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OXIDIZED GroEL CAN FUNCTION AS A CHAPERONIN

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TABLE OF CONTENTS

1. ABSTRACT

Here, we report on the facilitated reactivation (85%) of oxidatively inactivated rhodanese by an oxidized form of the molecular chaperone GroEL (ox-GroEL). Reactivation by ox-GroEL required a reductant, and the enzyme substrate, sodium thiosulfate. Also, we found that ox-GroEL formed a complex with oxidatively inactivated rhodanese as shown by differential centrifugation and fluorescence spectroscopy. Ox-GroEL was obtained upon incubation of native GroEL for 16 h with 5 mM hydrogen peroxide. Under these conditions, GroEL was shown to retain its quaternary and secondary structures, but it displayed an increased exposure of hydrophobic surfaces as detected with 1,1'-bis(4-anilino) naphthalene-5,5'-disulfonic acid (bisANS) fluorescence. Additionally, ox-GroEL was significantly more sensitive towards proteolysis with trypsin compared to the native form of the protein. The oxidatively inactivated form of rhodanese, also had an increased exposure of hydrophobic surfaces, as previously reported. Thus, the proteins binding appeared to be mediated by hydrophobic interactions. Unlike in prior reactivation studies that involved native GroEL or alpha-crystallin, we have clearly shown that an oxidized form of GroEL can function as a molecular chaperone in the reactivation of oxidatively inactivated rhodanese suggesting that GroEL retains the ability to protect proteins during oxidative stress.

2. INTRODUCTION

The chaperonins for E.coli known as GroE proteins are represented by GroEL and GroES, a subclass of molecular chaperones, and are known to facilitate the folding of many polypeptides (1,2). GroEL was found to assist the refolding of chemically-denatured rhodanese (3,4) by stabilizing incompletely folded intermediates through the formation of a binary complex with those species (5). It has been suggested that exposed hydrophobic surfaces in folding proteins are likely to be involved in the interaction with GroEL (6). This was further supported by the detection of hydrophobic surfaces on GroEL (3), the observed correlation of higher affinity to the chaperonin by peptides with increasing hydrophobicity (7), and calorimetric studies demonstrating that hydrophobic interactions are an important driving force for the association of substrate proteins with GroEL (8). Increases in the number of hydrophobic surfaces have been

previously reported upon oxidation of protein (9). Although, it has been reported that Hsp33, alpha-crystallin, and Hsp90 can function as molecular chaperones under the conditions of oxidative stress (10-12), there are no reports concerning GroEL's performance under such conditions. For studying the structural and functional properties of those proteins, under oxidative stress, relatively large hydrogen peroxide (H_2O_2) concentrations (5-150 mM) have been employed (13-15).

Rhodanese, inactivated with H_2O_2, could be reactivated in the presence of a reductant and the substrate thiosulfate if these were added soon after inactivation provided the oxidant was also removed (16). We had previously reported that hydrophobic interactions were needed for alpha-crystallin and native GroEL to interact transiently with oxidatively-inactivated rhodanese facilitating its reactivation (17,18). We hypothesized that more exposed hydrophobic surfaces on GroEL were needed for the formation of a complex with rhodanese, since the hydrophobic exposure displayed on rhodanese, upon incubation with H_2O_2, was not sufficient for complex formation. We observed that upon incubation with moderate concentrations of H_2O_2, GroEL had an increased amount of hydrophobic surfaces, which we hypothesized, could lead to a stronger interaction with H_2O_2-inactivated rhodanese leading to the formation of a complex. Thus, these two proteins comprised an ideal protein model for investigating the interaction of GroEL with other proteins under oxidative conditions similar to those employed for other molecular chaperones (13-15).

In the present study, the facilitated reactivation (85%) of H_2O_2-inactivated rhodanese by an oxidized form of GroEL (ox-GroEL) is reported. Reactivation still required a reductant and the enzyme substrate, but not GroES or adenosine triphosphate (ATP). Without ox-GroEL, but in the presence of the reductant and thiosulfate, the inactivated enzyme regained about 51% of its original activity. Also, we found, that ox-GroEL formed a complex with H_2O_2-inactivated rhodanese. Ox-GroEL was obtained upon incubation of native GroEL for 16 h with a moderate concentration (5 mM) of H_2O_2. Under these conditions, GroEL underwent structural changes, without disruption of its quaternary and secondary structures, that led to an increased exposure of hydrophobic surfaces as detected with bisANS fluorescence. Additionally, this form of GroEL was significantly more sensitive towards proteolysis with trypsin compared to the native form of the protein. The oxidatively inactivated form of rhodanese, also had an increased exposure of hydrophobic surfaces, as previously reported. Thus, the binding of these proteins appeared to be mediated by hydrophobic interactions. This is the first report in which an oxidized form of GroEL (ox-GroEL) is shown to function as a molecular chaperone.

3. MATERIALS AND METHODS

3.1. Reagents and Proteins

Rhodanese from bovine liver was prepared as previously described (19) and stored at -70 ^{o}C as a crystalline suspension in 1.8 M ammonium sulfate. Rhodanese concentration was determined spectrophotometrically using a molecular mass of 33 kDa (20) and an absorption coefficient of 57,750 $M^{-1}cm^{-1}$. The chaperonins, GroEL and GroES were purified as described (21) from lysates of *E.coli* cells bearing the multicopy plasmid pGroESL (22) (which was kindly provided by Dr. Carl Frieden). With this purification method, the contaminating fluorescent species of GroEL were completely removed. After purification, the chaperonins were dialyzed against 50 mM Tris-HCl, pH 7.8, and 1 mM dithiothreitol. Glycerol was added at a final concentration of 10% (v/v) and the proteins were rapidly frozen in liquid nitrogen and stored at –70 ^{o}C. Prior to their utilization, the chaperonins were dialyzed against 50 mM Tris-HCl, pH 7.8 and kept at 4 ^{o}C. The protomer concentrations of GroEL and GroES were estimated by their absorbance at 280 nm using an extinction coefficient of 12,200 $M^{-1}cm^{-1}$ for GroEL (23), and 3,440 $M^{-1}cm^{-1}$ for GroES (24), and molecular masses of 57,259 Da and 10,368 Da, respectively (25). All the reagents used were of analytical grade unless stated otherwise. A fresh bottle of 3% H_2O_2 (Longs Drugs) was used in each experiment.

3.2. Oxidation of GroEL with H_2O_2

Unless indicated otherwise, GroEL (5.83 uM; monomers) was incubated for 16 h with 5 mM H_2O_2 in 50 mM Tris-HCl, pH 7.8 at 37 ^{o}C. Excess oxidant was removed from the protein by dialysis into 50 mM Tris-HCl, pH 7.8, followed by centrifugation on a bio-spin column from Bio-Rad.

3.3. Inactivation of Rhodanese with H_2O_2

Rhodanese (5.45 uM) in 120 mM sodium phosphate, pH 7.5, was incubated with a 10-fold molar excess of KCN and H_2O_2 at 25 ^{o}C, as previously described (18). The excess oxidant and KCN was removed from the enzyme by centrifugation with a bio-spin column from Bio-Rad.

3.4. Detection of Hydrophobic Surfaces on ox-GroEL

Exposed hydrophobic surfaces on native GroEL (control) or in GroEL (5.83 uM; monomers) incubated with H_2O_2, as indicated in the figure legends, was detected with bisANS (10 μM) in 50 mM Tris-HCl pH 7.8, and their fluorescence spectra measured separately at 25 ^{o}C. The bisANS fluorescence emission spectra of all the samples were obtained by using an excitation wavelength of 394 nm. Fluorescence emission was recorded between 430 and 600 nm using an SLM Aminco Series 2 Chemiluminescence Spectrophotometer. The appropriate blanks were subtracted.

3.5. Complex Formation Between ox-GroEL and H_2O_2-Inactivated Rhodanese

Rhodanese (109 nM) that had been previously inactivated with H_2O_2 was mixed with either native GroEL or ox-GroEL in 50 mM Tris-HCl, pH 7.8, at a 1:1 molar stoichiometric ratio of rhodanese to GroEL, and incubated for 15 min at 25 ^{o}C. The solution was made 5 mg/ml in dextran by adding 50 μL of 25 mg/mL dextran in a final volume of 250 μL. A 200-uL sample was centrifuged in an

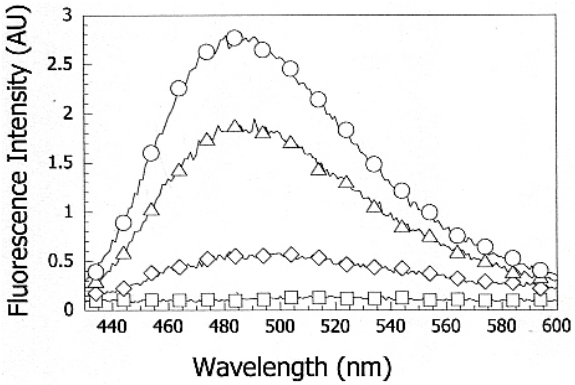

Figure 1. Fluorescence emission spectra of bisANS in the presence of GroEL or ox-GroEL. BisANS (10 uM) was incubated with GroEL (5.83 uM; monomers) (triangles) or with 5 mM H_2O_2 (circles) in 50 mM Tris-HCl, pH 7.8 in a final volume of 500 uL. The fluorescence spectra of GroEL alone (squares) or bisANS alone (diamonds) in 50 mM Tris-HCl are shown. The excitation wavelength for bisANS was taken at 394 nm and fluorescence spectra were recorded between 430-600 nm.

Optima Ultracentrifuge Beckman using a TLA-100 rotor for 19 minutes at 100,000 rpm (436,000g). After centrifugation, the sample was fractionated into four equal aliquots, which were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli system (26).

3.6. Reactivation of H_2O_2-Inactivated Rhodanese with ox-GroEL

After centrifugation of the mixtures of rhodanese (109 nM) and GroEL (at the same molar ratios described above), bottom fractions (fraction # 4 of 50 uL) of each sample were supplemented with 50 mM sodium thiosulfate, and 200 mM beta-mercaptoethanol (BME), and incubated at 25 °C. The rhodanese activity for the samples was determined every 30 min (up to 1.5 h) using a discontinuous method that quantifies the product, thiocyanate, based on the absorption of the ferric thiocyanate complex (19). Additionally, rhodanese activity was measured, separately, from samples that were similarly prepared, but without centrifugation. An equal amount of rhodanese without inactivation with H_2O_2 was used as a control, and rhodanese inactivated with H_2O_2 in the absence of ox-GroEL was used as a blank for calculating the percent reactivation.

3.7. Tryptophan Fluorescence

The intrinsic fluorescence emission spectra of rhodanese and ox-GroEL-rhodanese complex were obtained by using an excitation wavelength of 295 nm. Fluorescence emission was recorded between 310 and 370 nm using an SLM Aminco Series 2 Chemiluminescence Spectrophotometer. The appropriate blanks were subtracted.

3.8. Limited Proteolysis of Native and ox-GroEL

Samples of native GroEL or ox-GroEL were incubated at 37 °C with trypsin at a 40/1 (w/w) ratio of GroEL/trypsin in a 200 uL volume, and the reaction monitored by withdrawing 30 uL-fractions at 0, 10, 20, 30, 45, and 60 min as previously reported (27). The reaction of trypsin was stopped by adding, phenylmethylsulfonyl fluoride (PMSF) (in methanol) to a final concentration of 3.5 mM. The samples were then incubated for 10 min, at 25 °C, supplemented with SDS-gel loading buffer, and heated for 4 min, at 100 °C. Analysis of the digested material was carried out in SDS-containing 12% polyacrylamide gels (26). Gels were Coomassie-stained.

3.9. Circular Dichroism Spectroscopy

Circular dichroism measurements were made using an Aviv 62DS spectrophotometer at 37 °C. The spectra shown are the average of 5 scans. Buffer blanks were subtracted from the corresponding samples. Ellipticity is expressed as degrees cm^2 $(dmol\ amino\ acid)^{-1}$.

4. RESULTS AND DISCUSSION

4.1. Effect of H_2O_2 on GroEL Hydrophobicity

Changes in protein conformation induced by oxidation are often associated with a change in surface hydrophobicity (9). As we previously reported (17,18), H_2O_2-inactivated rhodanese contains extensive exposed hydrophobic surfaces. To determine if incubation of GroEL with H_2O_2 also increases hydrophobic surfaces on the chaperonin, we incubated GroEL with increasing concentrations of the oxidant. The hydrophobicity of GroEL after incubation with H_2O_2 was measured by using the fluorescence probe, bisANS. This dye has a very weak fluorescence, which is significantly increased when it binds to proteins containing exposed hydrophobic surfaces (28). The results of these measurements are summarized in Figure 1, which shows that native GroEL alone does not display any fluorescence as expected (squares); while bisANS alone displayed a very weak fluorescence (diamonds), which was significantly increased when bisANS was added to GroEL (triangles) indicating a significant exposure of hydrophobic surfaces on GroEL. The fluorescence intensity of bisANS, however, was increased even more when this was added to a sample of GroEL that had been previously incubated with 5 mM H_2O_2 (circles) indicating a higher exposure of hydrophobic surfaces on this oxidized form of GroEL (ox-GroEL) compared to that of native GroEL.

4.2. Reactivation of H_2O_2-Inactivated Rhodanese by ox-GroEL

It was previously reported that GroEL requires hydrophobic surfaces for binding to its substrate polypeptides (4). Also, previously, we reported that native GroEL interacts with H_2O_2-inactivated rhodanese (18) without forming a complex, leading to the speculation that more hydrophobic surfaces were required for a stronger interaction. Therefore, we hypothesized that GroEL that had been previously incubated with 5 mM H_2O_2 which contained extensive hydrophobic surfaces, would interact more strongly with rhodanese that had also been exposed to the oxidant and which contained extensive hydrophobic surfaces too. This interaction, we hypothesized, would perhaps facilitate the reactivation of the H_2O_2–inactivated rhodanese.

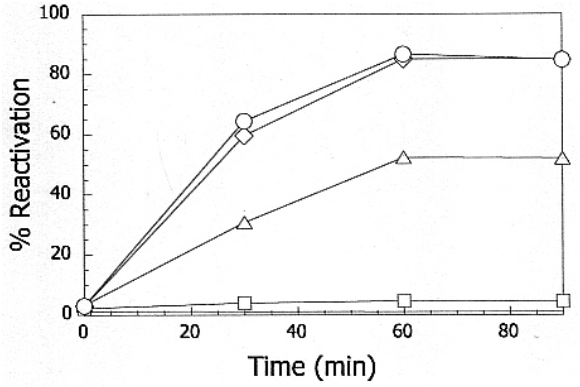

Figure 2. Time course of ox-GroEL-facilitated reactivation of H_2O_2-inactivated rhodanese. Rhodanese (5.45 uM) in 120 mM sodium phosphate, pH 7.5, was incubated with a 10-fold molar excess of KCN and H_2O_2 in a final volume of 100 uL uL at 25 ^oC. Excess KCN and H_2O_2 were removed and regain of rhodanese activity was determined every 30 min for up to 1.5 h. Reactivation was carried out in a Tris buffer (50 mM, pH 7.8) containing 200 mM BME, 50 mM sodium thiosulfate, the RF, and the absence (triangles) or the presence of 109 nM GroEL (circles) or 109 nM ox-GroEL (diamonds). Squares represent the reactivation of rhodanese in the absence of the BME, and the enzyme substrate, sodium thiosulfate. The final concentration of rhodanese in the reactivating reaction was 109 nM in a final volume of 300 uL. Rhodanese activity is expressed as a percentage of the activity of an equal quantity of native non-inactivated enzyme.

Figure 2 shows the reactivation of rhodanese that had been inactivated by exposure to H_2O_2. Previously, H_2O_2-inactivated rhodanese was shown to not be able to regain activity without a reductant and its substrate, sodium thiosulfate, even in the presence of GroEL (16-18). In agreement with these results, in the absence of the reductant, BME, and sodium thiosulfate, rhodanese reactivation was limited to only 4% after 90 min (squares). Also, as previously shown (18), the sample of rhodanese containing BME, and sodium thiosulfate, but without GroEL, had the ability to reactivate itself to approximately 51% after 60 min (triangles). Again, as it was previously shown (18), the sample of rhodanese, containing BME, sodium thiosulfate, and native GroEL had the ability to reactivate to about 85% after 60 min (circles). Interestingly, however, the oxidized form of GroEL (ox-GroEL) was also able to support the reactivation to a similar extent (diamonds).

Thus, the reactivation process observed here is different than that seen with chemically- (5) or thermally-inactivated rhodanese (29) in the presence of GroEL in which hydrolysis of ATP and GroES were required to discharge the bound enzyme from GroEL. We (18), as well as, other groups have recently reported (30-32) that GroEL alone could facilitate the reactivation or refolding of other proteins in the absence of GroES and/or ATP. Although, virtually the same yields of active rhodanese were obtained from H_2O_2-inactivated enzyme in the presence of native GroEL or ox-GroEL, it was not clear from these results if an ox-GroEL-rhodanese complex had formed during the reactivation of rhodanese assisted by ox-GroEL.

4.3. Ox-GroEL Forms a Complex with H_2O_2-Inactivated Rhodanese

The chaperonin-mediated refolding of chemically-denatured proteins is a process characterized by the formation of a binary complex between GroEL and a partially-folded polypeptide. The direct formation of a binary complex between GroEL and urea-denatured rhodanese was previously demonstrated by using high-speed centrifugation (5,18). Here, we utilized a similar assay to test the possible formation of a complex between ox-GroEL and H_2O_2-inactivated rhodanese. First, a sample containing native GroEL and H_2O_2-inactivated rhodanese was subjected to centrifugation under conditions in which only GroEL would completely sediment. Differential sedimentation of the two proteins can be achieved due to the large difference in their sedimentation coefficients: 23S (33) and 3S (5) for GroEL and rhodanese, respectively. After centrifugation of the sample, the tube was fractionated into four equal aliquots, each of which was analyzed by SDS-PAGE. Figure 3A shows that native GroEL (G) was found almost exclusively in the bottom fraction (lane 4 of the left panel); while rhodanese (R) was found distributed in all four fractions (Figure 3A, lanes 1-4 of left panel), as it was previously shown (18). However, when a sample containing ox-GroEL and H_2O_2-inactivated rhodanese was centrifuged under similar conditions, both GroEL (G) and rhodanese (R) were found in the bottom fraction (lane 4 of right panel). As it is shown in Figure 3A (lanes 1-3 of right panel), no rhodanese was found in the upper fractions (1-3), indicating that all rhodanese had co-sedimented and, therefore, was bound to GroEL. Overall, these results clearly show that the interaction of ox-GroEL with H_2O_2-inactivated rhodanese led to the formation of a binary complex that could be detected by differential centrifugation and which could be mediated by the significant exposed hydrophobic surfaces detected on H_2O_2-inactivated rhodanese (4,9,17-18), and on ox-GroEL. This is in contrast to the interaction between native GroEL or a-crystallin and H_2O_2–inactivated rhodanese, where the former proteins were observed to form a transient complex with rhodanese that could not be detected by differential centrifugation (17,18) as it was shown here for ox-GroEL. The complex detected here must be structurally different from that formed between native GroEL and chemically- or thermally-denatured rhodanese since it does not require GroES and ATP for the discharge of active rhodanese (4,5). Since, in the reactivation of H_2O_2-inactivated rhodanese by ox-GroEL observed here, the release of rhodanese from the complex occurred in the absence of the co-chaperonin GroES and/or ATP, it is possible that the enzyme could be released from ox-GroEL after being repaired and its folding induced by the reducing agent and its substrate until it eventually achieves its folded active state.

4.4. Characteristics of the Complex of ox-GroEL and H_2O_2-Inactivated Rhodanese

The intrinsic tryptophan fluorescence of rhodanese was used to detect changes in the enzyme tertiary structure during its interaction with GroEL. Figure

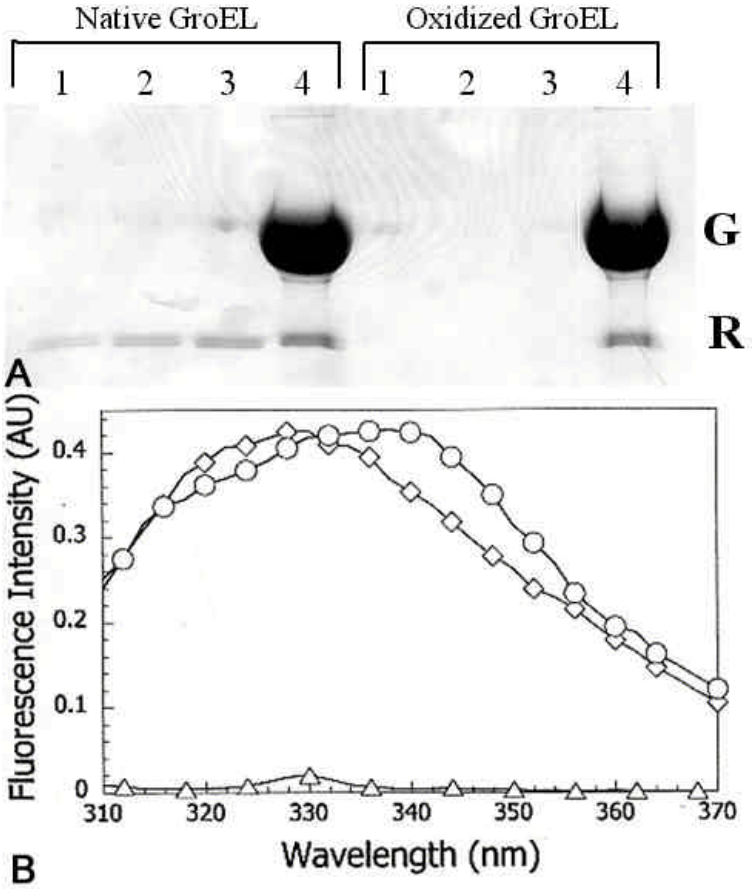

Figure 3. (A) Centrifugation of ox-GroEL and H_2O_2-inactivated rhodanese. H_2O_2-inactivated rhodanese (109 nM) was incubated for 15 min with ox-GroEL (109 nM; 14-mers) at 25 ^oC in a final volume of 250 uL. The mixture was treated, centrifuged, and fractionated as described under "MATERIALS AND METHODS". Aliquots were subjected to SDS-PAGE. Lanes 1-4 of the left panel correspond to the fractions 1-4 (from top to bottom, respectively) when H_2O_2-inactivated-rhodanese was incubated with native GroEL. Lanes 1-4 of the right panel correspond to fractions 1-4 (from top to bottom, respectively) when H_2O_2-inactivated-rhodanese was incubated with ox-GroEL under similar conditions. (B) Change in the tryptophans fluorescence emission spectrum of H_2O_2-inactivated rhodanese in the absence or presence of ox-GroEL. Rhodanese (5.45 uM) was inactivated with H_2O_2 as described in Fig. 2 and diluted into a buffer containing 50 mM Tris-HCl, pH 7.8 to a final concentration of 109 nM in a final volume of 500 uL. The sample was incubated at 25 ^oC and the fluorescence emission recorded (diamonds). The lower spectrum (triangles) corresponds to GroEL alone (109 nM; 14-mers). A similar spectrum was also obtained for the buffer sample. Circles corresponds to the sample of rhodanese (5.45 uM) inactivated with H_2O_2 bound to ox-GroEL in the form of a binary complex (109 nM; 14-mers) diluted into a buffer containing 50 mM Tris-HCl, pH 7.8 to a final concentration of 109 nM in a final volume of 500 uL. For all the samples, the excitation wavelength was taken at 295 nm and fluorescence spectra were recorded between 310-370 nm.

3B shows the fluorescence emission spectra of H_2O_2-inactivated rhodanese and ox-GroEL. The lower spectrum in Figure 3B corresponds to ox-GroEL (triangles), which exhibits negligible tryptophan fluorescence since this protein does not have any tryptophans. As previously reported (18), the fluorescence spectrum of rhodanese that was inactivated with H_2O_2 (diamonds) was virtually identical to that of untreated native rhodanese indicating that the tryptophan residues environment was not affected by H_2O_2 treatment (not shown). Interestingly, when H_2O_2-inactivated rhodanese was incubated with ox-GroEL, a significant red shift in the maximum fluorescence emission wavelength was observed (from 328 to 342 nm), indicating that ox-GroEL interacted with H_2O_2-inactivated rhodanese (Figure 3B, circles). Similar results were previously observed for the chemically-denatured rhodanese bound to native GroEL and for the complex of GroEL with unfolded creatine kinase (5,34). Previously, when we reported that native GroEL transiently interacted with H_2O_2-inactivated rhodanese, the tryptophan fluorescence intensity of rhodanese decreased with time (18). No change, however, in the fluorescence intensity was observed here with time, indicating that rhodanese readily bound to ox-GroEL.

4.5. Proteolysis of GroEL with Trypsin

As shown in Figure 1, the incubation of GroEL with H_2O_2 led to an increased exposure of hydrophobic surfaces. Here, H_2O_2–induced conformational changes of

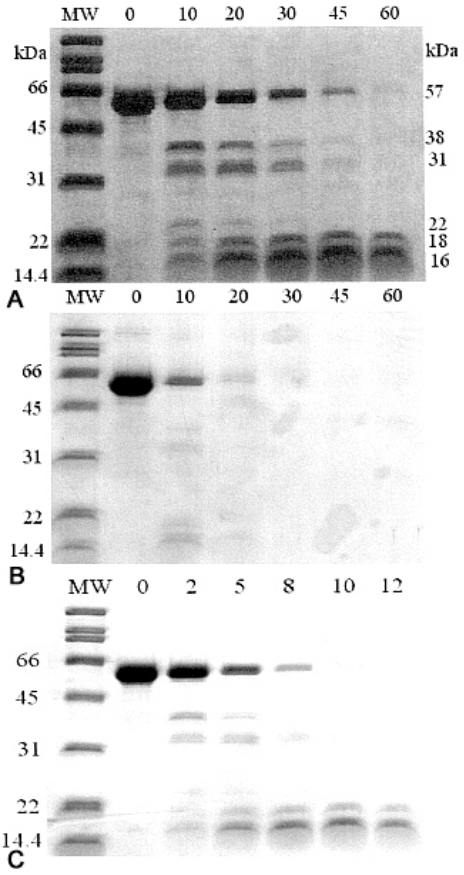

Figure 4. Time course of digestion of GroEL with trypsin. Native GroEL (5.83 uM; monomers) was incubated for 16 h without H_2O_2 and digested for up to 60 min (A), or with 5 mM H_2O_2 and digested for 60 min (B) or with 5 mM H_2O_2 and digested for up to 12 min (C) in 50 mM Tris-HCl, pH 7.8, and treated as described under "MATERIALS AND METHODS". The first lane (MW) corresponds to molecular mass standard proteins (Bio-Rad). Lanes 2-7 correspond to samples incubated with trypsin for the indicated times in min.

GroEL were studied by limited proteolysis in an attempt to characterize further the ox-GroEL. Figure 4 represents a 12% SDS-PAGE showing the time course for trypsin digestion of GroEL. As previously reported (27), digestion of native GroEL was complete after 60 min, and resulted in the formation of six fragments of 38, 32, 31.5, 22, 18, and 16 kDa (Figure 4, top panel). The band with a molecular mass of 28 kDa was found, by amino acid sequencing, to correspond to trypsin as previously reported (27). Proteolysis of ox-GroEL was also carried out to determine if conformational transitions, in the trypsin-sensitive regions of GroEL, could be induced by H_2O_2. GroEL was, therefore, incubated for 16 h with 5 mM H_2O_2 and digested under the same conditions used for native GroEL. As shown in the figure, a significantly higher rate of degradation of ox-GroEL was observed (Figure 4, middle panel). Digestion of ox-GroEL was repeated for shorter times so that a fragmentation pattern could be determined. As shown in the Figure 4 (bottom panel), digestion of ox-GroEL was virtually complete after only 10 min and the obtained digestion pattern was similar to that obtained for native GroEL. Therefore, our data indicate that significant structural changes in the trypsin-sensitive regions of GroEL occur as a result of the oxidation with H_2O_2, which are accompanied by an increased exposure of hydrophobic surfaces as shown above.

4.6. Effect of H_2O_2 on the Quaternary Structure of GroEL

Figure 5A represents a native gel showing the effects of increasing concentrations of H_2O_2 on the native structure of GroEL. As shown in Figure 5A (lanes 1-5), the native structure of GroEL remained intact upon incubation of the chaperonin for 16 h with H_2O_2 at concentrations of up to 5 mM. GroEL, however, began to dissociate after it was incubated for the same time but with higher concentrations of H_2O_2 (i.e., 7 or 8 mM, lanes 6 and 7, respectively), and then to completely lose its native structure (lane 8) when it was incubated with 10 mM H_2O_2. Analysis of the samples by SDS-PAGE showed that the integrity of the monomeric chain of GroEL was not affected by these oxidative conditions (not shown).

4.7. Effect of H_2O_2 on the Secondary Structure of GroEL

The effect of the exposure of GroEL to H_2O_2 on the secondary structure was determined by circular dichroism. As shown in Figure 5B, the circular dichroism spectra of native GroEL (circles) and of ox-GroEL (triangles) were virtually identical indicating that the secondary structure of GroEL remained intact under these conditions. The secondary structure of GroEL, however, was lost when it was incubated for 16 h with 10 mM or higher concentrations of H_2O_2 (not shown).

In summary, our results demonstrate that rhodanese inactivated with H_2O_2 containing exposed hydrophobic surfaces, interacted with ox-GroEL, which also contains significant exposed hydrophobic surfaces. Our results also show that ox-GroEL was able to perform its chaperonin function without the assistance of GroES and ATP by forming a complex with H_2O_2-inactivated rhodanese. The ability of ox-GroEL to efficiently refold H_2O_2-inactivated rhodanese is important in light of the fact that under *in vivo* conditions of oxidative stress, such as during inflammation and other diseases, a significant amount of peroxide is generated by neutrophils, breakdown of superoxide anion and ischemia-reperfusion injury (35) and thus many oxidatively modified proteins may actually exist in the conformations we have duplicated in this study. Although the concentrations needed to produce oxidized chaperonin are not physiological, the final product may well be. High amounts of oxidants were used to hasten the oxidation of the proteins studied. Using a similar non-physiological approach to study biomolecular interactions has many precedents. In a recent study of Hsp60, concentrations of 8 mM peroxide were used in cultured cells to accelerate reactions and show the protective effects of the chaperone (36). The increased amounts of cellular GroEL produced in response to elevated concentrations of H_2O_2 (37) might reflect and could be related to a peroxide

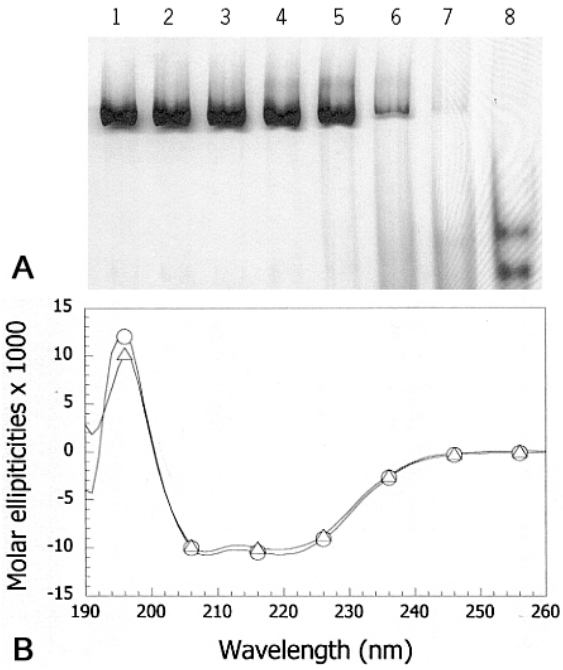

Figure 5. (A) Native gel electrophoresis of ox-GroEL. GroEL (5.83 uM; monomers) was incubated for 16 h in the absence (lane 1) and presence of 2, 3, 4, 5, 7, 8 and 10 mM H_2O_2 (lanes 2-8, respectively) at 37 ^oC. Excess H_2O_2 was removed by dialysis of the samples into 50 mM Tris-HCl, pH 7.8, followed by centrifugation on a bio-spin column from Bio-Rad. The samples were then analyzed by 5 % native gel electrophoresis, using the Laemmli system without SDS. The gels were stained with Coomassie Blue. (B) Circular dichroism spectra of GroEL. GroEL (30 uM; monomers) was incubated for 16 h without (circles) or with 5 mM H_2O_2 (triangles). Excess oxidant was removed as described above and circular dichroism spectra were recorded between 190 and 260 nm, as described under "MATERIALS AND METHODS".

incubated GroEL-facilitated reactivation of oxidatively-inactivated enzymes, such as that observed here for rhodanese.

5. ACKNOWLEDGMENTS

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