Redox-regulated chaperone function and conformational changes of *Escherichia coli* Hsp33

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Abstract We have studied the chaperone activity and conformation of *Escherichia coli* heat shock protein (Hsp)33, whose activity is known to be switched on by oxidative conditions. While oxidized Hsp33 completely prevents the heat-induced aggregation of ζ-crystallin at 42°C at a ratio of 1:1 (w/w), the reduced form exhibits only a marginal effect on the aggregation. Far UV–circular dichroism (CD) spectra show that reduced Hsp33 contains a significant α-helical component. Oxidation results in significant changes in the far UV–CD spectrum. Near UV–CD spectra show changes in tertiary structural packing upon oxidation. Polarity-sensitive fluorescent probes report enhanced hydrophobic surfaces in the oxidized Hsp33. Our studies show that the oxidative activation of the chaperone function of Hsp33 involves observable conformational changes accompanying increased exposure of hydrophobic pockets.

Key words: Molecular chaperone; Heat shock protein 33; Oxidative activation; Conformation; Hydrophobic surface

1. Introduction

Molecular chaperones are a class of proteins, which are important in cellular functions including correct folding and assembly of proteins [1–4]. A common property that molecular chaperones possess is their ability to differentiate between native and non-native partially folded/unfolded proteins: they selectively bind and stabilize partially folded/unfolded proteins, which are otherwise prone to aggregation [5–7]. Some prokaryotic and eukaryotic heat shock proteins (Hsps) whose chaperone functions have been the subject of extensive studies include the members of the Hsp60 (GroEL), Hsp70 (DnaK), Hsp90, Hsp100 and small Hsp families [1–7]. The up-regulation of these proteins under stress is rather well established. However, modulation of their function by post-translational events is not well understood. Temperature appears to act as one such factor, as the activities of some chaperones are enhanced under non-permissible temperatures. Mammalian Hsp70 has been shown to exhibit enhanced chaperone activity above 40°C involving a conformational change [8,9]. We have shown that the eye lens α-crystallin, a member of small Hsp family, exhibits temperature-dependent chaperone-like activity involving a structural change that leads to several fold enhanced activity [10–13]. Hsp90 has chaperone activity, which is latent under normal conditions but is induced by heating (43°C) to bind substrates and prevents their aggregation [14]. In some cases, the chaperone function of the GroEL/ES machinery has been shown to be modulated by temperature [15,16].

Until recently, no molecular chaperone was known to be turned 'ON' or 'OFF' by a post-translational event acting as a switch. Jakob et al. [17] have shown that a member of highly conserved cytoplasmic prokaryotic molecular chaperones, Hsp33, one among the 26 identified heat-inducible bacterial gene products whose functions are not known so far [18], is regulated by the redox state of the medium. This class of proteins contains a conserved motif, $CXC(X)_{27-30}$ CYZC, where the cysteine residues are in their reduced form and coordinated with a single Zn^{2+} ion in the environment of a high reduction potential of cytosol [17,19]. The reduced form of Hsp33 does not exhibit chaperone activity. The high affinity zinc coordination (K_a of the order of 10^{17} M^{-1}) is believed to assist fast and efficient oxidation of the thiols under stress [19]. Under oxidative conditions, these conserved cysteines get oxidized to form intramolecular disulfide bonds (between C232 and C234; C265 and C268), which leads to activation of the molecule [20]. Both the reduced and oxidized forms exist as monomer [20]. Oxidized Hsp33 (O-Hsp33) has been shown to exhibit chaperone-like activity in preventing heat-induced aggregation of citrate synthase and luciferase [17] as well as the refolding-induced aggregation of luciferase [20] in vitro. It has been shown to protect cell viability from oxidative stress [17].

Thus, gain of function by disulfide formation appears to be one way of responding to cellular stress [21]. However, the molecular mechanism of oxidative activation of the chaperone Hsp33 is not yet clear. The structure of the molecule per se, as well as structural changes, if any, accompanying the oxidative activation of Hsp33 are not known. In order to understand these aspects, we have cloned, overexpressed and purified Hsp33 from *Escherichia coli*, studied its chaperone activity, and investigated its structure in the inactive, reduced form and the active, oxidized form. Our results clearly demonstrate that conformational changes leading to increased exposure of hydrophobic surfaces are involved in the oxidative activation of Hsp33.

2. Materials and methods

2.1. Materials

1,1′-bi(4-anilino)Naphthalene-5,5′-disulfonic acid (bis-ANS), Nile

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Red, dithiothreitol (DTT), reduced and oxidized glutathione and isopropyl-β-D-thiogalactopyranoside were purchased from Sigma Chemical Co., USA. 8-Anilinonaphthalene-1-sulfonic acid (ANS) was obtained from Aldrich Chemical Co., USA. *Pfu* DNA polymerase was purchased from Stratagene, USA. T-vector pCR2.1 was purchased from Invitrogen, USA. Expression vector pET21a was purchased from Novagen, USA. Pre-packed PD-10 desalting columns and Sephadex G-75 were obtained from AP Biotech., USA. Restriction enzymes were procured from New England Biolabs, UK. Hydrogen peroxide and other chemicals used in the study were of analytical grade.

2.2. Cloning of E. coli Hsp33

The Hsp33 coding region from the yrfI gene (section 305 of the *E. coli* genome [22]) was PCR-amplified from the *E. coli* genomic DNA using *Pfu* DNA polymerase. A 986 bp product containing the Hsp33 cDNA was amplified using 5′-d(CTGTCACCTGCAAGACATATGATTATGCC)-3′ as forward and 5′-d(GAATGTGAAGCTTAATGAGTGACAAAGCG)-3′ as reverse primer. The forward primer binds at the start codon and has an engineered *Nde*I site and the reverse primer, with a *Hin*dIII site, binds 201 bp downstream the stop codon. The amplified product was cloned into a T-vector pCR2.1 to generate pCR2.1-Hsp33 plasmid.

2.3. Sequencing and subcloning

Sequencing was done with M13 forward and reverse primers using the Big dye™ terminator cycle sequencing kit (Perkin-Elmer) in an Applied Biosystems 9600 automated DNA sequencer. The coding region of Hsp33 was found to be mutationless. The coding region of Hsp33 was excised from pCR2.1 after digestion with *Nde*I and *Hin*dIII and ligated to *Nde*I–*Hin*dIII linearized expression vector pET21a to produce pET21a-Hsp33.

2.4. Overexpression and purification

The expression plasmid pET21a-Hsp33 was transformed into competent *E. coli* BL21(DE3) cells. A 1% inoculum of an overnight culture of the transformed cells was subcultured into 1 l of Luria Bertani medium containing ampicillin (100 μg/ml) and 0.5 mM zinc sulfate. The culture was grown at 37°C with vigorous shaking at 250 rpm and was induced with 1 mM isopropyl-β-D-thiogalactopyranoside when the optical density reached midlog phase (0.7 at 600 nm). After 3 h of induction, the cells were harvested by centrifugation at 6000 rpm for 10 min. The harvested cells were stored at −70°C until further processing.

The cell pellet was resuspended in 50 mM Tris–HCl buffer pH 7.4 containing 100 mM NaCl and 0.2 mM $ZnSO_4$ (Buffer A) and lysed by two cycles at 12 000 psi on a SLM Aminco french pressure cell press. Cell debris was removed by centrifugation at 12 000 rpm for 30 min at 4°C. The supernatant was loaded on to a Sephadex G-75 gel filtration column. The fractions containing Hsp33 were pooled and loaded on to Mono Q FPLC anion exchange column (AP Biotech.). The protein was found to elute at around 350 mM NaCl in a gradient of 0–2 M NaCl. The eluted protein was found to be highly homogeneous and was desalted and concentrated by ultrafiltration using an Amicon ultrafiltration unit. The protein concentration was estimated using the extinction coefficient at 280 nm of 0.54 for a 1 mg/ml solution [23]. The absorbance ratio of the preparation at 280 to 260 nm was found to be >1.8.

2.5. Preparation of O-Hsp33 and reduced Hsp33 (R-Hsp33)

O-Hsp33 was prepared essentially following the method described by Barbirz et al. [20]. Hsp33 (2 mg/ml) in 50 mM sodium bicarbonate (pH 8.0) was incubated in the presence of 4 mM hydrogen peroxide for 3 h at room temperature. We have also studied the glutathione redox system O-Hsp33. To prepare this form, Hsp33 (2 mg/ml) in 50 mM sodium bicarbonate (pH 8.0) was incubated in the presence of 2 mM GSSG and 0.5 mM GSH (equilibrium constant $K=[GSH]^2/[GSSG]=0.125$) for 3 h. Under this redox condition, Hsp33 is known to exist in the fully oxidized form [17]. The oxidized samples were then loaded on to a pre-packed PD-10 desalting column equilibrated with buffer A. The void volume fractions containing the protein were pooled and concentrated using a Centricon unit with 10 kDa cut off filter (Amicon). Both the hydrogen peroxide-oxidized and GSSG-oxidized forms exhibit almost the same properties. The data shown in this paper are pertaining to the hydrogen peroxide O-Hsp33 as prepared above.

To obtain R-Hsp33, the protein in buffer A was incubated with DTT at room temperature for 1 h. At low concentration of the protein (0.1 mg/ml) used for fluorescence study and chaperone assay, 1 mM DTT was present in the buffer. At higher concentrations of the protein (1.5 or 3 mg/ml) used for the circular dichroism (CD) study, 10 mM DTT was present in the buffer.

2.6. Chaperone assay

ζ-Crystallin from guinea pig eye lens was isolated and purified as described by Rao et al. [24] except that the isolation buffer did not contain β-mercaptoethanol. ζ-Crystallin (0.1 mg/ml), in buffer A in the absence and in the presence of 0.1 mg/ml Hsp33, was placed in the cuvette compartment of the Hitachi F-4000 fluorescence spectrophotometer thermostated at 42°C using a Julabo circulating waterbath. The temperature inside the cuvette (42°C) was measured by Physitemp type-T microthermocouple thermometer. The aggregation of the protein sample was monitored as a function of time by light scattering with the excitation and emission wavelengths set at 360 nm. The excitation and emission band passes were set at 1.5 nm.

2.7. CD spectroscopy

The CD spectra of Hsp33 were recorded using a Jasco J-715 spectropolarimeter. All spectra shown are the average of six accumulations. To record far UV–CD spectra, a 1.5 mg/ml solution of the protein in buffer A was used in a 0.01 cm path length cell. Near UV–CD spectra were recorded using a 3 mg/ml solution of the protein in the above-mentioned buffer in a 1 cm path length cell. All spectra were corrected for the appropriate blanks.

2.8. Fluorescence spectroscopy

To record the tryptophan fluorescence spectra of Hsp33, 0.1 mg/ml solution of the reduced or oxidized protein was taken in buffer A and the sample was excited by 295 nm light. A Hitachi F-4000 fluores-

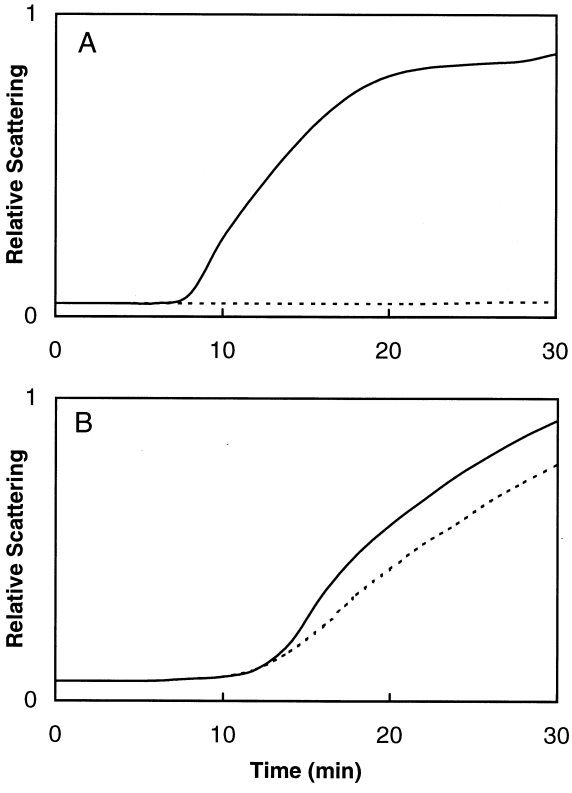

Fig. 1. Chaperone activity of oxidized (A) and reduced (B) Hsp33 towards heat-induced aggregation of ζ-crystallin. The figure shows the aggregation of ζ-crystallin (0.1 mg/ml) as monitored by light scattering at 360 nm as a function of time in the absence (—) and in the presence of 0.1 mg/ml Hsp33 (- - - - - -). A 50 mM Tris–HCl buffer (pH 7.4) containing 100 mM NaCl was used. In the case of R-Hsp33 (B), the buffer also contained 1 mM DTT.

cence spectrophotometer was used. To probe the hydrophobic surfaces of the protein, polarity-sensitive fluorescent probes such as bis-ANS, ANS and Nile Red were used. Stock solutions of bis-ANS and ANS were prepared in methanol. Nile Red was dissolved in dimethyl sulfoxide. The probes of these stock solutions were added to the Hsp33 samples in buffer A such that the final concentration of the organic solvents never exceeded 1%. The final concentrations of the probes, excitation wavelengths and other details are furnished in the figure legends.

3. Results and discussion

In order to investigate the molecular mechanism of the redox-regulated chaperone activity of Hsp33, we have cloned the hsp33 gene and overexpressed the protein in *E. coli* (see Sections 2.2–2.4 for details). The purified Hsp33, in its reduced and oxidized forms, is studied for its chaperone activity towards heat-induced aggregation of ζ-crystallin, a quinone oxido-reductase [24] from guinea pig eye lens. This protein, in the absence of its cofactor, NADPH, aggregates at 42°C, a temperature close to the heat shock temperature and, therefore, serves as a good target protein for the chaperone assay. Fig. 1A shows the heat-induced aggregation of ζ-crystallin in the absence and in the presence (at 1:1 w/w ratio) of O-Hsp33 as monitored by light scattering at 360 nm. Aggregation of ζ-crystallin is completely prevented by O-Hsp33. The aggregation profile of ζ-crystallin is altered in the presence of 1 mM DTT and this aggregation is only marginally decreased by R-Hsp33 (Fig. 1B). These results are in line with the earlier reports on the chaperone activity of the reduced and oxidized forms of Hsp33 towards heat- and refolding-induced aggregation of citrate synthase and luciferase [17,20].

As mentioned earlier, the structure of Hsp33 per se as well as its change, if any, associated with its activation is not known. Since there is no X-ray crystallographic structure or solution structure of the protein derived from nuclear magnetic resonance studies available so far, theoretical structural predictions might be useful despite the limitations associated with them. We have analyzed the sequence of Hsp33 for the propensity of its residues to adopt the known secondary structural elements of a protein using the programs PSIPRED [25,26] and GOR IV [27] as shown in Fig. 2. The predictions made by the two programs are, in general, comparable with some differences in specific regions of the sequence (Fig. 2). Overall, the Hsp33 is predicted to have significant α-helix (37–40%) and extended strands (15–26%). A major portion of the sequence is predicted to be randomly coiled (36–46%). We have compared this secondary structural propensity prediction with the estimated values obtained from the CD spectra of Hsp33.

The far UV–CD spectra of Hsp33 show that it contains significant α-helices (Fig. 3A). Analysis of the CD spectrum of R-Hsp33 (curve 1, Fig. 3A) using the program K2d [28,29] shows that it contains 44% α-helix, 25% β-sheet and 31% random coil. O-Hsp33 exhibits a significantly different far UV–CD spectrum (curve 2, Fig. 3A) and the analysis shows that it contains 35% α-helix, 16% β-sheet and 49% random coil. These values are close to the sequence-based predicted values shown in Fig. 2. Two cautionary aspects to bear in mind in this context are: (i) sequence-based prediction algorithms do not take in to account the oxidized or reduced state of the protein, (ii) despite considerable effort, analysis of CD spectra for secondary structural elements is associated with

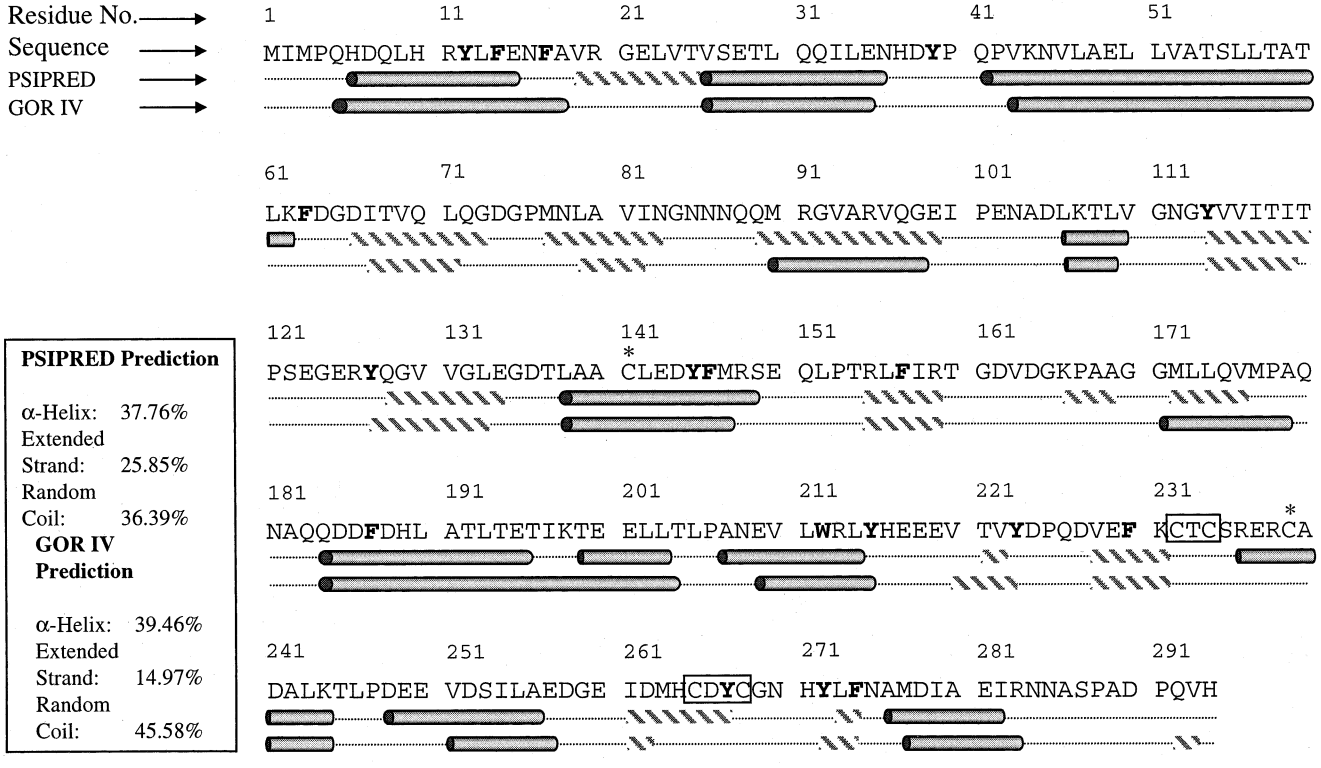

Fig. 2. Sequence-based secondary structure prediction of *E. coli* Hsp33 using the programs PSIPRED and GOR IV. ▭, α-helix; ⟍⟍⟍, extended strand and - - -, random coil. Aromatic amino acid residues are shown bold. Rectangular boxes highlight the conserved motifs, CXC and CYZC. Asterisk denotes the non-conserved cysteine residue.

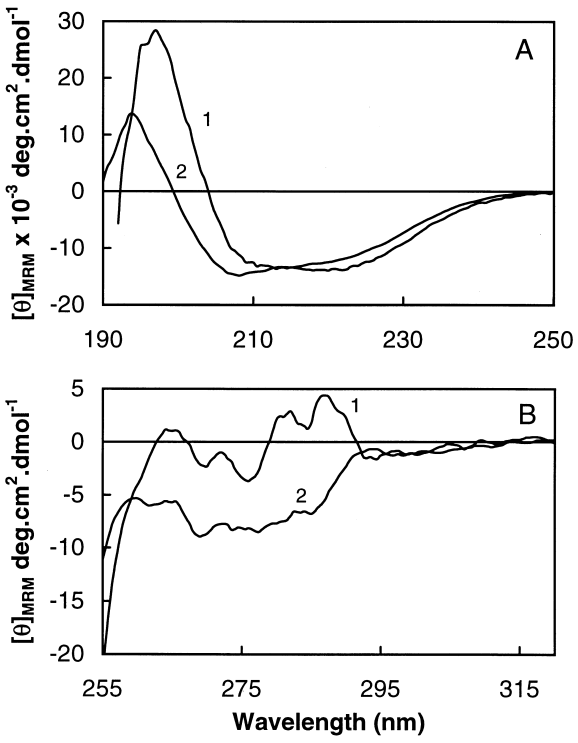

Fig. 3. Far (A) and Near (B) UV–CD spectra of reduced (curve 1) and oxidized (curve 2) Hsp33. $[\theta]_{MRM}$ is mean residue mass ellipticity.

some degree of uncertainty. However, qualitative differences between the far UV–CD spectra of reduced and O-Hsp33 clearly demonstrate that significant conformational changes even at the secondary structural level are associated with the oxidative activation of the chaperone.

The tertiary structure of the protein is studied by near UV–CD and fluorescence spectroscopy. Fig. 3B shows the near UV–CD spectra of R-Hsp33 and O-Hsp33. Besides the expected differences in the 255–270 nm region where disulfide contributes to the CD signal [30], appreciable differences can be observed in the 270–295 nm region of the spectra of reduced and oxidized forms (curve 1 and 2 respectively in Fig. 3B) where aromatic amino acid residues contribute to the CD signal. The O-Hsp33 exhibits enhanced ellipticity, suggesting changes in tertiary structural packing as compared to the reduced form. The aromatic amino acid residues of Hsp33 (see Fig. 2) include eight phenylalanines (at positions 14, 17, 63, 146, 157, 230 and 274), nine tyrosines (at positions 12, 39, 114, 127, 145, 215, 223, 267 and 272) and one tryptophan (at position 212). Fig. 4 shows the tryptophan fluorescence spectra of the protein in its reduced and oxidized forms by selectively exciting the tryptophan residue by 295 nm light. The spectrum of R-Hsp33 shows the emission maximum at 351 nm indicating that the tryptophan residue is in a polar environment as in bulk water. The fluorescence spectra of O-Hsp33 almost overlap with that of the reduced form showing that the microenvironment around the single tryptophan residue does not change significantly upon oxidation of the protein. These results show that the tryptophan residue of Hsp33 is completely exposed to the bulk solvent (probably located on the water-accessible surface of the protein) and its microenvironment is not altered upon oxidation. Thus, the observed differences seen in the near UV–CD spectra of R-Hsp33 and O-Hsp33 could be due to the changes in the packing and microenvironment of tyrosine and phenylalanine residues.

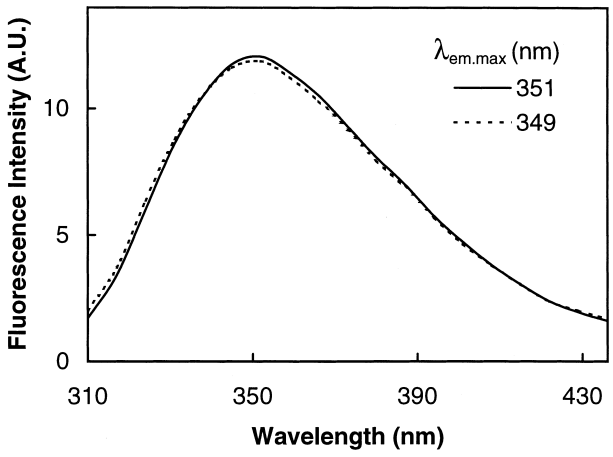

Fig. 4. Tryptophan fluorescence spectra of reduced (—) and oxidized (- - - -) Hsp33. The excitation wavelength was 295 nm. Excitation and emission band passes were set at 5 nm.

Molecular chaperones, in general, recognize and bind to non-native states of target proteins and prevent their aggregation. Hydrophobic interactions between the chaperone molecules and the target protein are known to play a major role in the chaperone–target protein interactions. We have, therefore, probed the hydrophobic surfaces of the Hsp33 in its reduced and oxidized forms. Fluorescent probes such as bis-ANS [31,32], ANS [33,34] and Nile Red [35,36] are widely used to probe the hydrophobic surfaces of proteins. Upon binding to the less polar surfaces, their fluorescence intensity increases several fold accompanying a blue shift in their emission maximum, albeit, to different extents depending on the fluorescence property of the probe as well as on the magnitude of the apolarity of the binding surfaces. Fig. 5 shows the binding of bis-ANS to R-Hsp33 and O-Hsp33. The probe reports a remarkable difference in the hydrophobic surfaces of reduced and oxidized forms of Hsp33. The fluorescence spectrum of bis-ANS bound to R-Hsp33 shows an emission maximum of 502 nm. The fluorescence spectrum of the probe

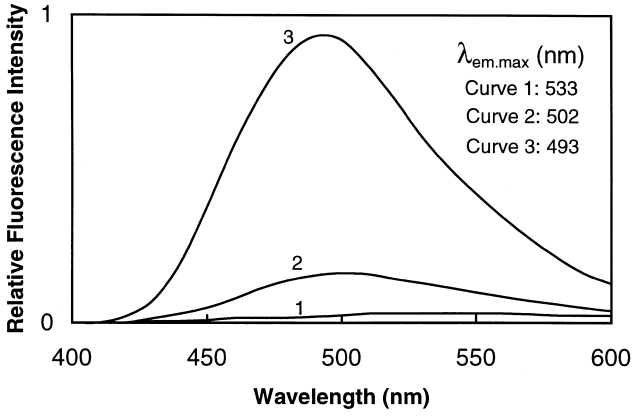

Fig. 5. Fluorescence spectra of bis-ANS (10 μM) in the absence (curve 1) and in the presence of 0.1 mg/ml reduced (curve 2) and oxidized (curve 3) Hsp33. The excitation wavelength was set at 390 nm. Excitation and emission band passes were set at 5 nm.

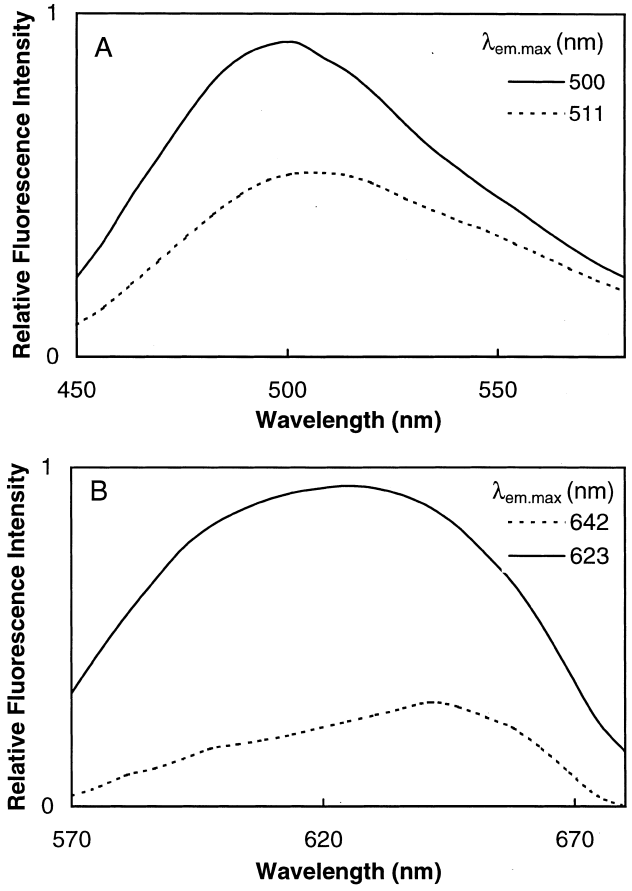

Fig. 6. A: Fluorescence spectra of ANS bound to reduced (- - - - -) and oxidized (—) Hsp33. The spectral contribution by ANS in buffer alone was subtracted from the spectra of the probe in the presence of protein. The final concentrations of ANS and Hsp33 used were 50 μM and 0.1 mg/ml respectively. The excitation wavelength was set at 365 nm. Excitation and emission band passes were set at 5 nm. B: Fluorescence spectra of Nile Red bound to reduced (- - - - - - -) and oxidized (—) Hsp33. The spectral contributions from protein sample alone and Nile Red in buffer alone were subtracted from the spectra of Nile Red in the presence of the protein. The final concentrations of the probe and the protein used were 1.5 μM and 0.5 mg/ml respectively. The excitation wavelength was set at 550 nm. Excitation and emission band passes were set at 5 nm.

bound to O-Hsp33 shows an increase in fluorescence intensity with the emission maximum shifted to 493 nm. The difference in the fluorescence intensities of the probe bound to O-Hsp33 and R-Hsp33 is about 6-fold as can be seen from the fluorescence spectra.

Fig. 6A shows the fluorescence spectrum of ANS bound to R-Hsp33 and O-Hsp33. The spectra of the probe bound to the reduced and oxidized forms of Hsp33 exhibit emission maxima of 511 and 500 nm respectively. The fluorescence intensity of the probe bound to O-Hsp33 is significantly more than that of the probe bound to R-Hsp33. It is to be noted that the difference in the intensities is not as many fold as observed in the case of bis-ANS. This apparent difference may be because of the different modes of their interactions and/or their different effects on the fluorescence properties of these two probes.

Nile Red is a neutral molecule whose fluorescence property is altered by the polarity of the medium: its fluorescence intensity increases with a blue shift in the emission maximum upon binding to the apolar surfaces of proteins [35,36]. Fig. 6B shows the fluorescence spectra of the probe bound to R-Hsp33 and O-Hsp33. Enhanced fluorescence intensity (about 5-fold) with a blue-shifted emission maximum is seen when it bound to O-Hsp33 compared to the R-Hsp33. Thus, all these fluorescent hydrophobic probes consistently show that the hydrophobic surfaces of Hsp33 are enhanced upon its oxidation. This enhancement in the hydrophobic surfaces may represent the creation of binding site(s) for the target protein upon turning on the oxidative switch.

E. coli appears to employ at least three different strategies [37] to alleviate the toxicity of oxidative stress: (i) using counter oxidative stress involving the maintenance of a reducing environment in the cytoplasm, (ii) de novo synthesis of protective enzymes like Sox R and Oxy R whose activities are regulated by redox-sensitive mechanisms, and (iii) oxidative activation of the cytoplasmic molecular chaperone, Hsp33. *E. coli* Hsp33, a member of a new family of highly conserved cytoplasmic prokaryotic Hsps, is the first candidate identified whose chaperone function is regulated by the redox potential of the medium [17,37]. Cellular heat shock is known to be accompanied by oxidative stress [38,39]; oxidative stress is also found to induce the expression of Hsps [40,41]. Hsp33 is found to play a protective role both in heat and oxidative stress in vivo [17]. This class of proteins contains a conserved motif, $CXC(X)_{27-30}$ CYZC, where the cysteine residues are in their reduced form and coordinated with a single Zn^{2+} ion [17,20] in the environment of a high reduction potential as in cytosol. The reduced form of Hsp33 does not exhibit chaperone activity. Under oxidative conditions, these conserved cysteines get oxidized to form intramolecular disulfide bonds, which leads to activation of the molecule [17,19,20]. Hsp33, when oxidized either by hydrogen peroxide or by a glutathione redox system with a high oxidation potential, has been shown to exhibit chaperone activity in preventing heat-induced aggregation of citrate synthase and luciferase [17] as well as the refolding-induced aggregation of luciferase [20] in vitro. Our results on the chaperone activity of Hsp33, in its reduced and oxidized forms, towards heat-induced aggregation of ζ-crystallin corroborate well with the earlier findings. The data shown in this paper are pertaining to the hydrogen peroxide O-Hsp33. We have also studied Hsp33 oxidized by the glutathione redox system (see Section 2.5). It is found to behave similar to the hydrogen peroxide-O-Hsp33 (data not shown). Our fluorescence and CD studies show remarkable differences in the conformations of the reduced and oxidized forms of Hsp33. Our results clearly demonstrate that conformational changes leading to increased exposure of hydrophobic surfaces are involved in the activation of Hsp33 by the oxidative switch.

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