 nm.

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their active sites (60). The mutation of 4 His residues from the iron coordination center of cytochrome c₅₅ leads to the accumulation of Met sulfones (61).

GroEL inactivation by HOCl quantitatively correlates with the conversion of oligomeric into monomeric GroEL (Fig. 6A) suggesting that the inactive form of HOCl-treated GroEL is monomeric. This is in contrast to ONOO⁻, where 0.25 mM oxidant does not cause any significant change in oligomeric structure (Fig. 6B), while ~30% of the activity is lost (Fig. 1A).

Oxidation of GroEL by HOCl and ONOO⁻ generates some inactive GroEL oligomer. This difference between HOCl and ONOO⁻ may be caused by the different nature of Cys oxidation products (no cysteic acid detected for ONOO⁻ up to 1.0 mM) and/or a differential selectivity for specific Met residues. For example, ONOO⁻ appears neither to react with Met¹¹¹ nor with Met¹¹⁴. In the tetradecameric structure of GroEL (Protein Data Bank file 1PCQ) these 2 Met residues are located at the interface between the two heptamers, and their oxidation by HOCl may promote the loss of oligomeric structure.

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Potential Role of Methionine Sulfoxide in the Inactivation of the Chaperone GroEL by Hypochlorous Acid (HOCl) and Peroxynitrite (ONOO⁻)
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