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In vivo oxidation-reduction kinetics of OxyR, the transcriptional activator for an oxidative stress-inducible regulon in *Escherichia coli*

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Abstract The OxyR protein is a transcriptional activator for a subset of peroxide stress-inducible genes, most of which are involved in defense systems against oxidative stress. Recently, it was demonstrated that purified OxyR has one intramolecular disulfide bond, which led to the proposal that the reversible disulfide bond formation regulates the activity of OxyR as a transcription factor in response to peroxide stress. In this study, I demonstrated by SDS-PAGE under non-reducing conditions that an intramolecular disulfide bond is formed in OxyR upon exposure of the cells to hydrogen peroxide in vivo. Experiments using strains expressing mutant OxyR proteins with Cys to Ser single amino acid substitutions confirmed that the disulfide bond is formed between the Cys-199 and -208. Kinetic analyses indicated that the formation of the disulfide bond is rapid and transient, oxidized within 30 s and re-reduced within 5 min after the addition of hydrogen peroxide in the wild-type strain. These results provide evidence for the regulatory role of the reversible oxidation of dithiol to disulfide in sensing peroxide stress in vivo and signal transduction to the transcription apparatus by OxyR.

Key words: Transcription activator; Redox regulation; Disulfide bond; Oxidative stress; *Escherichia coli*

1. Introduction

Exposure of exponentially growing *Escherichia coli* cells to low concentrations of hydrogen peroxide activates the expression of at least 40 proteins, and makes the cells resistant to subsequent challenges with much higher concentrations of hydrogen peroxide [1,2]. The *oxyR* gene is required for the induction of a subset of hydrogen peroxide-inducible genes [3], including *katG* (encoding catalase hydroperoxidase I), *ahpC* (alkylhydroperoxide reductase), *gor* (glutathione reductase) and *grxA* (glutaredoxin 1), which are directly involved in defense against oxidative stress [1–3]. In addition, some *oxyR*-activated genes function as regulators for gene expression, thereby controlling additional genes under oxidative stress conditions [4,5].

OxyR is a member of the LysR family of activators, and acts both as a sensor of peroxide stress and as a transcriptional activator for genes induced by the stress [6,7]. The inactive form of the OxyR protein present in unstressed cells is oxidized upon exposure to peroxide stress and converted to its active conformation as a transcriptional activator [8]. The activated form binds just upstream of the promoter −35 sequence of target genes, and stimulates transcription initiation by directly making contact with RNA polymerase [9,10]. The molecular nature of the oxidative modification leading to OxyR activation had been considered to be oxidation of a Cys thiol to sulfenate or sulfinate, because only Cys-199 among the six Cys residues was reported to be essential for sensing peroxide stress [8]. However, there has been no biochemical evidence directly demonstrating the presence of such oxidatively modified Cys residues in the active form of OxyR. Recently, Zheng et al. [11] demonstrated by mass spectrometric analysis that there is one intramolecular disulfide bond in purified OxyR4C-A protein, an OxyR mutant in which four of the six Cys residues other than those at positions 199 and 208 were substituted with Ala. They also reported that the remaining two Cys residues are essential for transcriptional activation of the target genes upon exposure to peroxide stress. Furthermore, these two Cys residues are conserved among the OxyR homologues identified in other bacterial species. Based on these observations, they proposed that formation and reduction of the disulfide bond between Cys-199 and -208 regulates the activity of OxyR as a transcriptional activator [11].

In this study, I examined the formation of the intramolecular disulfide bond in the wild-type OxyR protein in vivo by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions, and found that the disulfide bond identified in vitro is transiently formed in peroxide-stressed *E. coli* cells. The results indicate that the disulfide bond formation in OxyR is relevant to the regulation of the OxyR activity as a transcriptional activator.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

E. coli K12 strain MC4100 was used as the wild-type strain. TA4112 is an *oxyR* deletion strain described previously [3]. pRS100 carries the promoterless *oxyR* gene from pKT823 [7] in the *Eco*RI-*Hin*dIII site of pKK223-3 (Pharmacia LKB). pRS100 derivatives (pRS101–pRS106), each carrying a Cys to Ser mutation in OxyR, were constructed by oligonucleotide-directed mutagenesis [12]. Cells were aerobically grown in Luria-Bertani medium [13] at 37°C.

2.2. Determination of OxyR oxidation states by non-reducing SDS-PAGE

To avoid artificial reduction of oxidized proteins during sample preparation by cellular factors, proteins were rapidly denatured with trichloroacetic acid at various time intervals after the addition of hydrogen peroxide, and trapped in the oxidized form [14]. Briefly, exponentially growing cultures of *E. coli*, with or without hydrogen peroxide treatment, were directly mixed with an equal volume of 10% trichloroacetic acid solution, and incubated for 1 h on ice. The trichloroacetic acid-insoluble fraction was precipitated by centrifugation, washed twice with cold acetone, and air-dried. The precipitates were solubilized in the sample buffer (100 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% SDS and 2% iodoacetamide), and incubated for 15 min at 25°C. Then samples were subjected to electrophoresis on a polyacrylamide gel (10% T, 3.3% C) as described previously [15], except that the separation gels contained 8 M urea. After electrophoresis, the

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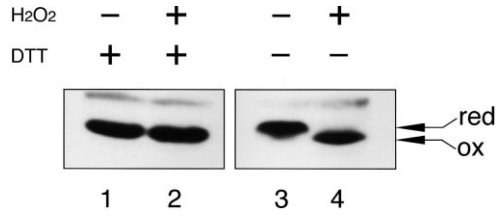

Fig. 1. Identification of disulfide-bonded forms of OxyR by SDS-PAGE under non-reducing conditions. Exponentially growing cells (MC4100) were exposed to (lanes 2 and 4) 100 μM of hydrogen peroxide for 1 min (lanes 1 and 3, not exposed). Proteins were rapidly denatured and precipitated by ice-cold tricholoroacetic acid, washed with acetone, and dissolved in SDS buffer containing iodoacetamide. They were then electrophoresed on a polyacrylamide gel containing 8 M urea, after (lanes 1 and 2) or without (lanes 3 and 4) dithiothreitol treatment (35 mM). OxyR was visualized by Western blot analysis. The reduced and oxidized forms of OxyR are indicated by arrowheads.

proteins were electroblotted onto a polyvinylidene difluoride membrane [16], and OxyR was visualized with anti-OxyR antiserum [17] and a chemiluminescence detection system.

3. Results and discussions

Proteins having intramolecular disulfide bond(s) often migrate faster than their reduced forms on SDS-PAGE under non-reducing conditions, because of their more compact conformations in gels [18]. Based on this principle, I initially attempted to identify the oxidized form of OxyR in cells exposed to peroxide stress. When proteins were electrophoresed on a standard gel system [15] without any reducing treatment, no reproducible difference in electrophoretic mobility was observed between OxyR exposed to oxidative stress and that not exposed to oxidative stress (data not shown). Therefore, I attempted to identify the oxidized form by using urea-containing separation gel instead of a standard gel. Inclusion of urea

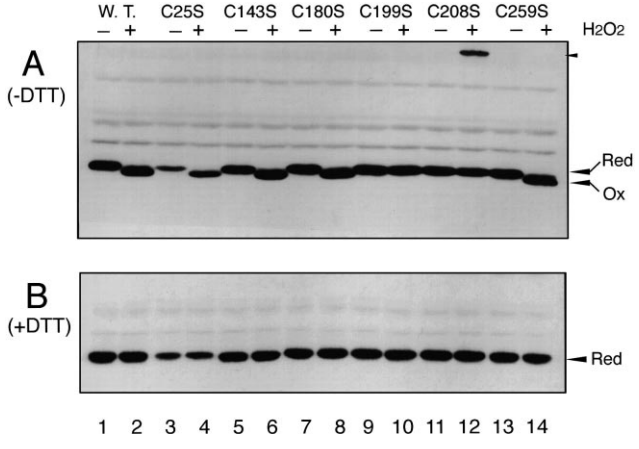

Fig. 2. Disulfide bond formation in the mutant OxyR with Cys to Ser single amino acid substitutions. An *oxyR* deletion strain (TA4112) was transformed with pRS100 (lanes 1 and 2), pRS101 (lanes 3 and 4), pRS102 (lanes 5 and 6), pRS103 (lanes 7 and 8), pRS104 (lanes 9 and 10), pRS105 (lanes 11 and 12) and pRS106 (lanes 13 and 14), and was examined for the formation of the disulfide-bonded form of OxyR after exposure to hydrogen peroxide (100 μM) for 1 min as in Fig. 1. Electrophoresis was carried out under reducing (A) or non-reducing conditions (B). The oxidized and reduced forms of OxyR are indicated by arrowheads on the right. For the C208S mutant, an additional high molecular weight band is seen, as indicated by the small arrowhead.

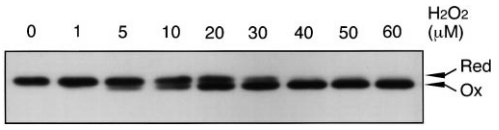

Fig. 3. Disulfide bond formation of OxyR following exposure to increasing concentrations of hydrogen peroxide. A strain expressing the wild-type OxyR (TA4112 carrying pRS100) was grown to mid-log phase, and then exposed to increasing concentrations of hydrogen peroxide for 1 min. Redox states of OxyR were examined as described in Fig. 1.

in the separation gel might enhance the structural difference between the reduced and disulfide-bonded forms of proteins during electrophoresis by the denaturing effect of urea, and would allow separation of the disulfide-bonded form of OxyR. Fig. 1 shows the results of an immunoblotting after electrophoresis on urea-containing gel. OxyR from hydrogen peroxide-treated cells migrated distinctly faster than that from untreated cells on urea-containing gel under non-reducing conditions (Fig. 1, lanes 3 and 4). After reduction with dithiothreitol, they migrated identically (Fig. 1, lanes 1 and 2). Thus, the faster-migrating band corresponds to the oxidized form of OxyR.

Next, I examined the formation of the faster-migrating species of OxyR after hydrogen peroxide treatment in strains expressing mutant OxyR proteins, carrying Cys to Ser single amino acid substitutions. A series of plasmids expressing mutant OxyR proteins were constructed by site-directed mutagenesis, and transformed into an *oxyR* deletion strain (TA4112). In agreement with a previous report [11], two mutants, Cys-199 to Ser (C199S) and C208S, could not complement the hydrogen peroxide sensitivity of the *oxyR* deletion strain (data not shown). Furthermore, they were totally defective in *oxyR*-dependent *katG* induction in response to peroxide stress as measured by the expression of the β-galactosidase activity encoded in the *katG′-′lacZ* fusion gene (data not shown). These observations confirm that the Cys residues at 199 and 208 are essential for peroxide stress sensing by OxyR. Using the same strains, I examined the formation of the faster-migrating form of OxyR after hydrogen peroxide treatment. Under non-reducing conditions (Fig. 2B), the faster-migrating form of OxyR was observed in hydrogen peroxide-treated cells expressing the wild-type, and the C25S, C143S, C180S and C259S mutant OxyR proteins. In contrast, the faster-migrating form was not detected for the C199S and C208S mutants. In the C208S mutant, a slowly migrating band was detected in addition to the band corresponding to the reduced form (Fig. 2B, lane 12). This high molecular weight band may correspond to an OxyR dimer crosslinked between two Cys-199 residues observed in vitro [11]. Again, the wild-type and all the mutant OxyR proteins migrated identically under conditions where the samples were reduced with dithiothreitol before being subjected to electrophoresis

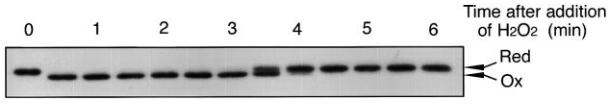

Fig. 4. Oxidation-reduction kinetics of OxyR. A strain expressing the wild-type OxyR (TA4112 carrying pRS100) was grown to mid-log phase, and exposed to 200 μM hydrogen peroxide. Samples were withdrawn at indicated time points after the addition of hydrogen peroxide, and analyzed as described in Fig. 1.

(Fig. 2A). Taken together, these results indicate that the faster-migrating species identified on urea-containing gels represents the disulfide-bonded form of OxyR, and that an intramolecular disulfide bond between Cys-199 and -208 is formed upon exposure of the cells to low concentrations of hydrogen peroxide in vivo.

The OxyR response is activated by micromolar levels of hydrogen peroxide in exponentially growing cells [19,20]. If the disulfide bond formation is relevant to the activation of OxyR as a transcriptional activator, it should be formed upon exposure to a similar range of hydrogen peroxide concentrations. In addition, transcriptional activation of the OxyR regulon is rapid and transient. Upon exposure to hydrogen peroxide, transcription of an *oxyR*-regulated gene is induced within 1 min, and the maximum accumulation of transcripts is observed between 2.5 and 5 min [4]. Pulse-labeling experiments have also shown that the *oxyR*-regulated proteins are synthesized during the first 10 min after treatment with hydrogen peroxide [3]. These results predict that OxyR is possibly oxidized immediately after the addition of hydrogen peroxide, and re-reduced after the removal of the added hydrogen peroxide by cellular factors. To examine these issues, I first examined the redox states of OxyR after exposure of the cells to increasing concentrations of hydrogen peroxide (Fig. 3). Without hydrogen peroxide treatment, most OxyR molecules are observed in the reduced form in aerobically growing cells (Fig. 3, lane 1, see also Fig. 1, lane 3), although the possibility of the presence of small amounts of the oxidized form cannot be excluded because of the limitations of electrophoretic separation of the reduced and oxidized forms under the experimental conditions used in this study. When sampling was carried out at 1 min after the addition of hydrogen peroxide, a significant fraction of OxyR was found to be in the disulfide-bonded form. The amount of the oxidized form increased with exposure to increasing hydrogen peroxide concentration, and finally, virtually all the OxyR was found to be in the oxidized form at 40 μM hydrogen peroxide. These results are consistent with the notion that the OxyR protein acts as a sensitive biological sensor of hydrogen peroxide in the cells. Next, I examined the redox states of OxyR at 30 s intervals after the addition of hydrogen peroxide, to directly measure the lifetime of the oxidized form of OxyR in vivo (Fig. 4). After exposure to 200 μM hydrogen peroxide, most of the OxyR was observed in its disulfide-bonded form even at the earliest time point examined (30 s), and then rapidly converted back to the reduced form during subsequent culture. Reduction of the oxidized form was complete within 5 min in the wild-type strain. The time course and hydrogen peroxide concentration-dependency of the oxidation and reduction of OxyR in vivo were consistent with previously observed kinetics of the OxyR response[1]. Together with in vitro observations [11], these results indicate that reversible intramolecular disulfide bond formation between Cys-199 and -208 regulates the activity of OxyR as a transcriptional activator.

In this study, I demonstrated that the oxidized form of OxyR can be separated from the reduced form by SDS-PAGE under non-reducing conditions. This method allows us to directly determine the redox states of OxyR during stress response in vivo. A remarkable feature of the response was the rapid oxidation-reduction switch. A similar rapid response has been observed in *E. coli* for another well-characterized response to oxidative stress, namely the superoxide stress response regulated by SoxR [1,2]. SoxR functions as both a sensor for increased superoxide anion radicals and as a transcriptional activator for the genes responding to the stress. The activity of SoxR as a transcriptional activator is regulated by one electron oxidation-reduction of the [2Fe-2S] cluster responding to the increase in superoxide radicals [21,22]. Similar to the OxyR response, SoxR is rapidly activated (within 2 min) by redox agents, and is rapidly deactivated after the removal of the stress [23]. OxyR and SoxR protect cells against oxidative stress mainly by inducing the expression of important frontline defense enzymes, such as catalase hydroperoxidase I and Mn-containing superoxide dismutase, respectively. Therefore, the ability to quickly respond to changes in the environment is essential for these sensor/activator proteins.

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References

[1] Hidalgo, E. and Demple, B. (1996) in: Regulation of Gene Expression in *Escherichia coli* (Lin, E.C.C. and Lynch, A.S., Eds.), pp. 435–452, R.G. Landes, Austin, TX.
[2] Jamieson, D.J. and Storz, G. (1997) in: Oxidative Stress and the Molecular Biology of Antioxidant Defenses (Scandalios, J.G., Ed.), pp. 91–115, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[3] Christman, M.F., Morgan, R.W., Jacobson, F.S. and Ames, B.N. (1985) Cell 41, 753–762.
[4] Altuvia, S., Weinstein-Ficher, D., Zhang, A., Postow, L. and Storz, G. (1997) Cell 90, 43–53.
[5] Mukhopadhyay, S. and Schellhorn, H.E. (1997) J. Bacteriol. 179, 330–338.
[6] Christman, M.F., Storz, G. and Ames, B.N. (1989) Proc. Natl. Acad. Sci. USA 86, 3484–3488.
[7] Tao, K., Makino, K., Yonei, S., Nakata, A. and Shinagawa, H. (1989) Mol. Gen. Genet. 218, 371–376.
[8] Storz, G., Tartaglia, L.A. and Ames, B.N. (1990) Science 248, 189–194.
[9] Tao, K., Fujita, N. and Ishihama, A. (1993) Mol. Microbiol. 7, 859–864.
[10] Tao, K., Zou, C., Fujita, N. and Ishihama, A. (1995) J. Bacteriol. 177, 6740–6744.
[11] Zheng, M., Åslund, F. and Storz, G. (1998) Science 279, 1718–1721.
[12] Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82, 488–492.
[13] Miller, J.H. (1992) A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[14] Kishigami, S., Akiyama, Y. and Ito, K. (1995) FEBS Lett. 364, 55–58.
[15] Lammeli, E.K. (1970) Nature 227, 680–685.
[16] Towbin, H., Staehelin, T. and Gordon, J. (1978) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[17] Tao, K. (1997) J. Bacteriol. 179, 5967–5970.
[18] Pollitt, S. and Zalkin, H. (1983) J. Bacteriol. 153, 27–32.
[19] Demple, B. and Halbrook, J. (1983) Nature 304, 466–468.
[20] Gonzalez-Flecha, B. and Demple, B. (1997) J. Bacteriol. 179, 382–388.
[21] Gaudu, P. and Weiss, B. (1996) Proc. Natl. Acad. Sci. USA 93, 10094–10098.
[22] Ding, H., Hidalgo, E. and Demple, D. (1996) J. Biol. Chem. 271, 33173–33175.
[23] Ding, H. and Demple, D. (1997) Proc. Natl. Acad. Sci. USA 94, 8445–8449.

[1] Recently, Åslund et al. (Proc. Natl. Acad. Sci. USA 96, 6161–6165) have also reported similar oxidation-reduction kinetics of OxyR during oxidative stress response in vivo.