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Original Contribution

DIFFERENT MECHANISMS OF THIOREDOXIN IN ITS REDUCED AND OXIDIZED FORMS IN DEFENSE AGAINST HYDROGEN PEROXIDE IN *ESCHERICHIA COLI*

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Abstract—The present experiments were done to elucidate the roles of thioredoxin and thioredoxin reductase system in defense against hydrogen peroxide (H_2O_2) in *Escherichia coli*. The thioredoxin-deficient mutant (*trxA*) was more sensitive to H_2O_2 than was the wild-type strain, when challenged in the stationary and exponentially growing phase. Thioredoxin reductase-deficient mutant (*trxB*) in the stationary phase also exhibited increased sensitivity, compared with the wild-type strain. These results indicated that reduced form of thioredoxin is required for defense against H_2O_2, possibly by scavenging radicals generated in the cells. In contrast, the *trxB* mutant in the growing phase had higher survival after exposure to H_2O_2 than the wild-type strain. The acquirement of resistance related to increased capacity for removing H_2O_2 in the *trxB* mutant and was not observed in a catalase-negative background. Furthermore, enhanced expression of the *katG : : lacZ* gene occurred in the mutant. Therefore, it was concluded that oxidized form of thioredoxin confers H_2O_2 resistance on *E. coli* cells by increasing activity to remove H_2O_2, which was brought about by enhanced induction of the *katG*-coded catalase/hydroperoxidase I at the transcriptional level. In addition, this resistance to H_2O_2 correlated well with reduced amount of DNA damage caused by H_2O_2, determined by the induction level of the *recA : : lacZ* fusion gene after treatment with H_2O_2. © 1998 Elsevier Science Inc.

Keywords—Thioredoxin, Thioredoxin reductase, Hydrogen peroxide, Redox, Catalase/hydroperoxidase I, *Escherichia coli*, Gene expression, Free radical

INTRODUCTION

Active oxygen species such as superoxide-radical, hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), are continuously produced in aerobic cells.[1–4] H_2O_2 is decomposed to produce nontoxic H_2O and O_2 by catalases and peroxidases.[3,4] However, in the presence of transition metal ions such as Fe^{2+}, H_2O_2 produces highly reactive $^{\bullet}OH$ via the Fenton or Haber/Weiss reaction.[1–4] Intracellular H_2O_2 is kept at low concentrations by the action of catalases and other peroxidases. In *Escherichia coli*, the steady-state concentration of H_2O_2 is estimated to be ~0.15 μM.[5] Oxidative stress refers to imbalances between the production and disposal of active oxygen species. An overabundance of H_2O_2 gives rise to $^{\bullet}OH$, which damages cellular DNA, protein, and lipid.[1–4] To reduce and repair the oxidative damage, genetic responses to such oxidative stress occur in bacteria.[6–9] *E. coli* cells possess a specific defense system against H_2O_2 mediated by the transcriptional activator OxyR.[10–13]

Thioredoxin is a small, heat-stable, redox-active protein present in high concentrations in *E. coli* cells.[14–16] This well-conserved protein contains a redox-active disulfide/dithiol active site within the structure Trp-Cys-Gly-Pro-Cys, which can be reversibly oxidized and reduced.[15,16] Thioredoxin reductase specifically reduces this disulfide group with NADPH.[15–18] Many biological functions of thioredoxin and thioredoxin reductase system have been characterized.[14–16,18,19] The reduced form of thioredoxin serves as a hydrogen donor for ribonucleotide reductase and for enzymes reducing sulfate or methionine sulfoxide in vitro. It is also a highly efficient and broadly specific protein disulfide reductase.[18,19] However, the thioredoxin- and thioredoxin reductase-deficient mutants of *E. coli*, *trxA*, and *trxB*, respectively,

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exhibit normal growth features.[20,21] The precise physiological functions of the thioredoxin system are not fully understood.

Thioredoxin has been demonstrated to play a role in defense against oxidative stress by scavenging active oxygen species and radicals.[22–25] Lunn and Pigiet[26] reported that thioredoxin protected *E. coli* cells against gamma-radiation. The protection was primarily due to its action of scavenging radicals generated by radiation. In addition, protein disulfide reductase activity of thioredoxin was observed to repair oxidatively damaged proteins and to regulate the activity of some enzymes by thiol redox control.[22–25]

In the present study, we examined the effect of thioredoxin- and thioredoxin reductase-deficiencies on the survival of *E. coli* cells after exposure to H_2O_2. The results obtained show that thioredoxin has different mechanisms in its reduced and oxidized forms for defending the cells against H_2O_2, i.e., the scavenging of radicals and the upregulation of the expression of catalase/hydroperoxidase I, respectively.

MATERIALS AND METHODS

Bacterial strains and plasmid

The *E. coli* strains used in this study are derivatives of *E. coli* K12, K38 [HfrC *sup*$^+$ (λ)], A179 (the same as K38 but *trxA* : : Tn*5*) and A237 (the same as K38 but *trxB zbj-1230* : : *Tn10*).[27] These strains were generous gifts from Dr. M. Russel of Rockefeller University. Transduction experiments with $P1_{vir}$ phage were performed according to the method of Miller.[28] *E. coli* cells were routinely grown at 37°C in LB medium[28] with aeration. Ampicillin, kanamycin, and tetracycline were added at 50, 50, and 25 μg/ml, respectively. The construction of plasmid pKT1033 (a single copy number plasmid carrying the *katG* : : *lacZ* fusion gene) was described previously.[12,13]

Survival assay after the treatment of E. coli *cells with* H_2O_2

For the treatment of exponentially growing cells, overnight cultures were diluted 100-fold in fresh LB medium and grown at 37°C until the optical density at 600 nm (OD_{600}) reached about 0.6. The cells were collected, washed, and resuspended in the original volume of phosphate-buffered saline (PBS, pH 7.2). For the treatment of cells in the stationary phase, the cells cultured for 20–24 h after inoculation were collected, washed, and resuspended in PBS to set the OD_{600} to about 0.6. Cell suspensions were then incubated at 37°C for 60 min with various concentrations of H_2O_2. After treatment, the cell suspensions were appropriately diluted in PBS and plated on LB plates. After incubation at 37°C for about 20 h, the number of viable colonies on the plates was counted to estimate the cell survival.

Measurement of glutathione

Overnight cultures of *E. coli* (0.2 ml) were added to 1.8 ml of ice-cold 5% TCA-20 mM EDTA solution and mixed thoroughly. Total glutathione in acid-soluble fractions was determined by the method of Tietze[31] using dithionitrobenzoic acid (DTNB).

Measurement of catalase activity

Overnight cultures of *E. coli* were diluted 100-fold in fresh LB medium and incubated at 37°C until the OD_{600} reached about 0.6. The cells were harvested by centrifugation, washed once and resuspended in 50 mM potassium phosphate (pH 7.8) containing 0.1 mM EDTA. The cells were lysed by an ultrasonic disrupter (Tomy UR-200P) five times for 10 s at 50% output power at 4°C. The resultant homogenates were centrifuged at 13,000 rpm for 30 min at 4°C, and the supernatants were used for measuring the activity of catalase. The protein content was measured according to the method of Lowry et al.[29] with bovine serum albumin as the standard. The catalase activity was measured according to the method described by Aebi[30] and represented as the decrease of OD_{240} (ΔOD_{240})/min/mg protein.

Measurement of the induction of the katG : : lacZ *and* recA : : lacZ *fusion genes by* H_2O_2

For the *katG* : : *lacZ* fusion gene, overnight cultures of *E. coli* with pKT1033[12,13] were diluted 100-fold in fresh LB medium with ampicillin and incubated at 37°C until the OD_{600} reached about 0.2. To exclude the effect of RpoS-dependent induction of the *katG* gene,[32,33] these experiments were done in the early growth phase. An aliquot of the cultures was mixed with 100 μM H_2O_2, and the cell suspensions were subsequently incubated at 37°C for up to 90 min with shaking. For the *recA* : : *lacZ* fusion gene, overnight cultures were diluted 100-fold in LB medium and incubated at 37°C until the OD_{600} reached about 0.6. The aliquots of the cultures were mixed with up to 2 mM of H_2O_2 and the cell suspensions were incubated at 37°C for 60 min with shaking. The β-galactosidase activity in the whole-cell extracts was determined according to the method of Miller.[28]

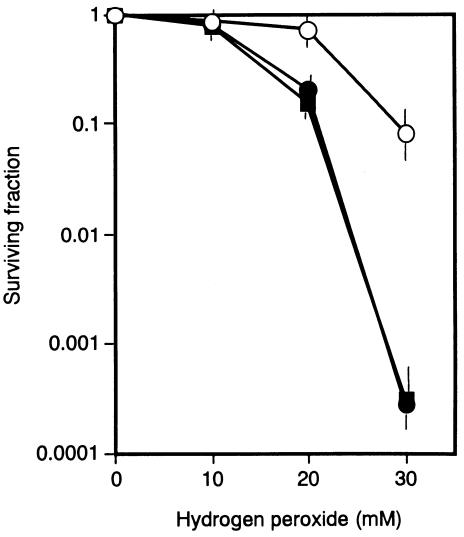

Fig. 1. Survival curves for *trxA* and *trxB* mutants of *E. coli* in the stationary phase after treatment with H_2O_2. Overnight cultures were collected, washed, and resuspended in PBS to set the OD_{600} to about 0.6, and then treated with H_2O_2 at 37°C for 60 min, followed by the survival assay. Results are expressed as mean ± SD (n = 3–4). ○, K38; ●, A179; ■, A237.

RESULTS

Sensitivity of the trxA *and* trxB *mutants to* H_2O_2

Lunn and Pigiet[26] reported that thioredoxin protects *E. coli* cells from damage caused by gamma-radiation at a certain stage of cell growth. The cells showed increased resistance, compared with the wild-type strain, when irradiated in the stationary phase. The survival correlated well with the level of thioredoxin in the cells.[26] However, exponentially growing cells showed almost the same sensitivity to gamma-radiation as the wild-type strain, regardless of the intracellular concentration of thioredoxin.[26] The reason for this has not been fully elucidated. It is of interest to know whether such a growth phase-dependent effect of thioredoxin is also present in cells treated with H_2O_2. We, thus, compared the sensitivity of thioredoxin- and thioredoxin reductase-deficient mutants with that of the wild-type strain.

In the stationary phase, both the *trxA* and *trxB* mutants were hypersensitive to H_2O_2 (Fig. 1). As shown in Table 1, there was no change in the concentration of glutathione by the mutations. Therefore, in this case, thioredoxin may serve to protect the cells against H_2O_2 by scavenging radicals generated in the cells.

Table 1. The Amount of Total Glutathione in the Stationary Phase *E. coli* Wild-Type, *trxA*, and *trxB* Strains

Strain	Relevant Genotype	Glutathione (nmol/10^8 Cells)
K38	*trx*+	2.2
A179	*trxA*	2.0
A237	*trxB*	2.3

The effects of H_2O_2 on exponentially growing cells were different between in *trxA* and *trxB* mutants (Fig. 2). The *trxA* mutant exhibited increased sensitivity to H_2O_2, as in the stationary phase. The *trxB* mutant was more resistant to H_2O_2 than was the wild-type strain. The results suggested that the oxidized form of thioredoxin protected the cells against H_2O_2 by a mechanism different from that of its reduced form.

Catalase activities in the trxA *and* trxB *mutants*

To determine how the *trxB* mutation confers increased resistance against H_2O_2, the capacity of the *trxB* mutant for decomposing H_2O_2 was compared with that of the wild-type and *trxA* strains. Overnight cultures were diluted 100-fold in fresh LB medium and incubated at 37°C until the OD_{600} reached about 0.6. The cells were centrifuged, washed once, and resuspended in 50 mM potassium phosphate (pH 7.8) containing 0.1 mM EDTA. Cell-free extracts were prepared as described above, and the catalase activities were measured as the capacity for decomposition of H_2O_2. The catalase activities were 0.39 ± 0.03, 0.40 ± 0.04, and 0.59 ± 0.04 ΔOD_{240}/min/mg protein in the wild-type, *trxA*, and *trxB* strains, respectively. In the exponentially growing phase, the *trxB* mutant had the highest level of catalase activity among these strains.

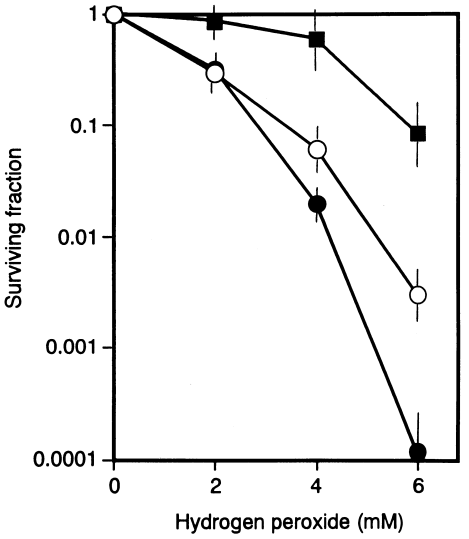

Fig. 2. Survival curves for *trxA* and *trxB* mutants of *E. coli* in the exponentially growing phase after treatment with H_2O_2. *E. coli* cells in the exponentially growing phase were collected, washed once, and resuspended in PBS to set the OD_{600} to about 0.6, and then treated with H_2O_2 at 37°C for 60 min. Results are expressed as mean ± SD (n = 3–5). ○, K38; ●, A179; ■, A237.

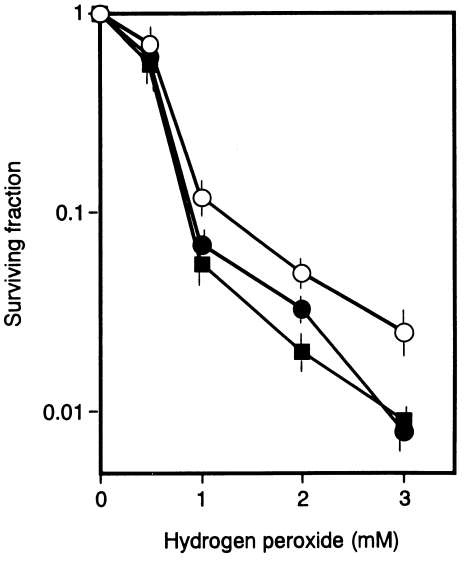

Fig. 3. Survival curves for *trxAkatEkatG* and *trxBkatEkatG* mutants after treatment with H_2O_2. *E. coli* cells in the exponentially growing phase were collected, washed once, and resuspended in PBS to set the OD_{600} to about 0.6, and treated with H_2O_2 at 37°C for 60 min. Results are expressed as mean ± SD (n = 3–5). ○, K38*katEkatG;* ●, A179*katEkatG;* ■, A237*katEkatG*.

Sensitivity to H_2O_2 of trxA *and* trxB *mutants deficient in catalase activities*

E. coli possess two catalase-hydroperoxidases called HPI and HPII, which are encoded by the *katG* and *katE* gene, respectively.[34,35] These enzymes remove intracellular H_2O_2 by catalyzing the conversion of H_2O_2 to water and oxygen. We investigated whether these catalase activities were directly related to an acquired resistance to H_2O_2 in the *trxB* mutant. The sensitivity of the *trxA* and *trxB* mutants to H_2O_2 was determined in a catalase-deficient background. The mutations of *katE* and *katG* were introduced into wild-type, *trxA* and *trxB* strains using a P1 phage.[28] As shown in Fig. 3, the H_2O_2 resistance of *trxB* mutant in the exponentially growing phase was not seen in cells deficient in catalase activities. Because of their deficiency in catalases, the strains became highly sensitive to H_2O_2, and the *trxA* and *trxB* mutants had increased sensitivity to H_2O_2. This was due to a loss of radical-scavenging ability and/or a protein disulfide reductase activity of reduced thioredoxin. Similar results were obtained with the *katG* single mutant (data not shown).

H_2O_2-induced expression of the katG *gene in* trxA *and* trxB *mutants*

The expression of both HPI and HPII is growth phase-dependently regulated at the transcriptional level.[32,33] The expression of HPI is also regulated by OxyR upon exposure to H_2O_2.[10–13] In the present study, HPI was expressed to a greater extent in the *trxB* mutant. Hence, it was of interest to determine whether the expression of the *katG* gene is enhanced by the *trxB* mutation. The H_2O_2-induced expression of the *katG : : lacZ* fusion gene was measured to determine the induction of HPI at the transcriptional level. Plasmid pKT1033, a single copy number plasmid carrying the *katG : : lacZ* fusion,[12,13] was introduced into the wild-type, *trxA*, and *trxB* strains. Because HPI is also regulated by RpoS,[32,33] the experiments were done in the early growth phase (OD_{600} = about 0.2) to exclude the effect of RpoS. As shown in Fig. 4, the *trxA* mutation resulted in a decrease in β-galactosidase activity. The *trxB* mutation was found to stimulate the induction of the *katG : : lacZ* fusion gene. It was evident that the level of β-galactosidase was much higher in the nontreated *trxB* mutant than in the wild-type and *trxA* strains. These results corresponded well to the enhanced activity of catalase and the increased resistance against H_2O_2.

H_2O_2-induced DNA damage in the trxA *and* trxB *mutants*

To compare the amounts of DNA damage caused by H_2O_2 in the *trxA* and *trxB* mutant cells, the expression of the *recA : : lacZ* fusion gene was measured after treatment with H_2O_2 in cells in the exponentially growing phase. Upon treatment with a variety of DNA-damaging agents, RecA protein acquires a catalytic ability to cleave the LexA repressor, which in turn induces a series of

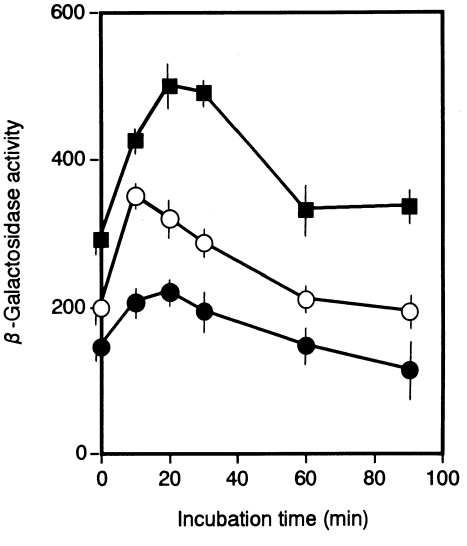

Fig. 4. Induction of the *katG : : lacZ* fusion gene of *E. coli* by H_2O_2. Exponentially growing cells were treated with 100 μM H_2O_2 at 37°C for up to 90 min under aerobic conditions, followed by the assay for β-galactosidase activity. Results are expressed as mean ± SD (n = 3–4). ○, K38/pKT1033; ●, A179/pKT1033; ■, A237/pKT1033.

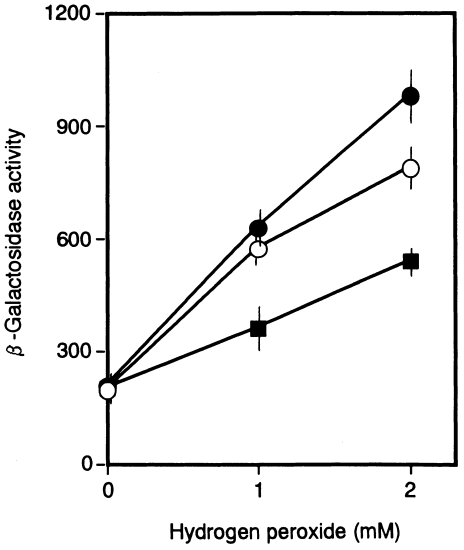

Fig. 5. Induction of the *recA : : lacZ* fusion gene of *E. coli* by H_2O_2. Exponentially growing cells of *E. coli* with the *recA : : lacZ* fusion gene in the genome were treated with H_2O_2 at 37°C for 60 min under aerobic conditions, followed by the assay for β-galactosidase activity. Results are expressed as mean ± SD (n = 3). ○, K38 with the *recA : : lacZ* fusion; ●, A179 with the *recA : : lacZ* fusion; ■, A237 with the *recA : : lacZ* fusion.

genes including the *recA* gene, termed the ''SOS response''.[36,37] The SOS response is also induced by H_2O_2.[38–40] As shown in Fig. 5, the level of β-galactosidase activity was increased in the *trxA* mutant. The *trxB* mutation was found to reduce the level of induction of the *recA : : lacZ* fusion gene by H_2O_2.

DISCUSSION

The present experiments demonstrated that the thioredoxin present in *E. coli* has at least two different mechanisms of defense against H_2O_2. The thioredoxin-deficient mutant (*trxA*) was hypersensitive to H_2O_2 when treated in the stationary phase and in the growing phase (Figs. 1 and 2). The thioredoxin reductase-deficient *trxB* mutant, in which the oxidized form of thioredoxin cannot be recycled,[16,17,21,27] showed increased sensitivity to H_2O_2 in the stationary phase (Fig. 1). These findings indicated an important role of the reduced form of thioredoxin in the defense against H_2O_2. This phenotype did not result from changes in the concentration of the major nonprotein thiol glutathione (Table 1), which functions as a cellular scavenger of radiation- and chemically generated $^{\cdot}OH$ radicals.[41] Similar observations have been reported by Lunn and Pigiet with gamma-radiation.[26] The survival of *E. coli* after exposure to radiation correlated well with the level of thioredoxin within the cells.[26]

Resistance of the stationary phase wild-type and mutant cells to H_2O_2 (Fig. 1) was greater than that in the exponential phase cells (Fig. 2). Recent studies revealed that stationary phase cells intrinsically exhibit an increased resistance to a variety of stress conditions, including resistance to H_2O_2,[8] and both HPI and HPII are expressed constitutively when the cells enter the stationary phase.[32,33] The high level of HPI and HPII could confer resistance to H_2O_2 on the cells.

H_2O_2 produces $^{\cdot}OH$ radicals via a transition metal ion-mediated Fenton reaction or Harber/Weiss reaction in cells.[1–4] Hence, thioredoxin could protect *E. coli* cells against H_2O_2 by scavenging radicals. As Lunn and Pigiet[26] have also suggested, thioredoxin may react directly with a radical species or may interact with a radical induced on a critical target molecule. The ability of reduced thioredoxin to scavenge intracellular radicals has been suggested from its reaction with a radical intermediate during the reduction of ribonucleotides by ribonucleotide reductase[14–16] and with phenoxyl radicals.[25] Thioredoxin is an important cellular redox buffer. Not only the amount but also the redox state of thioredoxin might have a strong influence on the sensitivity of *E. coli* to H_2O_2. The redox cycling of such radicals is accompanied by the oxidation of thioredoxin sulfhydryls to disulfides. The thioredoxin plays an important role in defense against a variety of types of oxidative stress by scavenging active oxygen species.[22–25]

Recent studies have revealed that novel thiol peroxidases of *E. coli* act as an enzyme removing peroxides or H_2O_2 and is functionally linked to the thioredoxin.[42,43] Scavengase p20 is identified as a novel family of bacterial antioxidant enzymes possessing thioredoxin-linked thiol peroxidase activity.[44] In addition, thiol-specific antioxidant enzyme, which catalyzes the destruction of H_2O_2, is more active in the presence of thioredoxin.[45] Hence, the mutation in the *trxA* gene might result in a loss of such H_2O_2 removing activity, which leads to enhanced sensitivity to H_2O_2.

Another activity of thioredoxin as a powerful protein disulfide reductase might be to repair damaged proteins. These activities of reduced thioredoxin might contribute to the protection of cells from oxidative stress. In this case, the amount of intracellular thioredoxin and sufficient activity of thioredoxin reductase might influence the cell survival against H_2O_2, as reported for gamma-radiation.[26]

The exponentially growing cells of the *trxB* mutant, in contrast, showed a significant resistance to H_2O_2 (Fig. 2). To explore the mechanisms of this phenomenon, the possible abilities of cells to decompose H_2O_2 and/or to repair damage caused by H_2O_2 were examined. The extracts from the *trxB* mutant showed increased activity for removing H_2O_2 compared with the wild-type strain. This was brought about by an enhanced expression of the

katG gene, which encodes HPI,[34,35] at the transcriptional level (Fig. 4). *E. coli* possesses two types of catalase/hydroperoxidase, HPI and HPII,[34,35] and only the HPI expression is inducible through the adaptive response against H_2O_2.[6–8,10–13] Because the resistance of the thioredoxin reductase-deficient mutant (*trxB*) was not seen in the catalase-deficient background (Fig. 3), the induction of HPI could be a direct cause of the enhanced activity of catalase and the increased resistance against H_2O_2.

Reduced thioredoxin regulates the activity of some enzymes by thiol redox control. Recent studies of mammalian cells revealed that thioredoxin can serve as a redox signal and regulate a variety of cellular functions via redox control.[46,47] The precise mechanisms of the involvement of thioredoxin in the transcriptional induction of the *katG* gene are under investigation in our laboratory.

Ionizing radiation and H_2O_2 induce a wide variety of types of DNA damage. These include strand breaks, purine, and pyrimidine base damage, and damage to the deoxyribose moiety.[48–50] If left unrepaired, these types of damage can have lethal and mutagenic consequences. This DNA damage might initiate a regulatory signal causing the simultaneous $recA^+$ $lexA^+$-regulated expression of a number of genes including the *recA* gene in *E. coli*.[36,37] In the exponentially growing phase, the level of β-galactosidase activity induced by H_2O_2 correlated well to the cell survival after treatment with H_2O_2 (Fig. 2). The results demonstrated that an increased activity of HPI reduced the intracellular concentration of H_2O_2, which decreased the amount of DNA damage. It is also possible that thioredoxin exerts its effect by influencing one of the DNA repair systems. The involvement of thioredoxin redox system in the DNA repair pathways merits further investigation. The H_2O_2-inducible repair of oxidatively damaged DNA may also be relevant.[51,52]

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