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# Activation of the *Escherichia coli Sox*RS-Regulon by Nitric Oxide and Its Physiological Donors

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**Abstract**—Activation of the *Escherichia coli Sox*RS-regulon by nitric oxide (NO) and its physiological donors (S-nitrosothiol (GS-NO) and dinitrosyl iron complexes with glutathione ($DNIC_{glu}$) and cysteine ($DNIC_{cys}$) ligands) has been studied. To elucidate the molecular mechanisms of signal transduction via nitrosylation of Fe-S-centers in SoxR, the ability of pure NO and NO-producing agents to activate the *Sox*RS-regulon in *E. coli* cells bearing a *sox*S::*lac*Z operon (promoter) fusion has been compared. EPR spectroscopy of whole cells has been used to monitor the formation of inducible protein–DNIC complexes. $DNIC_{cys}$, GS-NO, and pure NO appeared to be potent inducers of *sox*S expression, whereas $DNIC_{glu}$ was considerably less efficient. Thus, lower *in vitro* stability of $DNIC_{cys}$ was in contrast with its higher biological activity. Pretreatment of the cells with *o*-phenanthroline, a chelating agent for iron, prevented *sox*S expression by GS-NO. Treatment of intact *E. coli* cells with DNIC, GS-NO, and NO at equimolar concentration 150 μM resulted in formation of a single EPR-detectable DNIC-type signal with $g = 2.03$. The initial stage in the SoxR transcription activity is supposed to include two steps: first, DNIC primers are formed from exogenous NO and free iron, and then these DNIC disintegrate SoxR [2Fe-2S] clusters and thus activate *Sox*RS-regulon transcription.

*Key words*: nitric oxide, *Sox*RS-regulon, S-nitrosothiols, dinitrosyl iron complexes, *Escherichia coli*

Nitric oxide (NO) and its metabolites are the main cytotoxic agents of macrophages that attack bacterial cells in the bodies of mammals. The resistance of *E. coli* to NO is regulated by the multifunctional *Sox*RS-regulon, which consists of at least ten promoters and is activated by coordinated action of *sox*R and *sox*S [1]. The *Sox*RS system demonstrates how dynamic modulation of gene expression allows resistance to metabolic oxidants and oxidative stress caused by exogenous agents to occur [2]. Along with its main function, oxidative stress protection, the *Sox*RS-regulon provides *E. coli* cells resistance to a wide variety of antibiotics [3, 4], blocking the synthesis of OmpF membrane protein and enhancing the synthesis of AcrA and AcrB, which prevent intracellular accumulation of antibiotics [5].

The *Sox*RS-regulon activation mechanism is not yet completely elucidated; it seems to be more complex than activation of a single transcription factor like a stress response [6].

Detailed study of the *E. coli Sox*RS-regulon began more than ten years ago with the identification of SoxR and SoxS [7]. Nevertheless, until recently these studies were performed almost without exception *in vitro* using $O_2^-$ and NO$^{\cdot}$ as the most efficient *Sox*RS-regulon inducers.

In the present view, *Sox*RS-regulon regulation is a double-stage process. First SoxR changes to an active form, which initiates *sox*S transcription; then increase in the SoxS level activates expression of the whole *Sox*RS-regulon [8, 9]. A structural change of SoxR containing [2Fe-2S] clusters as the starting point for *Sox*RS-regulon induction is the key to understanding the mechanism of *Sox*RS-regulon activation.

## MATERIALS AND METHODS

**Reagents.** The following reagents were used in this study: cysteine, glutathione, and Hepes from Sigma (USA); iron sulfate from Fluka (Switzerland). Dinitrosyl

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iron complexes with cysteine and glutathione were used in the form of dimers and obtained by treatment of 5.4 mM $FeSO_4$ and 10.8 mM glutathione or cysteine (iron/thiol = 1 : 2 w/w) with gaseous NO in a Thunberg vessel under 200-300 mm Hg pressure in 15 mM Hepes solution, pH 7.6, under vacuum. S-Nitrosoglutathione (GS-NO) was synthesized in a Thunberg vessel by treatment of 50 mM glutathione with a mixture of air and gaseous NO for 5 min and subsequent removal of excess NO under vacuum. Nitroso-adduct concentration was determined spectrophotometrically at 340 nm (molar extinction coefficient 980 $M^{-1}\cdot cm^{-1}$). All these reagents were synthesized immediately before an experiment.

**Cell growth and storage.** For bacterial growth and storage and suspension preparation, L-medium was used (composition given in [10]). *E. coli* night culture was grown on L-medium for 3 h to cell titer $(1\text{-}5)\cdot 10^8$ cells/ml ($A_{600}$ = 0.36). For EPR spectroscopy, a suspension was concentrated by centrifugation to titer $(1\text{-}5)\cdot 10^{10}$ cells/ml ($A_{600}$ = 0.42).

**Cell treatment with NO.** Gaseous NO was synthesized by reaction of $FeSO_4$ with $NaNO_2$ in 0.1 M HCl with subsequent purification under vacuum. NO-saturated aqueous solution was prepared by passage of NO into a Thunberg vessel. The limiting NO concentration in solution was 300 μM and that during cell treatment was 150 μM. NO was injected into the cell suspension by syringe taking care that other nitrogen oxides (e.g., $N_2O_3$) did not form.

***sox*S expression.** The *Sox*RS-regulon induction was regulated via *sox*S expression using specially constructed *E. coli* strain TN530 ($F^-$, Δ(*lac*U-*arg*F) U169, rpsL179, *sox*$RS^+$, λφ(Δ*sox*S′::*lac*Z) bearing a *sox*S::*lac*Z operon fusion; in the latter β-galactosidase gene, *lac*Z, is inserted under the control of *sox*S promotor, and in the genome there is a deletion of *lac*Z structural genes so that *lac*Z expression can be performed only simultaneously with *sox*S expression [11]. *sox*S expression was monitored indirectly via β-galactosidase activity according to Nunoshiba el al. [11]. After treatment of bacteria with corresponding reagents for 30 min, 2.7 ml of buffer for β-galactosidase and 0.6 ml of *o*-nitrophenyl-β-D-galactopyranoside (chromogen for β-galactosidase) were added to 0.3 ml of suspension. Cells were incubated for 1 h at 37°C. Color development was stopped by addition of 2 ml of 1 M $Na_2CO_3$.

β-Galactosidase activity was monitored spectrophotometrically at 420 nm. The activity of β-galactosidase (in units) was calculated by the equation:

$$U = 1000 \cdot A_{420}/t,$$

where $A_{420}$ is absorption at 420 nm and $t$ is incubation time.

The buffer composition for determination of β-galactosidase activity was [10]: $Na_2HPO_4$ (32.2 g), $NaH_2PO_4\cdot H_2O$ (11.0 g), KCl (1.5 g), $MgSO_4\cdot 7H_2O$ (0.5 g), SDS (2.0 g), β-mercaptoethanol (5.4 ml), and distilled water (700 ml). After attaining pH 7.7, distilled water was added to final volume 1000 ml.

In comparative experiments, *E. coli* strain TN531 isogenous in relation with TN530, containing *sox*R deletion (TN531:TN530 Δ(*sox*RS)), was used. Negative results in experiments with *sox*R expression in TN531 indicate that color development in experiments with TN530 is caused by the presence of the active *sox*R in this strain [12].

To induce *sox*S expression, cells were treated with NO and its physiological donors, GS-NO and DNIC, containing various SH-ligands at concentrations from 0.01 to 3 mM. In some cases free thiols, glutathione, and cysteine, at concentrations from 10 to 100 mM, were added to the reaction mixture. It should be noted that glutathione is a monothiol, which decompose Fe-S clusters. In contrast, cysteine prevents the action of monothiols [13].

4-Nitroquinolin-N-oxide (4-NQO), a powerful SOS-inducer, was used at concentration 2.63 nM as a standard (positive) control of induction of *sox*S expression.

Each experiment was repeated thrice.

**EPR spectroscopy.** Cells were grown on L-medium with aeration to $A_{600}$ = 0.4. To prepare one EPR sample, 250 ml of culture were centrifuged at 7,000*g* and concentrated to 5 ml. After incubation with NO donors or *o*-phenanthroline for 30 min, the cells were centrifuged again, resuspended in L-medium (0.3 ml), and frozen in calibrated tubes for EPR spectroscopy. X-Band EPR spectra were recorded using a Radiopan spectrometer (Poland) under the following conditions: 77 K, microwave power 5 mW, modulation amplitude 0.5 mT.

## RESULTS

The main results are as follows.

1. Pure NO as well as its physiological donors activated *sox*S expression of the *E. coli Sox*RS-regulon.

2. $DNIC_{cys}$ appeared to be as potent as an inducer of *sox*S expression as NO and GS-NO, whereas $DNIC_{glu}$ was considerably less effective.

3. Treatment of intact *E. coli* cells with DNIC, GS-NO, and NO at equimolar concentration (150 μM) resulted in formation of a single EPR-detectable DNIC-type signal with $g$ = 2.03.

The dose dependence of induction of *sox*S expression with GS-NO and DNIC with various SH-ligands is presented in Fig. 1. As shown, DNIC with cysteine ligands ($DNIC_{cys}$) caused larger *sox*S expression than DNIC with glutathione ligands ($DNIC_{glu}$). The quantitative characteristics of *sox*S expression by GS-NO are comparable with induction by $DNIC_{cys}$.

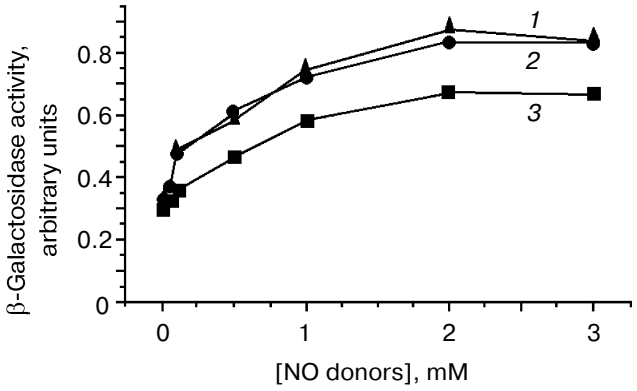



**Fig. 1.** Induction of *E. coli* TN530 *sox*S expression by NO donors. β-Galactosidase activity versus GS-NO (*1*), $DNIC_{cys}$ (*2*), and $DNIC_{glu}$ (*3*) concentration. β-Galactosidase activity was determined as described in "Materials and Methods". All the results are average values of three measurements.

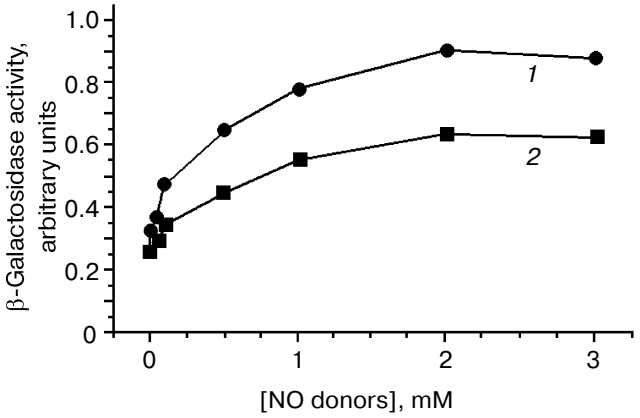



**Fig. 2.** *E. coli* TN530 *sox*S expression versus $DNIC_{glu}$ concentration in the presence of 10 mM free cysteine (*1*) and $DNIC_{glu}$ (*2*). β-Galactosidase activity was determined as described in "Materials and Methods". The results presented are average values of three measurements.

Cell pretreatment with free cysteine increased the level of *sox*S induction on subsequent treatment with $DNIC_{glu}$ (Fig. 2). The maximal positive effect (*sox*S expression increased twice) was observed on cell pretreatment with 10 mM cysteine. Further increase in free cysteine concentration to 100 mM decreased *sox*S induction (Fig. 3).

For more detailed study of the role of iron in induction of *sox*S expression, cells were pretreated with *o*-phenanthroline, an iron chelating agent. On subsequent treatment of these cells with GS-NO, *sox*S expression decreased practically to the base level in the concentration range 0.01-3 mM (Fig. 4).

Treatment of *E. coli* cells with pure NO in the concentration range 0.05-0.15 mM caused *sox*S expression twice as large as a control and equal to *sox*S expression by $DNIC_{cys}$ and GS-NO at the given concentrations.

When *E. coli* TN531 cells with *sox*RS deletion were treated with various inducers, the level of *sox*S expression was the same as the control (table).

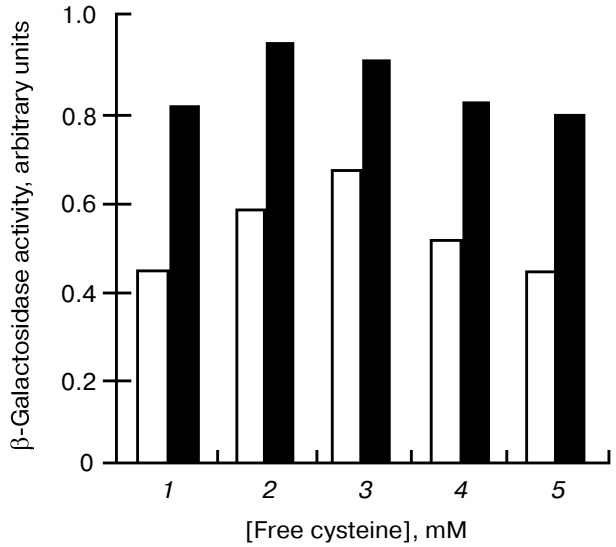



**Fig. 3.** *E. coli* TN530 *sox*S expression by $DNIC_{glu}$ (light columns, 1 mM; dark columns, 2 mM) versus free cysteine concentration (mM): *1*) 5; *2*) 10; *3*) 25; *4*) 50; *5*) 100. The results presented are the average values of three measurements.

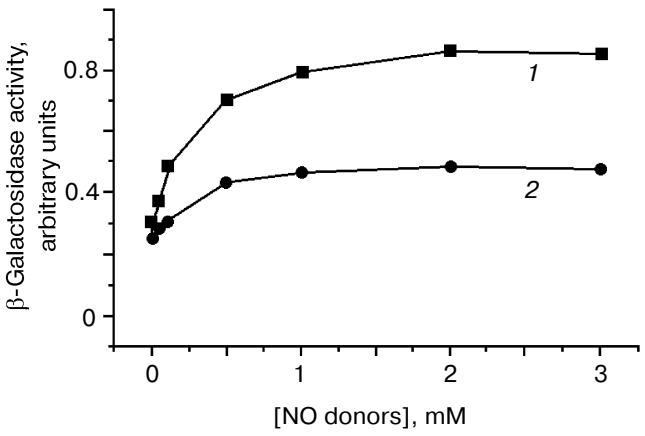



**Fig. 4.** Suppression of S-nitrosoglutathione-induced *sox*S expression (0.01-3.0 mM) (*1*) by *o*-phenanthroline (10 mM) (*2*). β-Galactosidase activity was determined as described in "Materials and Methods". The data shown are average values of three measurements.

Activation of *E. coli sox*S′::*lac*Z expression by NO and 4NQO donors (β-galactosidase units)

| Inducer (concentration) | Strain/expression level | |
|---|---|---|
| | TN 530 λφ (Δ*sox*S′ :: *lac*Z) | TN 531 (TN530 Δ*sox*RS) |
| Control | 0.28 | 0.3 |
| 4NQO (2.63 nM) | 1.20 | 0.3 |
| $DNIC_{glu}$ (10 nM) | 0.33 | 0.3 |
| $DNIC_{cys}$ (10 nM) | 0.35 | 0.3 |
| $DNIC_{glu}$ (1 mM) | 0.55 | 0.3 |
| $DNIC_{cys}$ (1 mM) | 0.70 | 0.3 |
| $DNIC_{glu}$ (2 mM) | 0.60 | 0.3 |
| $DNIC_{cys}$ (2 mM) | 0.86 | 0.3 |

Interesting results were obtained in experiments with *sox*S expression induced by 4NQO. The induced expression was four times as large as the basic level. 4NQO at 2.63 nM concentration was a more potent *sox*S inducer than other inducers at higher concentrations. Comparing the levels of expression induced by equimolar solutions of $DNIC_{cys}$ or $DNIC_{glu}$ we noted that cysteine-containing complexes were more effective than glutathione-containing ones (table).

Incubation of bacterial cells with NO (at the maximal possible NO concentration 150 μM) resulted in formation of a single EPR-detectable DNIC-type signal with $g$ = 2.03. Consequently, DNIC was formed in the cells by the interaction between NO and intracellular iron (Fig. 5).

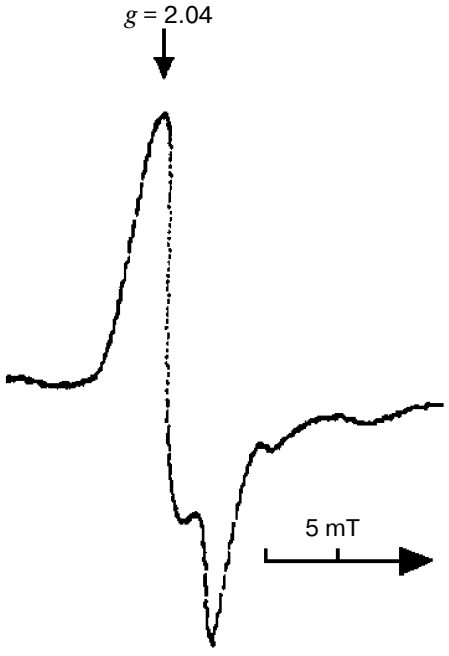



**Fig. 5.** EPR spectrum of *E. coli* cells incubated with 150 μM NO. Experimental conditions: 77 K, microwave power 5 mW, modulation amplitude 0.5 mT, spectrometer amplification $0.5 \cdot 10^4$.

## DISCUSSION

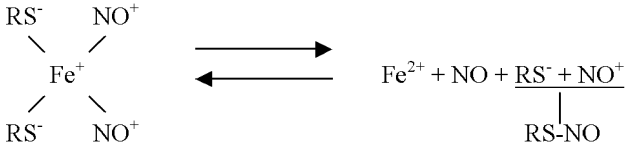
These results confirm earlier data on the activation of the *sox*RS-system in *E. coli* by nitric oxide and NO-producing agents [11, 14]. We first demonstrated that DNIC with thiol-containing ligands also possess *Sox*RS-inducing activity: GS-NO and $DNIC_{cys}$, the NO-producers used by us, can enhance *sox*S expression as much as free NO does. As for $DNIC_{glu}$, its activity appeared to be somewhat lower: it increased to the level observed in experiments with $DNIC_{cys}$ only when cysteine was added to the reaction medium; this was obviously caused by conversion of $DNIC_{glu}$ to $DNIC_{cys}$.

SoxR is a sensor in the development of the *Sox*RS oxidative response and a regulator of *Sox*RS-regulon transcription. SoxR is known to be a homodimer containing two [2Fe-2S] centers per protein monomer. Activation of *sox*R transcription is supposed to be induced by oxidation of [2Fe-2S] clusters, which remain intact. As a result, the state of iron ions in the cluster changes from ($Fe^{2+}/Fe^{3+}$) to ($Fe^{3+}/Fe^{3+}$). The protein thus becomes transcriptionally active. The opposite is also true: the protein becomes inactive on reduction of [2Fe-2S] centers by dithiols.

Genotoxic efficiency of DNIC with various SH-ligands is influenced by the nature of these ligands and their concentration. These are features of the cluster (its instability and ability for redox transitions) that allow flexible regulation of activity of anti-stress mechanisms.

Considering the dependence of *Sox*RS-regulon induction on iron metabolism, we used *o*-phenanthroline, an iron chelating agent. The very important role of iron in prooxidant cell protection was confirmed. We described earlier a significant suppression of NO-donor-induced SOS-response induction by pretreatment of cells with iron chelators [15]. Thus, cellular iron is the most important element in development of *Sox*RS- and SOS-repair responses in *E. coli*.

The release of NO from DNIC is determined by the chemical equilibria between these complexes and their components:

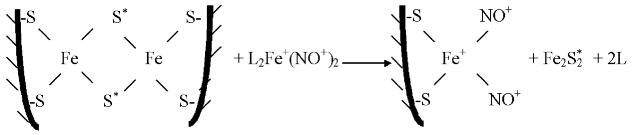


When contacting cells, free NO released from DNIC penetrates into the cells and, on reacting with iron–sulfur-containing protein SoxR, the *sox*S transcription factor, activates the former, thus promoting *sox*S expression [14]. It is obvious that the easier and faster NO releases from DNIC, i.e., the lower the stability of this complex, the more efficiently it can induce this process. Our results are in complete agreement correspond with this suggestion. Of all the DNIC we studied, the least stable $DNIC_{cys}$ effected the *sox*RS-system the most. The equal activities of $DNIC_{cys}$ and free NO in induction of expression indicate essentially complete decomposition of the complex.

$DNIC_{glu}$ is much more stable [16]; this seems to be the reason for its lower activity with the *sox*RS-system. Retaining most of its NO in the bound state, $DNIC_{glu}$ transfers NO in the $Fe^{+}(NO^{+})_2$ groups to thiol groups of cell proteins, forming more stable DNIC, i.e., weaker NO donors [16, 17].

The question arises why on increase in $DNIC_{glu}$ concentration, when NO release should also increase, enhanced activation of the *sox*RS-system was not observed. The only explanation is the toxic action of DNIC on the bacterial cells that we described earlier [15]; this toxic action increases at high DNIC concentrations.

Our earlier studies [18] demonstrated that decomposition of iron–sulfur complexes in the presence of NO seems to occur only when a primer—a low-molecular-weight DNIC able to attack thiol groups of proteins in the composition of iron–sulfur complexes—appears. The resulting formation of protein DNIC releases iron from the "iron–sulfur" center:




In accordance with this scheme, subsequent inclusion of this iron into the low-molecular-weight DNIC in the presence of NO results in decomposition of other iron–sulfur centers via a self-catalyzing mechanism. Primers, low-molecular-weight DNIC, can be formed by transfer of protein DNIC arising in the cells on their treatment with free NO and NO donors (DNIC and GS-NO) into low-molecular-weight form through the action of low-molecular-weight thiols.

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