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Effects of Cystine and Hydrogen Peroxide on Glutathione Status and Expression of Antioxidant Genes in *Escherichia coli*

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Abstract—Cysteine or cystine was earlier shown to multiply enhance the toxic effect of hydrogen peroxide on *Escherichia coli* cells. In the present work, the treatment of *E. coli* with H_2O_2 in the presence of cystine increased fivefold the level of extracellular oxidized glutathione ($GSSG_{out}$) and decreased fivefold the $GSH/GSSG_{out}$ ratio (from 16.8 to 3.6). The same treatment of cells with deficiency in glutathione oxidoreductase (GOR) resulted in even more severe oxidation of GSH_{out}, so that the level of oxidized glutathione exceeded that of reduced glutathione and the $GSH/GSSG_{out}$ ratio decreased to 0.4. Addition of cystine to the GOR deficient cells resulted in significant oxidation of extracellular glutathione even in the absence of oxidant and in tenfold increase in intracellular oxidized glutathione along with a decrease in the $GSH/GSSG_{out}$ ratio from 282 to 26. However, in the cytoplasm of wild type cells, the level of oxidized glutathione ($GSSG_{in}$) was changed insignificantly and the $GSH/GSSG_{in}$ ratio increased by 26% (from 330 to 415). Data on glutathione status and cystine reduction in the *E. coli gsh* and *gor* mutants suggested that exogenous cystine at first should be reduced with extracellular GSH outside the cells and then imported into them. The high toxicity of H_2O_2 in the presence of cystine resulted in disorders of membrane functions and inhibition of the expression of genes including those responsible for neutralization of oxidants and DNA repair.

Key words: hydrogen peroxide, cystine, gene expression, reduced and oxidized glutathione, *Escherichia coli*

Addition of H_2O_2 to aerobic culture of *Escherichia coli* resulted in the dose-dependent inhibition of the bacteria growth and their death because of DNA damage [1]. The mechanism of the damaging effect of H_2O_2 on DNA is well studied. Hydrogen peroxide itself does not oxidize DNA, but during the Fenton reaction it rapidly interacts with Fe^{2+} and generates an extremely reactive hydroxyl radical which causes multiple damages to this macromolecule. Addition of cysteine or cystine prior to treatment of *E. coli* with H_2O_2 multiply enhanced the cell death. The effect of cysteine (cystine) was prevented in the presence of iron chelators 2,2′-dipyridyl and desferrioxamine and was associated with increase in the concentration of intracellular cysteine upon addition of cystine into the medium [2, 3]. Intracellular cysteine reduced Fe^{3+} and thus maintained the high level of Fe^{2+} and promoted intense production of hydroxyl radicals in the cytoplasm in the presence of H_2O_2. As distinguished from cysteine, glutathione (GSH) also capable of reducing Fe^{3+} *in vitro*, although at a lower rate, did not induce oxidative damages. Nevertheless, *E. coli* cells deficient in synthesis of glutathione and glutathione oxidoreductase (GOR) were more resistant to H_2O_2-induced death in the presence of cystine due to lower concentration of intracellular cysteine in these strains. The authors concluded that the transport of cystine was likely to require reduced glutathione as a cofactor, although the mechanism of this interrelationship remained obscure [3].

The status of glutathione during oxidative stress strongly depended on the kind of oxidant [4, 5]. However, glutathione itself can influence the activity of antioxidant systems [6-8]. The combined effect of H_2O_2 and cystine is an interesting example of oxidative stress differentiated from that induced by H_2O_2 alone. The present work was mainly designed to determine the status of glutathione and its role in the transport of cysteine and in the expression of antioxidant genes in *E. coli* under the influence of H_2O_2 in the presence of cystine.

MATERIALS AND METHODS

Bacteria and culture conditions. Bacterial strains used in the present work are listed in Table 1. Aerobic cultures of *E. coli* were grown on minimal M9 medium [9] supplemented with 0.2% glucose, 0.2% casaminic acids,

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Table 1. *Escherichia coli* strains used in the work

Strain	Genotype	Source or reference
AB1157	F⁻ *thr-1 leu*B6 *pro*A2 *his-4 thi-1 arg*E2 *lac*Y1 *gal*K2 *rps*L *sup*E4 *ara-14 xyl-15 mtl-1 tsx-33*	Coli Genetic Stock Center
JTG10	similarly to AB1157, but *gsh*A (*gsh*A20::*Tn10 km* ⁻)	B. Demple
NM23	similarly to AB1157, but pKT1033 (*kat*G::*lac*Z)	[Oktyabrsky et al. 2001]
NM11	similarly to JTG10, but pKT1033 (*kat*G::*lac*Z)	[Oktyabrsky et al., 2001]
GE397	(MC4100) Δ(*gal-G*[λ]), *b2*(λ) ::[*Φ*(*rec*A-*lac*Z⁺)1 *lac*Y⁺]	G. Weinstock
DM4000	*his*G4 *arg*E3 *thr-1 ara-14 xyl-5 mtl-1 rps*L31 *tsx-33 ilv* TS *sfi*A::*Mu-d1(bla lac) cam*	M. Volkert
CH1366	F⁻ *ara*D139 Δ(*arg*F-*lac*) U169 *rps*L150 *rel*A1 *flb*B5301 *pts*F25 *deo*C1 *pro*U1706::*Mu- dl1681(kanr) pro*U::*lac*Z	J. Booth
AE1318	*ara*D139 Δ(*arg*F-*lac*)169 *flh*D5301 Δ(*his-grd*)295 *rel*A1 *rps*L150 *deo*C1	A. Eisenstark
AE1319	similarly to 1318, but (*gor* ⁻¹::*Muc*)	A. Eisenstark
SH646	F⁻ *ggt-2 recA56 rps*L *sr1300* :: *Tn10*	H. Suzuki
SH641	similarly to SH646, but *ggt*	H. Suzuki
RI89	MC1000 *pho*R Δ*ara-714 leu*⁺	J. Beckwith
RI242	similarly to RI89, but *dsb*D::mini-Tn10 Kanr	J. Beckwith
RI385	similarly to RI89, but *dsb*ABD	J. Beckwith

and thiamine (1 μg/ml). Night culture cells were centrifuged, resuspended in 100 ml of fresh medium, and then grown at 37°C in 250-ml flasks on shakers at 150 rpm. The cell growth was followed by changes in the absorption at 670 nm. H_2O_2 or menadione (MD) was added to the medium when the culture density became 0.4 g dry cells per liter. Cystine was added 20 min before the addition of H_2O_2.

All reagents for determination of GSH and GSSG, casaminic acids, and thiamine were from ICN Biomedicals, Inc. (USA) or from Sigma (USA). All other reagents used in this work were of analytical purity.

Determination of GSH, GSSG, and total acid-soluble thiols. Intra- and extracellular glutathione was determined by spectrophotometry [10] with modifications described in [5]. Extracellular total acid-soluble thiols were determined with DTNB as described in [11] using cysteine as a standard for calibration.

Other methods. Intracellular K^+ was determined with a flame photometer in samples taken by rapid filtration across a membrane filter [12]. Gene expression was assessed by determination of β-galactosidase activity in the strains carrying fusions of the corresponding gene promoters with the structural gene of β-galactosidase [9]. Protein was determined by the Lowry method with BSA as a standard [13]. If necessary, the cells were broken by ultrasonication at 0°C (six 30-sec cycles). The H_2O_2 concentration in the medium was determined by changes in the absorption at 240 nm [14] in the samples taken by filtration across a membrane filter.

Statistics. Each result presents the mean value of at least three independent experiments ± standard error of the mean. Student's *t*-test was used for analysis. Data were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Growth of bacteria in the presence of H_2O_2 and cystine. Addition of 0.1 mM cystine or cysteine to the culture medium had virtually no affect on the growth rate of the aerobic bacterium *E. coli* AB1157. Addition of H_2O_2 (1 mM) alone decreased the growth rate by 25%, whereas treatment of the cells with 1 mM H_2O_2 plus 0.1 mM cystine or cysteine fully inhibited the cell growth. In the last case, cystine was added 20 min before the addition of H_2O_2. Treatment with 1 mM H_2O_2 of *E. coli* JTG10 (*gsh*A) cells with glutathione synthesis deficiency in the

presence of 0.1 mM cystine did not arrest the growth, although it markedly inhibited it. The combined treatment with cystine and H_2O_2 of *E. coli* AE1319 (*gor*) cells with glutathione oxidoreductase deficiency decreased the growth rate nearly fivefold as compared to the control, but, similarly to the case of *gsh*A mutants, the growth was not suppressed completely. These findings are consistent with earlier data on the bacteriostatic effect of H_2O_2 in the presence of cystine or cysteine and unfavorable role of glutathione in resistance to the combined effect of these compounds [2, 3].

Changes in extracellular thiols. The level of thiols in the extracellular medium of *E. coli* AB1157 before the addition of cystine did not exceed 0.01 mM, which corresponded to the concentration of external reduced glutathione. The addition of 0.1 mM cystine rapidly increased the level of thiols; after 10 min it was about 0.2 mM, and then it began to gradually decrease (Fig. 1). As the complete reduction of 0.1 mM cystine had to produce 0.2 mM cysteine, it was suggested that *E. coli* could reduce cystine outside the cells and then transport cysteine into the cytoplasm. As differentiated from the parental strain, in the culture of *E. coli* JTG10 (*gsh*A) the addition of cystine only slightly increased the level of thiols in the medium (by no more than 0.03 mM). *E. coli* strain AE1319 (*gor*) retained the ability for reducing cystine, although at a lower rate than the wild type cells. The maximum concentration of thiols in the medium of the gene *gor* mutants was significantly lower than in the culture of *E. coli* AB1157 and occurred only 30-40 min after the addition of cystine.

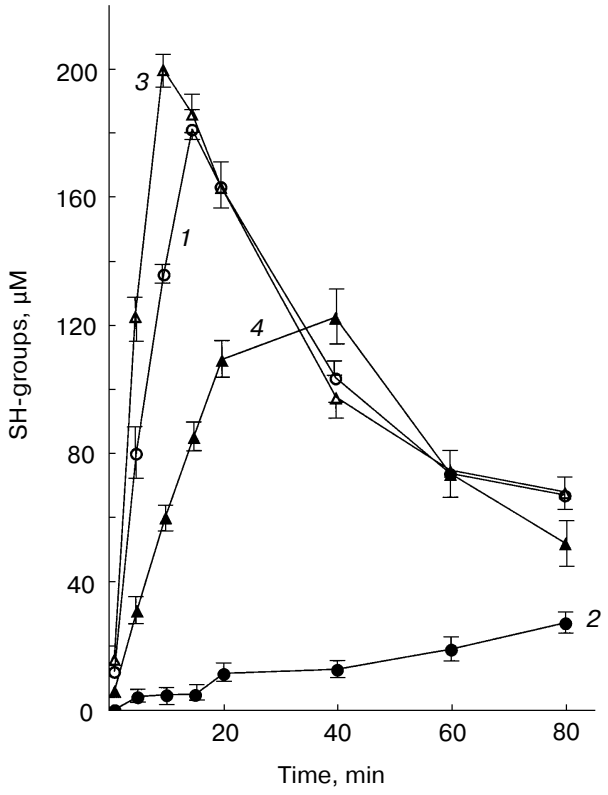

Fig. 1. Reduction of cystine by *E. coli* cells: *1*) AB1157; *2*) JTG10 (*gsh*); *3*) SH641 (*ggt*); *4*) AE1319 (*gor*). At moment "0", the culture was supplemented with 0.1 mM cystine. The first sample was taken immediately after the addition of cystine.

γ-Glutamyl transpeptidase (GGT) in *E. coli* is an enzyme involved in metabolism of glutathione. The cells with the *ggt* gene mutation and also wild type bacteria treated with inhibitors of GGT accumulated in the medium an increased amount of glutathione [15, 16]. In our experiments, the GGT-deficient *E. coli* SH641 (*ggt*) reduced cystine at a higher rate than *E. coli* AB1157 (Fig. 1). It is interesting that reduced cysteine was taken up by *ggt* mutants at the same rate as by the parental strain cells. Consequently, under the experimental conditions GGT was not directly involved in the transport of cysteine. These finding suggested that reduction of cystine should occur with involvement of extracellular reduced glutathione.

The addition of H_2O_2 after the introduction of cystine dramatically decreased the concentration of thiols in the medium to the basal level owing to rapid oxidation of cysteine to cystine under the influence of H_2O_2. The ability of H_2O_2 for oxidizing cysteine or preventing cystine reduction to cysteine with involvement of extracellular GSH seemed to explain the earlier observation: the addition of H_2O_2 before introduction of cysteine or cystine did not accelerate the death of *E. coli* cells [2]. Although the *E. coli* bacteria have a CysB-regulated system of cystine transport, it seemed that in our experiments the bulk of cystine entered the cells as cysteine through the system of transport of the latter.

Status of extracellular glutathione. Because cystine was reduced with involvement of external GSH, it was interesting to follow changes in the pool and redox status of glutathione inside and outside the cells.

In the growing aerobic *E. coli* cells, extracellular glutathione is mainly reduced, and the concentration of its oxidized form outside the cell is an order higher than inside. And respectively, the GSH/GSSG in the medium is 20-fold lower than inside the cell (Table 2) [5]. In the wild type bacteria, the addition of 0.1 mM cystine increased 1.7-fold the concentration of external reduced glutathione (GSH_{out}). Treatment with 1 mM H_2O_2 slightly decreased the level of GSH_{out}, and addition of 100 mM cystine 20 min before the introduction of 1 mM H_2O_2 decreased at first the GSH_{out} pool 2.4-fold and then increased it to the control level. If 0.1 mM cystine or 1 mM H_2O_2 were added separately, the concentration of external oxidized glutathione ($GSSG_{out}$) remained the same as in the control. However, the pretreatment of *E. coli* AB1157 cells with cystine succeeded by addition of 1 mM H_2O_2 increased the level of $GSSG_{out}$ 4.7-fold (Fig. 2). The GSH/$GSSG_{out}$ ratio increased 1.6-fold in the cul-

Table 2. Status of intra- and extracellular glutathione upon treatment of *E. coli* AB1157 cells (wt) with cystine and H_2O_2

Treatment	Intracellular glutathione[1]			Extracellular glutathione[1]		
	GSH	GSSG	GSH/GSSG	GSH	GSSG	GSH/GSSG
Control	18.8 ± 0.6	0.057 ± 0.005	330	6.9 ± 0.5	0.41 ± 0.02	16.8
0.1 mM cystine[2]	16.8 ± 0.15	0.059 ± 0.011	285	10.3 ± 0.3	0.38 ± 0.01	27.1
1 mM H_2O_2[3]	20.8 ± 2.3	0.043 ± 0.005	484	6.4 ± 0.3	0.46 ± 0.03	13.9
1 mM H_2O_2 + 0.1 mM cystine[4]	28.2 ± 3.1	0.068 ± 0.009	415	7.0 ± 1.3	1.92 ± 0.12	3.6

[1] μmol/g dry weight.
[2] 80 min after the addition of cystine.
[3] 60 min after the addition of H_2O_2.
[4] 80 min after the addition of cystine. The bacteria were treated with cystine for 20 min and then with H_2O_2.

tures treated with cystine alone and decreased 4.7-fold after the *E. coli* AB1157 were subjected to combined treatment with 0.1 mM cystine and 1 mM H_2O_2 (Table 2).

Thus, in wild type cells the reduction of cysteine with involvement of glutathione was not accompanied by exhaustion of GSH_{out} or accumulation of $GSSG_{out}$. According to calculations, the concentration of GSH_{out} was 60-fold lower than required for the complete reduction of exogenous cystine. Consequently, the glutathione oxidized during reduction of cystine was to be continuously reduced by the cells. External GSSG could be reduced by several pathways. First, through a transmembrane transfer of reducing equivalents onto external GSSG from the cytoplasm, which could involve known periplasmic redox proteins or a hypothetical redox carrier. Second, through a rapid transmembrane circulation of glutathione itself when oxidized glutathione, on entering the cells, was reduced in the cytoplasm with involvement of glutathione oxidoreductase. In this case, the diminution of glutathione in the medium was compensated at the cost of the reduced glutathione export from the cytoplasm.

Proteins Dsb catalyze SH–SS transitions in proteins of the *E. coli* periplasm. To test the possible involvement of DsbD, which usually acts as a donor of reducing equivalents for DsbC disulfide isomerase in reduction of $GSSG_{out}$ [17, 18], we determined the rate of cystine reduction in cultures of *E. coli* RI242 (*dsb*D) and RI385 (*dsb*ABD). The rate of cystine reduction in both strains was the same as in the wild type cells, which indicated a minor role of these redox proteins in reduction of $GSSG_{out}$. On the contrary, the *E. coli* AE1319 mutant in the gene *gor* reduced cystine at a significantly lower rate than wild type cells (Fig. 1). This suggested that GOR was involved in reduction of external oxidized glutathione. Because GOR was located inside the cell cytoplasm, a rapid transmembrane circulation of glutathione was likely to occur, although a hypothetical transmembrane carrier could also exist responsible for exchange of reducing equivalents between intracellular GSH and external

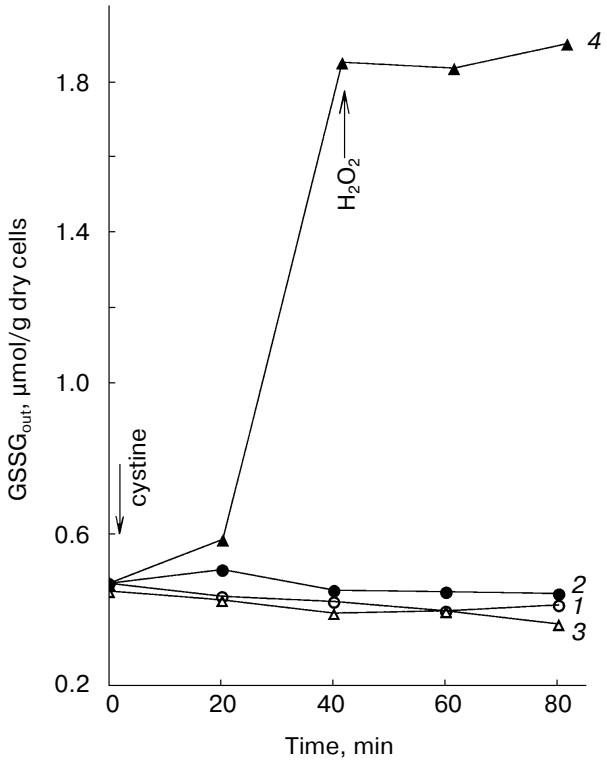

Fig. 2. Effect of H_2O_2 and cystine on the extracellular level of GSSG in aerobically cultured *E. coli* AB1157: *1*) control; *2*) 0.1 mM cystine; *3*) 1 mM H_2O_2; *4*) 1 mM H_2O_2 + 0.1 mM cystine. In the last case, cystine was added 20 min before the cell treatment with H_2O_2.

Table 3. Status of intra- and extracellular glutathione upon treatment of *E. coli* AE1319 cells (*gor*) with cystine and H_2O_2

Treatment	Intracellular glutathione[1]			Extracellular glutathione[1]		
	GSH	GSSG	GSH/GSSG	GSH	GSSG	GSH/GSSG
Control	22.6 ± 0.7	0.08 ± 0.009	282	4.8 ± 0.3	2.8 ± 0.2	1.7
0.1 mM cystine, 20 min[2]	27.1 ± 1.7	1.05 ± 0.2	26	2.7 ± 0.15	7.5 ± 0.06	0.36
0.1 mM cystine, 80 min[3]	48.4 ± 3.2	0.15 ± 0.03	323	7.9 ± 0.5	9.5 ± 0.16	0.83
1 mM H_2O_2[4]	25.5 ± 0.7	0.09 ± 0.02	283	4.4 ± 0.2	3.6 ± 0.18	1.2
1 mM H_2O_2 + 0.1 mM cystine[5]	36.3 ± 1.2	0.14 ± 0.01	259	4.8 ± 0.4	12.0 ± 0.2	0.4

[1] μmol/g dry cells.
[2] 20 min after the addition of cystine.
[3] 80 min after the addition of cystine.
[4] 60 min after the addition of H_2O_2.
[5] 80 min after the addition of cystine. Cystine was added into the medium 20 min before H_2O_2.

GSSG. To study in more detail the role of GOR in reduction of cystine, we examined changes in the level and redox status of glutathione in culture of *E. coli* AE1319.

The treatment of *E. coli* AE1319 with 1 mM H_2O_2 caused no significant changes in the levels of GSH_{out} and $GSSG_{out}$ (Table 3). And whereas addition of 0.1 mM cystine to the wild type cells increased the concentration of external reduced glutathione, in the *E. coli* AE1319 culture cystine decreased the pool of GSH_{out} and increased the level of oxidized glutathione. In 20 min after the addition of cystine into the medium GSSG was the bulk of external glutathione, and the ratio $GSH/GSSG_{out}$ decreased sixfold as compared to the control. During the next 60 min, the concentration of GSH_{out} was increasing and by the culture termination was twofold higher than in the control. Thus, reduction of cystine by *E. coli* AE1319 was accompanied by rapid oxidation of external glutathione even in the absence of the oxidant.

The treatment of *E. coli* AE1319 cells with 1 mM H_2O_2 in the presence of cystine resulted in complete oxidation of external glutathione in 20 min after the addition of H_2O_2. Then the level of GSH_{out} began to increase, but $GSSG_{out}$ remained the bulk of glutathione. The $GSH/GSSG_{out}$ ratio decreased to 0.4, which was the lowest level observed in our experiments.

Status of intracellular glutathione. Concentration of intracellular glutathione in *E. coli* was shown to increase on addition into the medium of cysteine, cystine, or casaminic acids [19]. This effect was explained by limitation of the GSH synthesis caused by availability of L-cysteine, and an increase in the concentration of this amino acid or its precursors should accelerate the synthesis of GSH. The addition of 0.5 mM cystine to the culture of *E. coli* AB1157 growing on medium supplemented with sulfate as the only source of sulfur, increased twofold the level of glutathione in the cytoplasm [3]. In our experiments *E. coli* cells grew on M9 medium in the presence of casaminic acids, and the addition of 0.1 mM cystine to the culture caused only a transient 16% increase in the concentration of intracellular glutathione. By the termination of the experiment, the level of glutathione became lower than the control value (Table 2). The treatment with 1 mM H_2O_2 caused nearly the same increase in the pool of cytoplasmic glutathione. However, combined treatment with cystine and H_2O_2 increased 1.5-fold the content of intracellular glutathione. Under all treatments, the level of oxidized glutathione was changed slightly, and upon treatment of *E. coli* AB1157 cells with 1 mM H_2O_2 in the absence and in the presence of 0.1 mM cystine the $GSH/GSSG_{in}$ ratio was higher than in the control.

Introduction of 1 mM H_2O_2 into the culture of *E. coli* AE1319 (*gor*) slightly increased the concentration of intracellular glutathione, whereas addition of 0.1 mM cystine increased the GSH_{in} level more than twofold (Table 3). The treatment of *E. coli* AE1319 cells with 1 mM H_2O_2 in the presence of 0.1 mM cystine significantly decelerated the cystine-induced increase in the cytoplasmic glutathione pool. It should be emphasized that the cystine-caused increase in the concentration of GSH_{in} was a response of the *gor*-mutant bacteria to the 13-fold increase in the level of $GSSG_{in}$. In 20 min after the addition of cystine, the $GSH/GSSG_{in}$ ratio was 11-fold lower than that in the control. The GSH_{in} level increased with the decrease in $GSSG_{in}$. As a result,

80 min after the addition of cystine the $GSH/GSSG_{in}$ ratio returned to the normal value.

Thus, even the GOR-deficient *E. coli* strain cells retained the ability for maintaining the optimal ratio of $GSH/GSSG_{in}$, although it was achieved with greater difficulties than in the case of wild type bacteria, which failed to respond to addition of cystine by significant changes in the redox status of intracellular glutathione.

In general, the experimental findings lead to some conclusions. First, cystine added into the medium was reduced by *E. coli* with involvement of external GSH and glutathione oxidoreductase and then transported into the cells as cysteine. Second, the treatment of wild type cells with H_2O_2 in the presence of cystine shifted the $GSH/GSSG_{out}$ ratio to oxidized values at the cost of increase in $GSSG_{out}$, whereas in the cytoplasm this ratio was shifted to reduced values due to increase in the concentration of GSH_{in}. Third, severe oxidative damage of DNA caused by the combined treatment of *E. coli* cells with cystine and H_2O_2 [3] was not prevented in the presence of high concentrations of intracellular GSH which is thought to be important for destruction of hydroxyl radicals. On the contrary, in the above-described situation the high value of the $GSH/GSSG_{in}$ ratio was likely to promote the maintaining of the reduced state of intracellular cysteine and thus to accelerate the cyclic reduction of Fe^{3+} and production of hydroxyl radical during the Fenton reaction.

Along with the decelerated reduction of cystine outside the cells, the oxidative DNA damage in the bacteria *gor*-mutant could be, in particular, attenuated due to decrease in the redox status of glutathione and, as a result, the lower ability for maintaining the reduced state of the pool of intracellular cysteine. Based on the findings, a sequence of events was supposed during the combined exposure of *E. coli* cells to cystine and H_2O_2 (Fig. 3). This sequence is a development of the scheme proposed in [3] and includes the reduction of cystine to cysteine outside of the cells with involvement of external GSH and entrance of cysteine into the cells through the system of its transport.

The role of glutathione during the treatment of *E. coli* with H_2O_2 in the presence of cystine seems to be a manifestation of various and sometimes contradictory functions of this compound. On one hand, the ability of GSH for reducing cystine outside of the cells is favorable for bacteria because they get an available and easily assimilated source of sulfur. On the other hand, this leads to a temporary disorder of cysteine homeostasis in the cytoplasm, which normally is kept at a very low level [3]. In the presence of H_2O_2, this disorder of cysteine homeostasis can be fatal, resulting in cell death. The high concentration of intracellular GSH can be also favorable because it promotes destruction of reactive oxygen species, but in the above-described situation the high GSH/GSSG ratio can maintain intracellular cysteine in the reduced state

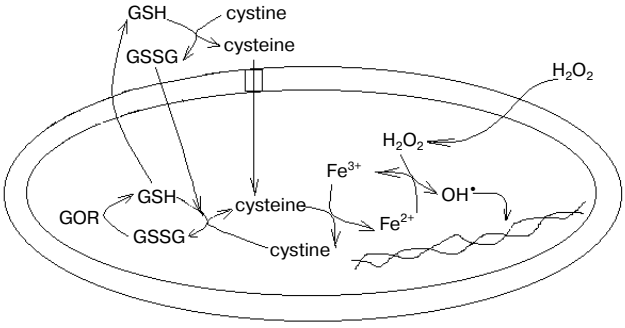

Fig. 3. Scheme presenting the mechanism of oxidative damage of *E. coli* under the influence of cystine and H_2O_2. It is supposed that cystine is reduced to cysteine in the medium with involvement of external GSH and then cysteine enters the cells through the system of its transport. GSSG produced during reduction of cystine is reduced with involvement of intracellular GOR as a result of the transmembrane circulation of glutathione or by transfer of reducing equivalents from intracellular GSH onto external GSSG with involvement of a hypothetical transmembrane redox carrier. Inside the cell, cysteine reduces Fe^{3+} and thus maintains the high pool of Fe^{2+} and in the presence of H_2O_2 provides for production of toxic hydroxyl radicals during the Fenton reaction.

with the resulting increased generation of hydroxyl radicals. Finally, glutathione can play a positive role as a reserve form of cysteine, an excess amount of this potentially toxic substance to be incorporated into GSH.

Note also that reduction of cystine with involvement of external glutathione seems to exemplify the general mechanism of extracellular reduction of various compounds capable of reacting with the SH-group of glutathione. Thus, external GSH was shown to protect the growth of *E. coli* and *S. typhimurium* cells against inhibition with micromolar concentrations of mercury, silver, cadmium, and iodoacetamide [20]. In our experiments, extracellular GSH was involved in reduction of the impenetrable oxidant ferricyanide. The ferricyanide reductase activity of the *E. coli* cells deficient in GSH synthesis was 41% decreased as compared to that of the wild type strain. Reduction of ferricyanide by the bacteria *E. coli* AB1157 was accompanied by the dose-dependent decrease in the concentration of intracellular glutathione [6, 21].

Expression of antioxidant genes. The effect of H_2O_2 on *E. coli* cells is associated with expression of many genes whose products influence activities of various metabolic pathways responsible for retaining biochemical homeostasis and survival under changes in the medium [22]. Some of the inducible genes including the *kat*G encoding catalase hydroperoxidase I (HPI) are regulated by the transcription factor OxyR. Genes of the SOS-regulon (*rec*A, *sfi*A, etc.), which play an essential role in repair of DNA damages, are also important for the cell defense against oxidative stress. It was interesting to clarify the effect of the cell treatment with H_2O_2 in the presence of cystine on the expression of antioxidant genes.

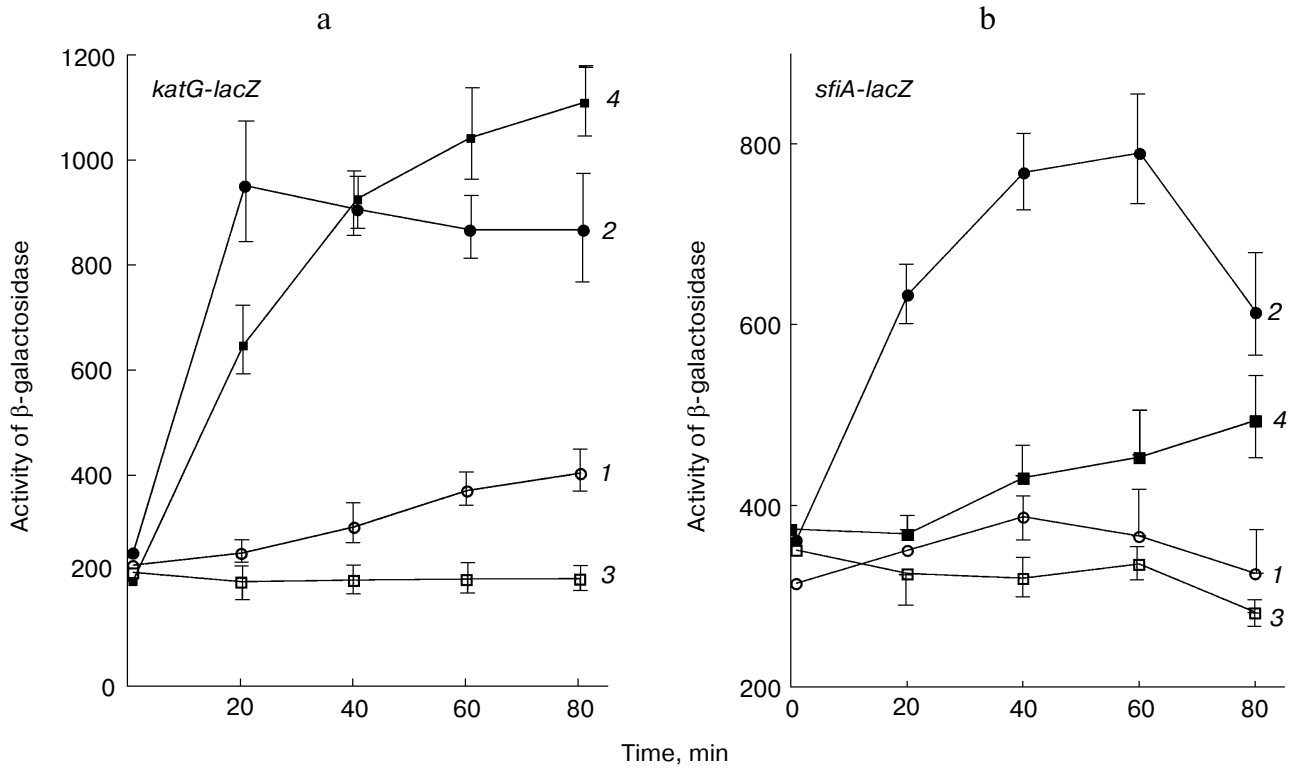

Fig. 4. Effects of H_2O_2 and cystine on expression of *kat*G::*lac*Z (a) and *sfi*A::*lac*Z (b) in *E. coli* cells: *1*) control; *2*) 1 mM H_2O_2; *3*) 1 mM H_2O_2 + 0.1 mM cystine; *4*) prior to addition of 1 mM H_2O_2 + 0.1 mM cystine the cells were treated with 0.2 mM chelator 2,2′-dipyridyl. a) Strain NM23 carrying the plasmid KT1033 with the fusion *kat*G::*lac*Z; b) DM4000 carrying the fusion *sfi*A::*lac*Z.

The level of gene expression was determined by changes in the activity of β-galactosidase in *E. coli* strains carrying fusions of promoters of the genes studied with the structural gene of β-galactosidase.

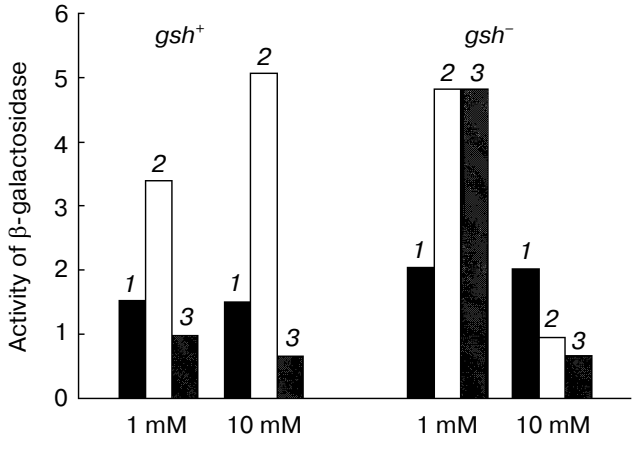

Fig. 5. Effect of *gsh*A mutation on *kat*G expression in aerobically growing *E. coli* cells. The strains NM23 (*gsh*$^+$) and NM11 (*gsh*$^-$) carrying the fusion *kat*G::*lac*Z were treated with 1 or 10 mM H_2O_2. The β-galactosidase activity 1 h after the cell treatment with H_2O_2 was considered to characterize the induction of *kat*G::*lac*Z. *1*) Control; *2*) cells treated with H_2O_2; *3*) treatment with H_2O_2 + 0.1 mM cystine.

The treatment of *E. coli* with cystine had virtually no effect on the activity of β-galactosidase in the *E. coli* strain NM23 carrying the fusion *kat*G::*lac*Z. As expected, the treatment with 1 mM H_2O_2 significantly increased the expression of *kat*G::*lac*Z, but the pretreatment of the cells with 0.1 mM cystine completely inhibited the expression of *kat*G induced by H_2O_2 (Fig. 4a). The addition to the medium of the chelator 2,2′-dipyridyl (0.2 mM) abolished the inhibitory effect of cystine on the expression of *kat*G::*lac*Z. These data suggest that the earlier observed inhibition of the catalase activity [3] in this case can occur on the level of transcription.

It was shown that the *gsh*-deficient *E. coli* exposed to moderate doses of H_2O_2 displayed both higher expression of *kat*G::*lac*Z and higher catalase activity [6, 8]. The same effect was also observed in the present work. In the *gsh*A-mutants treated with 1 mM H_2O_2 the expression of *kat*G::*lac*Z was markedly higher than in the parental type cells (Fig. 5).

The pretreatment of *gsh*A mutants with cystine did not inhibit the *kat*G::*lac*Z expression induced by 1 mM H_2O_2. The presence of cystine also influenced kinetics of the *kat*G::*lac*Z expression in the *E. coli* cells mutant in *gsh*A. Thus, the maximum activity of β-galactosidase on the treatment of *E. coli* NM11 with 1 mM H_2O_2 was observed even after 15-20 min, whereas in the presence of

cystine the maximum expression of *kat*G::*lac*Z was achieved only 80-100 min after the addition of H_2O_2. Thus, the deficiency of glutathione significantly changed the kinetics and level of the *kat*G::*lac*Z expression in the cells treated with cystine and H_2O_2.

At high concentrations of H_2O_2 (10 mM) the expression of *kat*G::*lac*Z in the glutathione-deficient *E. coli* cells was lower than in the parental type cells (Fig. 5) [8]. The addition of cystine prior to the treatment of the cells with 10 mM H_2O_2 inhibited the expression of *kat*G::*lac*Z in the *gsh*A mutant similarly to that observed in the parental type cells.

Note that the presence of 0.1 mM cystine in the medium had virtually no effect on the induction of *kat*G::*lac*Z by the superoxide producer menadione (0.2 mM). Cystine decreased 35% the induction by menadione of another antioxidant gene *sox*S involved in the response to superoxide.

However, the manifold increase in the toxicity of H_2O_2 in the presence of cystine cannot be ascribed only to decrease in the catalase activity [2]. The damages of DNA including those induced by H_2O_2 are repaired with involvement of a group of genes controlled by the protein RecA. The activity of this regulon is likely to be even more significant for resistance of *E. coli* to H_2O_2 than the activity of catalase [1, 23]. In our experiments cystine inhibited the expression of *rec*A::*lac*Z in response to the addition of 1 mM H_2O_2. The gene *sfi*A is controlled by *rec*A, and its expression increased in *E. coli* cells treated with H_2O_2 [24]. The expression of *sfi*A::*lac*Z induced by 1 mM H_2O_2 was fully inhibited in the presence of 0.1 mM cystine. The pretreatment of cells with 2,2′-dipyridyl (0.2 mM) partially recovered the expression of *sfi*A::*lac*Z (Fig. 4b).

The data presented above suggest that oxidative stress caused by H_2O_2 in the presence of cystine can suppress not only the abovementioned genes involved in the defense of *E. coli* against oxidative stress, but also other genes that are not directly associated with oxidative stress. To check this hypothesis, we determined the activity of β-galactosidase in the *E. coli* strain CH1366 carrying the fusion *pro*U::*lac*Z. The gene *pro*U encodes the transport system of glycine betaine, and its expression increases with increase in osmotic pressure of the medium. On addition of 0.3 mM NaCl into the medium M9 + CA the expression of *pro*U::*lac*Z increased during 1 h more than 11 times (from 84 to 926 Miller units). In the untreated cells, the activity of β-galactosidase did not change during this period. The pretreatment of the cells with 0.1 mM cystine or 1 mM H_2O_2 did not markedly influence the NaCl-induced expression of *pro*U::*lac*Z, whereas the pretreatment with 0.1 mM cystine + 1 mM H_2O_2 completely inhibited the NaCl-induced expression of *pro*U::*lac*Z. Therefore, it was suggested that stress caused by the combined influence of H_2O_2 and cystine should totally inhibit the expression of genes, including those responsible for neutralization of antioxidants and DNA repair.

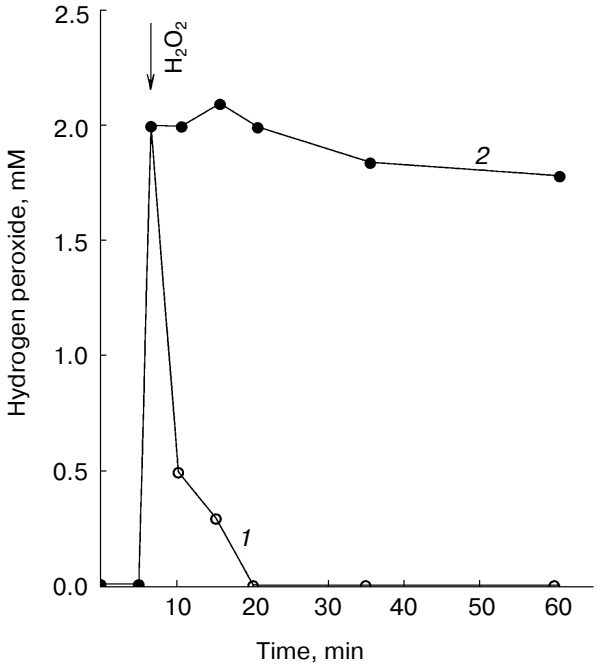

Fig. 6. Effect of cystine on destruction of H_2O_2 by *E. coli* AB1157 cells: *1*) control (the arrow indicates the moment of addition of 2 mM H_2O_2); *2*) 0.1 mM cystine was added to the medium 20 min before the cell treatment with H_2O_2.

Because chelating of iron and deficiency of cells in glutathione synthesis completely or partially prevented the inhibitory effect of cystine and H_2O_2 on the gene expression in *E. coli* cells, it was suggested that disorders in the gene expression should be originally caused by oxidative damage to DNA. Chelators bind free iron, and the lack of glutathione in *gsh*A$^-$ mutants prevent cystine reduction to cysteine in the medium and its accumulation in the cytoplasm lowering the rate of Fe^{3+} reduction to Fe^{2+} with involvement of cysteine. In both cases, the deficiency of free bivalent iron limits cyclic reactions between H_2O_2 and Fe^{2+} resulting in generation of hydroxyl radicals and damaging DNA. An intense oxidative damage of DNA can inhibit transcriptions with involvement of DNA and RNA polymerases. The decrease in expression of genes involved in DNA repair has, in its turn, to decelerate the repair, with the associated increased accumulation of oxidative damages. Moreover, inhibition of *kat*G expression can decrease the ability of cells for degrading H_2O_2. In fact, in the presence of cystine the growing *E. coli* cells nearly completely lost their ability for degrading H_2O_2 (Fig. 6).

Maintaining a relatively high level of H_2O_2 in the medium can also influence the structure and functions of cellular membranes. Thus, the treatment of *E. coli* cells with non-lethal doses of H_2O_2 inhibits transport processes both dependent and independent of the proton-motive force [25]. The mechanism of the inhibitory effect of H_2O_2 on transport remains unclear. In particular, oxida-

tion of essential sulfhydryl groups in membrane proteins can be important. The significant increase in the concentration of $GSSG_{out}$ in the culture of *E. coli* AB1157 upon the addition of cystine and H_2O_2 (Fig. 2) is likely to indirectly confirm a strong oxidation of SH-groups of proteins on the outer side of the cytoplasmic membrane.

The change in the membrane permeability for potassium ions under the combined treatment of *E. coli* cells with cystine and H_2O_2 also indicates disorders in the membrane properties. The addition of 1 mM H_2O_2 to the *E. coli* AB1157 cells had virtually no affect on the intracellular concentration of K^+. The introduction of 0.1 mM cystine into the medium even increased a little the potassium pool in the cells, whereas the addition of 1 mM H_2O_2 in the presence of cystine caused a stable 32% decrease in the cytoplasmic pool of K^+.

Disorders in transport seem to be a factor responsible for inhibition of processes of biosynthesis, which accentuates conditions of the *E. coli* cells treated with cystine and H_2O_2, prevents repair processes, and accelerates cell death caused by oxidative stress.

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