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Effect of Hydrogen Peroxide on the Activity and Structure of *Escherichia coli* Chaperone GroEL

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Abstract—Chaperone GroEL was treated with different concentrations of hydrogen peroxide. The conformational states of GroEL were monitored by protein intrinsic fluorescence, 8-anilino-1-naphthalene sulfonate fluorescence, and far-UV CD measurements. The results show that GroEL has unusual ability to resist oxidative stress. GroEL kept its quaternary structure and activity even when treated with 10 mM hydrogen peroxide. Two fragments were formed when GroEL was treated with high concentrations of hydrogen peroxide (more than 20 mM). It is suggested that GroEL, as a molecular chaperone, is related to oxidative process *in vivo*.

Key words: GroEL, hydrogen peroxide, structure, activity

Chaperone GroEL from *Escherichia coli* is an abundant and indispensable protein that assists folding of other proteins *in vitro* and *in vivo* [1, 2]. GroEL is a tetradecameric protein, consisting of two stacked rings with seven identical 57-kD subunits in each ring [3, 4]. A central cavity ~45 Å in diameter exists in each ring. Each subunit consists of three domains: apical, equatorial, and intermediate. The apical domain facing the channel shows a higher percentage of hydrophobic amino acid residues than the other domains and is presumed to bind directly to the substrate [5]. The equatorial domain, which provides most of the side-side contacts between subunits within the rings, primarily determines the GroEL assembly stability [6]. The intermediate domain connects the apical domain with the equatorial domain. GroEL protects non-native substrate proteins from irreversible aggregation and improves the final yield of productive folding in a co-chaperone GroES- and ATP-dependent manner [7, 8].

Recently, Zahn et al. [9] reported that the apical domain of GroEL shows high chaperone activity. Therefore, GroEL must have an intrinsic chaperone activity that is independent of the central cavity and its allosteric behavior. The apical domain that has chaperone activity is called a minichaperone [9]. The solved GroEL apical domain crystallographic structure shows that the polypeptides are bound by hydrophobic interactions and hydrogen bonds, and two core α-helices and their surrounding loops allow accommodation of a wide range of substrate proteins and protein conformations to the binding sites of the apical domains within the central cavity. But it has not been well understood how GroEL recognizes misfolded or unfolded proteins, folding intermediates, or native proteins.

The free radical hypothesis of aging postulates that the aging of cells is a consequence of the accumulation of oxidative damage caused by reactive oxygen species produced by normal metabolism [10, 11]. The native protein structure/folding has evolved to minimize the oxidative damage to proteins in a high oxidative potential environment. Oxidatively damaged proteins are more susceptible to proteolysis than their normal counterparts and it has been suggested that the carbonylation of amino acids, a tagging of aberrant proteins, serves to keep the macromolecular synthesis machinery of the cell free from mistranslated proteins [12]. Refolding with the help of GroEL is another pathway for misfolded proteins, GroEL must have more anti-oxidative ability. We confirmed that GroEL can indeed minimize the oxidative damage more effectively than common proteins and may be an important protector which keeps normal protein folding from being damaged by the oxidative environment.

Protein denaturation has been studied thoroughly [13-16], but few have investigated protein denaturation

Abbreviations: ANS) 8-anilino-1-naphthalene sulfonate; CK) creatine kinase.

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with hydrogen peroxide. In this paper, we report on the changes of conformation and activity of bacterial chaperone GroEL in the presence of different concentrations of hydrogen peroxide. The results suggest that GroEL resists oxidation at low concentrations of hydrogen peroxide, less than 10 mM. Higher hydrogen peroxide concentrations induce GroEL to break into two parts mainly.

MATERIALS AND METHODS

The chaperone GroEL was overexpressed in *E. coli* overproducing strain pOF39-JM101 provided by Dr. Jian-Guo Tang of the College of Life Science, Peking University. Chaperone GroEL was purified as described by Li [17].

The GroEL concentration was determined by measuring the absorption at 280 nm and using the absorption coefficient $A^{1\%}_{1cm} = 2.5$ [18]. Creatine kinase (CK) concentrations were determined using the absorption coefficient $A^{1\%}_{1cm} = 8.8$ [19]. GroEL was treated at 15°C with a solution containing different concentrations of hydrogen peroxide in 10 mM Tris-HCl (pH 7.5) for 12 h, the change of hydrogen peroxide concentration being less than 5% in 12 h. Denatured GroEL solutions were used for assays and for measurements of fluorescence, CD, activity, and gel electrophoresis. Catalase was used to remove the hydrogen peroxide in the solution before SDS-PAGE, native PAGE, and activity measurement because hydrogen peroxide will produce uncorrectable influences in these experiments. ATP was from Sigma (USA) and other chemicals were local products of analytical grade.

The intrinsic and 8-anilino-1-naphthalene sulfonate (ANS)-binding fluorescence signals were measured using a Hitachi 850 spectrofluorometer (Japan). The excitation wavelength for the intrinsic and ANS-binding fluorescence was 280 and 380 nm, respectively. The measured emission spectra of the intrinsic fluorescence were corrected spectra. A Jasco 500C CD spectrophotometer (Japan) was used for CD measurements in the far-UV region from 200 to 250 nm. The ATPase activity of GroEL was measured as described by Lill [20]. Nondenaturing gel electrophoresis was performed by the method of Neuhoff [21] using 6% polyacrylamide gel at constant 160 V for a 1-mm thick gel. Denaturing gel electrophoresis was performed using 12% polyacrylamide gel by the method of Laemmli [22]. As noted under "Results", both gels were stained with 0.05% Coomassie Brilliant Blue R-250, 25% alcohol, and 10% acetic acid.

Dilution of denatured CK caused aggregation. GroEL, being a molecular chaperone, can effectively prevent this aggregation. The aggregation prevention experiments monitored the light scattering intensity at 400 nm over time, using a Perkin Elmer Lambda Bio U/V spectrophotometer at 25°C. The cell diameter was 1 cm. All other measurements were carried out in 10 mM Tris-HCl buffer (pH 7.5) at 22°C.

RESULTS

Structural changes of GroEL in the presence of hydrogen peroxide. Hydrogen peroxide induced structural changes of GroEL. The data in Fig. 1 show the native and SDS gel fragments treated as described in "Materials and Methods". At low hydrogen peroxide concentrations, less than 10 mM, GroEL remained as a tetradecamer while at high hydrogen peroxide concentrations, higher than 10 mM, GroEL disassembled. With increasing hydrogen peroxide concentration, GroEL did not remain as a monomer, but broke into two fragments—a 27-28 kD

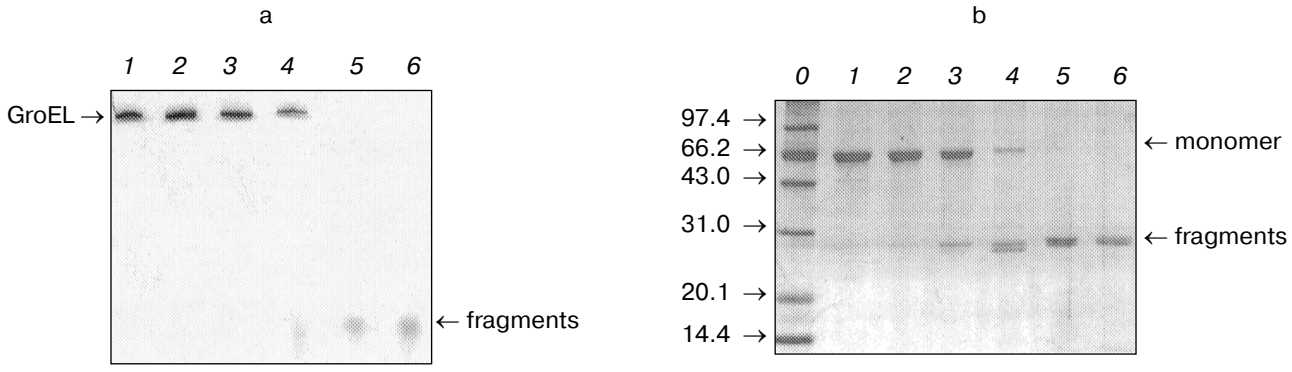

Fig. 1. Native and SDS gel electrophoresis of GroEL treated with various H_2O_2 concentrations. Chaperone GroEL was dissolved in 10 mM Tris-HCl buffer, pH 7.5, containing H_2O_2 at the desired concentrations. Before the samples were loaded, a trace amount of catalase was added to the solutions to decompose the remaining H_2O_2. a) Native PAGE (6% polyacrylamide gel) of the samples. The samples in the lanes from *1* to *6* were treated with H_2O_2 concentrations of 0, 5, 10, 20, 40, and 80 mM, respectively. Each lane contained 3 μg GroEL. b) SDS-PAGE (12% polyacrylamide gel) of the samples. The lane *0* contained a standard molecular marker, the other lanes (*1-6*) contained samples of GroEL treated with H_2O_2 at concentrations corresponding to those in Fig. 1a. Each lane contained 7 μg GroEL. Molecular masses of standard proteins are shown on the left in kD.

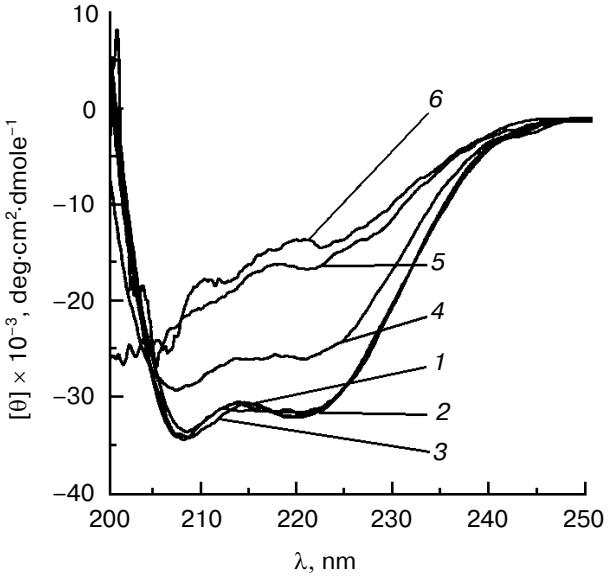

Fig. 2. Far-ultraviolet CD spectra of chaperone GroEL in solutions of different H_2O_2 concentrations. Experimental conditions were as for Fig. 1 except that no catalase was added to the solutions. The final GroEL concentration was 0.22 μM. The H_2O_2 concentrations for curves *1-6* were 0, 5, 10, 20, 40, and 80 mM, respectively.

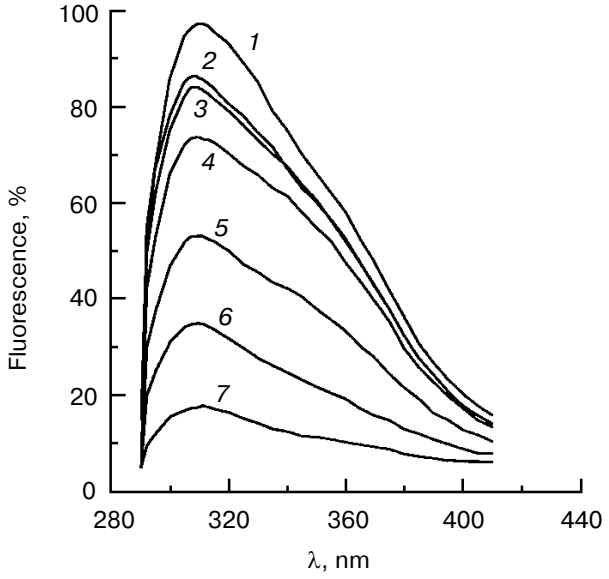

Fig. 3. Fluorescence emission spectra of chaperone GroEL in H_2O_2 solutions. Experimental conditions were as for Fig. 1. The final GroEL concentration was 0.22 μM. The H_2O_2 concentrations were 0 (*1*), 5 (*2*), 10 (*3*), 20 (*4*), 40 (*5*), 80 (*6*), and 160 (*7*) mM. The excitation wavelength was 280 nm.

fragment and a 29-30 kD fragment. Other small fragments may have also been produced which could not be detected by the methods used.

Figure 2 shows the CD spectra of chaperone GroEL for different H_2O_2 concentrations. There was no significant change of the secondary structure of GroEL at low hydrogen peroxide concentrations, less than 10 mM. However, the secondary structure content sharply decreased at higher hydrogen peroxide concentrations in accord with the disassembly of GroEL observed in the electrophoresis. Even at 80 mM H_2O_2, GroEL still retained some secondary structure.

The effect of the H_2O_2 concentration on the fluorescence emission spectra of GroEL was also studied. The data in Fig. 3 shows that increasing H_2O_2 concentrations caused the fluorescence emission intensity to decrease and the emission peak to red-shift 2-3 nm. A sharp decrease occurred between 20 and 40 mM H_2O_2 as the GroEL completely disassembled.

The ANS-binding fluorescence emission is known to increase when the dye binds to the hydrophobic regions of a protein [23]. In this work, the binding of ANS was used to monitor the hydrophobic surface exposure of GroEL in various H_2O_2 concentrations. The result in Fig. 4 shows a two-stage process. Below 10 mM H_2O_2, no marked changes occurred in the fluorescence intensity and peak position. Above 10 mM H_2O_2, increasing of hydrogen peroxide concentrations caused the fluorescence emission intensity of ANS bound by denatured GroEL to increase.

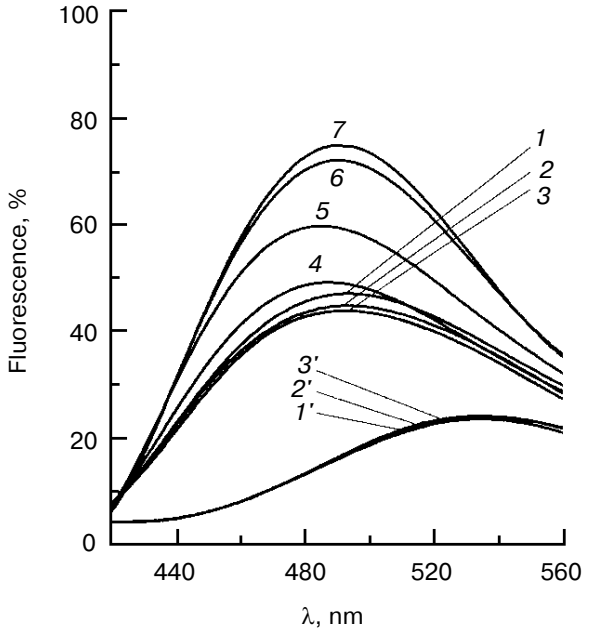

Fig. 4. ANS-binding characteristics of chaperone GroEL in H_2O_2 solutions. Experimental conditions were as for Fig. 1 except for the protein concentrations. The final concentrations of GroEL and ANS were 0.22 and 30 μM, respectively. The H_2O_2 concentrations for curves *1′-3′* (in the absence of GroEL) were 0, 20, and 40 mM and for curves *1-7* (in the presence of GroEL) were 0, 2.5, 10, 15, 20, 30, and 40 mM, respectively. The excitation wavelength was 380 nm.

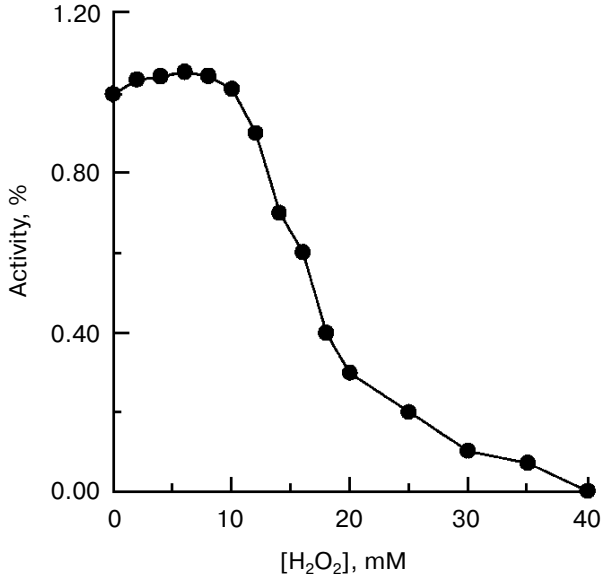

Fig. 5. Changes of ATPase activity of chaperone GroEL (in relative units) at different peroxide concentrations. The activity was monitored at 597 nm.

The activity changes of GroEL in hydrogen peroxide solutions. The changes of the ATPase activity of GroEL treated with different hydrogen peroxide concentrations, Fig. 5, showed that low hydrogen peroxide concentrations caused the ATPase activity to increase to a slight extent. Above 10 mM H_2O_2, the ATPase activity decreased sharply with increasing hydrogen peroxide concentration. When the concentration was further increased to 40 mM H_2O_2, the GroEL ATPase activity was completely lost.

The chaperone activity of GroEL treated with different hydrogen peroxide concentrations was measured using CK as the target protein (Fig. 6). Native GroEL strongly suppresses aggregation of creatine kinase (curve *2*). Low concentration of hydrogen peroxide (10 mM) has no marked effect on the GroEL chaperone activity (curve *3*). When treated with 40 mM peroxide, GroEL still retained 65% of its chaperone activity (curve *4*).

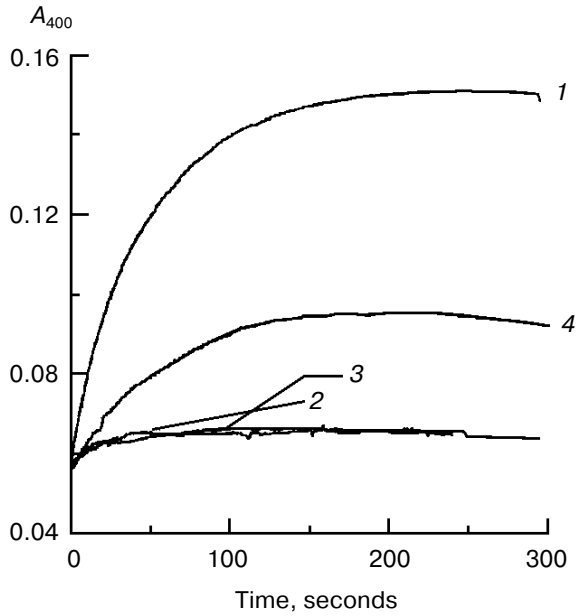

Fig. 6. Changes of chaperone activity of GroEL on hydrogen peroxide treatment. The experiments were performed at 25°C. The final concentrations of GroEL and CK are 0.5 and 2.5 μM, respectively. Curve *1* represents CK aggregation in the absence of GroEL. Curves *2-4* represent CK aggregation in the presence of GroEL treated with 0, 10, and 40 mM peroxide, respectively.

DISCUSSION

It is generally recognized that the accumulation of oxidative damage is the main reason for aging [10, 11]. Recent reports have shown that many kinds of prokaryotes, such as *Escherichia coli*, *Mycobacterium*, and *Francisella tularensis*, will increase syntheses of GroEL and its homologs when the cells are exposed to 5-15 mM hydrogen peroxide [24-26]. Figures 1-6 all show that the structure and activity of GroEL have no notable changes below 10 mM hydrogen peroxide which confirms that GroEL has a strong ability to protect itself from oxidative stress damage. These results suggest that high level overexpression of GroEL can help protect essential proteins *in vivo* in the presence of oxidative stress. And its antioxidative ability is decided by its function to protect other essential proteins.

With increasing hydrogen peroxide concentration, GroEL did not degrade into many fragments like other proteins, but formed two main fragments. Judging from its X-ray crystallographic structure [3], the two fragments may represent the apical domain and the equatorial domain respectively. Because the intermediate domain has the least secondary structure of the three domains, its peptide chain is more easily exposed to hydrogen peroxide than the other two domains. Comparing the native PAGE results with the SDS-PAGE results shows that the disassembly of GroEL and the breaking of its subunits happened in the same hydrogen peroxide concentration range. The whole process is shown as a scheme:

$$\textbf{Tetradecamer} \xrightarrow{\text{Disassembly}} \textbf{Monomer} \xrightarrow{\text{Oxidative breakage}} \textbf{Two fragments}$$

The external surface of GroEL is composed of amino acids whose groups are not easily damaged by oxidative stress. When the hydrogen peroxide concentration was high enough to break the interaction between the GroEL subunits, the inner GroEL surface was exposed and easily damaged, so the subunits could not exist together. It is

generally recognized that at low concentrations, hydrogen peroxide has three effects on protein: oxidative modification of some amino acids, changes of the quality of the hydrophobic groups of the protein, exposure of the hydrophobic surface and covalent cross-linking, mainly as the formation of disulfide bonds [27-31]. The formation of a disulfide bridge may inactivate the oxidized protein [32]. As hydrogen peroxide concentration increases, the reductive groups in GroEL were oxidized gradually. Since the amino acid mass generally increases when its groups are oxidized, the bands on SDS-PAGE that represent subunits shifted up slightly with increasing hydrogen peroxide concentration. GroEL maintained its whole quaternary structure in 10 mM hydrogen peroxide because the most important amino acids that involved the interactions of subunits and rings of GroEL are protected by its peripheral loop. For example Cys458, an important amino acid in the assembly of GroEL, is buried into the pocket locked by fragment Asn475-Asn487 [33].

Figure 3 shows that the peak position did not obviously shift but the fluorescence intensity decreased greatly with increasing hydrogen peroxide concentration. It is due to a change in the local environment of the loop containing Trp, with little influence on the main protein folding [32]. All these results show that oxidation of GroEL by low hydrogen peroxide concentrations causes limited changes in the secondary structure arrangement of the three domain cores so that GroEL still retains its normal functions. It is generally recognized that the oxidative modification of sulfhydryl group is related with the ATPase activity [34] and it has been reported that the binding and hydrolysis of ATP could involve, but not necessarily, all three Cys in each GroEL subunit [35, 36]. Crystallographic structure showed that the ATP binding site is located on top of the equatorial domain facing towards the central cavity [37]. Therefore, some unknown mechanism protects the central cavity from oxidative damaged but allows ATP to easily bind into the cavity.

It is thought that the aggregation of the renaturing protein is due to collision frequencies and hydrophobic interactions that drive the association of partially folded species [38]. Although oxidized GroEL cannot renature, it did not aggregate in our experiments. And interestingly, GroEL treated with 40 mM hydrogen peroxide still retained 60% activity of prevention of aggregation. Many recent papers have reported that the GroEL apical domain holds a part of chaperone activity and is, therefore, called a "minichaperone", it is generally thought that the polypeptide binding site of the GroEL apical domain interacts with folding intermediate to protect the hydrophobic surface of the folding intermediate [9, 39]. Figures 3 and 4 show that the apical domain structure has been damaged so that it does not retain its entire secondary structure. So it is likely that the aggregation prevention mechanism is similar to the effect of an "artificial chaperone". The fragments function by transient association with the non-native protein, which selectively diminishes the intermolecular attraction between peptide fragments that leads to aggregation, relative to the intramolecular attraction that drives folding [40-42].

The oxidative process is an important process with GroEL as an important protein involved in many processes. Although the structure of GroEL has been thoroughly researched, its function *in vivo* is not yet completely known. The present work has presented some evidence for the relation between GroEL and oxidation *in vivo*.

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