NADPH Inhibits Transcription of the Escherichia coli Manganese Superoxide Dismutase Gene (sodA) in Vitro*

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We have previously reported that the thiols glutathione, dithiothreitol, and β-mercaptoethanol suppress transcription of the Escherichia coli manganese-containing superoxide dismutase gene (sodA) in an in vitro coupled transcription plus translation system (Gardner, P. R., and Fridovich, I. (1987) J. Biol. Chem. 262, 17591–17595). We now report that NADPH, but not NADH, selectively decreases transcription of sodA in vitro and that an NADPH generating system utilizing glucose 6-phosphate and the corresponding dehydrogenase markedly augments this suppressive effect. A redox buffer containing various ratios of oxidized and reduced glutathione also modulated transcription of sodA thus demonstrating the existence of a redox-sensitive mechanism controlling sodA transcription. Fusion of a 120-base pair fragment, containing 90 base pairs of DNA upstream of the sodA transcription initiation site, to a promoterless galactokinase gene (galK) conferred redox-sensitivity to GalK synthesis. We propose that these redox effects act through a redox-sensitive regulator of sodA and that the anaerobic reduction charge, [NADPH]/([NADPH] + [NADP⁺]), is one cellular signal controlling sodA transcription.

The Escherichia coli manganese-containing superoxide dismutase provides a regulated defense against the toxic flux of superoxide (O₂⁻) produced by enzyme- and metal-catalyzed aerobic oxidations of cellular reductants. Conditions known to increase O₂⁻ production in E. coli such as exposure to hyperoxia (1) or to redox-cycling agents (2, 3) increase the expression of MnSOD. Other circumstances known to increase MnSOD synthesis include iron deprivation (4, 5), an oxidative metabolism (6), and anaerobic respiration with nitrate (7–11).

MnSOD is encoded by the sodA gene which is regulated by a complex set of transcriptional controls governed by the fur (ferric uptake regulation) (12, 13), arc (aerobic respiratory control) (13), fnr (fumarate nitrate reductase) (14), and the soxRS (superoxide response) (15, 16) regulons. Many of the conditions which are known to affect MnSOD synthesis can now be understood in terms of these regulators. The SoxRS activator increases sodA transcription in response to conditions which increase intracellular O₂⁻ production. The Fur repressor responds to the availability of Fe(II) while the ArcAB repressor system controls the expression of enzymes required for oxidative metabolism and aerobic respiration. Thus, the repression of sodA transcription observed when cells are grown in the presence of glucose (6) or sufficient Fe(II) (4, 5) can be explained by the actions of ArcAB and Fur (12, 13), respectively. The involvement of fnr, which controls the levels of enzymes involved in anaerobic respiration (17), may account for the modest levels of MnSOD synthesis observed in E. coli grown anaerobically with terminal electron acceptors such as nitrate and trimethylamine-N-oxide (7–11).

With the exception of Fur, little is known about the cellular signals controlling these sodA transcription regulators, although oxidation-reduction signals have been postulated for ArcAB, FnR, and SoxRS. The ability of redox-cycling agents and hyperoxia to increase MnSOD synthesis led to the early proposal that MnSOD synthesis was responsive to intracellular O₂⁻ levels (1–3), and the requirement for SoxRS for these inductions later led to the proposal of a direct role of SoxRS in sensing O₂⁻ levels (15, 16). However, overproduction of SOD, and thus diminution of O₂⁻ does not diminish the induction of MnSOD synthesis by hyperoxia (18), nor does overproduction suppress transcriptional activation of SoxRS-regulated genes by redox-cycling agents (19, 20). Thus, it is evident that activation of SoxRS must be due to some consequence of exposure to redox-cycling agents and hyperoxia, such as an altered cellular redox status, rather than to increases in O₂⁻ production per se.

We have previously described the specific inhibition of sodA transcription by glutathione, dithiothreitol, and β-mercaptoethanol in an in vitro coupled transcription plus translation system (21). It appeared likely that these thiols modulated the activity of the same redox-sensitive regulatory factor which affected sodA transcription within E. coli (7, 10, 21–24). Here we present results which show that in vitro sodA transcription can be modulated by a glutathione redox buffer and is strongly inhibited by NADPH in the presence of a glucose 6-phosphate driven NADPH regeneration system (22). These results suggest a role for the anabolic reduction charge, [NADPH]/([NADPH] + [NADP⁺]) (25), in the control of sodA transcription in E. coli. The possible role(s) of the putative redox-sensing sodA transcription regulators, ArcAB, FnR, and SoxRS are discussed.

MATERIALS AND METHODS

The plasmid pDT1-5 was provided by Dr. D. Touati (26). E. coli C600 galK and the plasmid pKO1 (27) were provided by Dr. D. Steege

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‡ The abbreviations used are: MnSOD, manganese-containing superoxide dismutase; MOPS, 4-morpholinepropanesulfonate; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; bp, base pair(s).

1 Part of this investigation was presented at the UCLA Symposium on Oxygen Radicals in 1988 and at the meeting of the American Society for Biochemistry and Molecular Biology in 1989.

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(Duke University). Plasmid DNA was isolated by alkaline lysis, followed by sedimentation in a gradient of cesium chloride and gel exclusion chromatography on Bio-Gel A50 m (Bio-Rad). [3H]L-Leucine (140 Ci/mmol), [α-32P]dATP (3000 Ci/mmol), and [γ-32P]ATP (3000 Ci/mmol) were purchased from Amersham. L-amino acids, rabbit muscle pyruvate kinase, sodium phosphoenolpyruvate, NADH, NADPH, GTP, GSH, GSTD, MSH, and 6-phosphate, ammonium formate, Pseudomonas ozalisute formate dehydrogenase, pyridoxine hydrochloride, FAD, β-aminobenzoic acid, folic acid, herring sperm DNA, Ficoll, and polyvinylpyrrolidone were from Sigma. E.coli RNA, S1 nuclease, and bovine serum albumin (fraction V) were purchased from Boehringer Mannheim. Dithiothreitol was from ICN (Cleveland, OH), and polyvinylpyrrolidone, 0.2% bovine serum albumin-fraction V was from Pharmacia LKB Biotechnology Inc. Bacterial growth media was from Difco.

Digests with restriction endonucleases, Klenow end-filling, phosphatase treatment, ligations, and kinase reactions were performed as described by Maniatis et al. (28). DNA fragments were isolated from restriction samples with agarose gel DNA after a short 10-min pre-equilibration at 37°C and were treated by electrophoresis and ion-exchange chromatography on NACS columns.

Plasmid Constructions—pD11c was made by inserting the end-filled 1,053-bp AflII fragment of pDT1-5 into the SmaI site of pUC9 (29). pPG1 was constructed by blunt-end ligation of the 120-bp EcoRI/SmaIII fragment of pD11c into the SmaI site of pBR322 (27), and pEG of E.coli G600 galK was selected as red colonies on galactose MacConkey agar containing 50 μg/ml ampicillin and were analyzed by restriction analysis.

In Vitro Transcription Plus Translation—The method of Zubay (30) was used with the following modifications. (a) Cultures of mid-log phase E.coli K12 (ATCC 27376) or E.coli B B87 (ATCC 29882) were chilled with crushed ice, concentrated in an Amicon hollow fiber cell concentrator at 4°C, and finally collected by centrifugation at 6000 × g for 20 min at 2°C. Smaller volume cultures (1 liter) were collected by centrifugation without an initial concentration step, and volumes were adjusted according to the Zubay protocol. (b) Reactions were performed in a total volume of 40 μl. (c) When protein synthesis was measured, 8.0 μCi of [3H]leucine with a specific activity of 140 Ci/mmol was used in place of the 0.22 mM L-leucine. (d) S-30 extracts were dialyzed prior to use to remove the dithiothreitol. This dialysis was performed for 4 h in an ice bath, while sweeping the dialysate with a stream of O2-free N2. The dialysate was 1000 volumes of 10 mM Tris acetate, 14 mM magnesium acetate, and 60 mM potassium acetate at pH 8.2, and it was changed twice during the dialysis. The dialyzed S-30 extract was rapidly frozen, in 120-μl aliquots, in a dry ice/ethanol bath and were stored at −70°C. Storage of dialyzed extracts for several weeks at −70°C did not affect to appear in the in vitro synthesis reactions. (e) Cysteine (150 μM) was added to reactions to oxidize traces of non-dialyzed DTT. (f) Transcription and translation reactions were normally initiated by adding template DNA after a short 10-min pre-equilibration at 37°C and were terminated by extraction with 40 μl of phenol, except when protein synthesis was being measured, in which case, reactions were terminated by freezing in a dry ice/ethanol bath. (f) When reduced pyridine nucleotides and thiols were being tested, freshly prepared solutions were added to reaction mixtures just prior to addition of the dialyzed extract.

Examination of mRNA Transcripts—Reaction mixtures were extracted with phenol and precipitated with ethanol. The nucleic acid precipitates were redissolved, with minimum agitation, in 80 μl of a solution containing 20 mM MOPS, 5.0 mM sodium acetate, 0.5 mM EDTA, 2.15 mM formaldehyde, and 50% deionized formamide, pH 7.0, and were incubated for 15 min at 55°C, 8.0 μl of loading buffer containing 50% glycerol, 1.0 mM EDTA, 0.4% xylene cyanol was added, and 20 μl of each was then loaded onto a 1% agarose-formaldehyde gel (28). After electrophoresis, the gel was blotted onto nitrocellulose (Northern blot analysis) which was then washed and then baked at 80°C, in vacuo, for 4 h. The nitrocellulose blot was then incubated for 24 h at 42°C in a solution containing 5 μSSE: 1 × SSE; 10 mM sodium phosphate, 0.18 M NaCl, 1.0 mM EDTA, 2.15% deionized formamide, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin-fraction V, and 100 μg/ml sonicated and heat-denatured herring sperm DNA. A 431-bp EcoRI/BstI fragment of plasmid pD11c was end-labeled with [α-32P]dATP and was used to probe for MnSOD gene transcripts. β-Lactamase transcripts were probed for with the 750-bp EcoRI/Part fragment of pBR322 which had been end-labeled with the Klenow enzyme and [α-32P]dATP. Probes were added to 0.5 μg in hybridization volumes of 25-50 ml, and hybridization on the nitrocellulose blots was allowed to proceed for 24 h at 42°C. Following hybridization, the blots were washed at 65°C with two successive 10-min washes of 1, 0.5, and 0.1 × SSSPE containing 0.1% SDS and were then dried and autoradiographed at −70°C on Kodak XAR5 film with an intensifying screen. The relative intensities of bands were determined by laser scanning densitometry, and exposure times were adjusted so that the darkening of the film remained within the linear response range.

S1 nuclease analysis of in vitro mRNA transcripts was performed essentially as described (28). Hybridization with the T4 polynucleotide kinase 5'-end-labeled 220-bp EcoRI/Ncol fragment of pD11c was performed at 50°C for 3 h with 10-20 ng of probe and varying amounts mRNA in a volume of 30 μl. S1 nuclease digestion products were separated on a 6% polyacrylamide-urea DNA sequencing gel with φX174-HaeIII end-labeled markers, and bands were visualized by radioautography.

Analysis of Polypeptide Products—Reaction mixtures were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (21) on 12% slab gel with 3.0 μg loaded/lane. Following electrophoresis, the gel was soaked in 7% acetic acid for 60 min and was then treated with En'Hance (Du Pont-New England Research Products) for 60 min prior to a final wash in water for 30 min. After drying, the gel was placed on Kodak XAR film with an intensifying screen at −70°C.

RESULTS

Effects of NADPH and NADH on sodA Transcription—NADPH and NADH were examined for their possible effects on MnSOD gene (sodA) transcription in vitro. In these experiments, the 30 μM NADP+ normally supplied to the in vitro synthesis reactions was omitted. NADPH at 3.0 mM elicited only a modest decrease in sodA transcripts as shown by the data in the figure, lanes 1 and 2. The relatively weak effect of NADPH on transcription, compared with that observed for thiols, may explain our previous failure to observe an effect of 1.0 mM NADPH on in vitro synthesis of the MnSOD polypeptide (21). However, the addition of glucose 6-phosphate plus NADPH, to promote the regeneration of the NADPH by double oxidation of glucose 6-phosphate dehydrogenase, caused a very marked decrease in sodA transcripts relative to β-lactam transcripts when compared to the reactions with glucose 6-phosphate alone in Fig. 1, lanes 4 and 3, respectively. NADH

FIG. 1. Effects of NADPH and NADH on sodA transcription. Transcription plus translation reactions (total volume 40 μl) directed by pDT1-5 were performed with the following additions; lane 1, none; lane 2, 3.0 mM NADPH; lane 3, 3.0 mM glucose 6-phosphate; lane 4, 3.0 mM NADPH plus 3.0 mM glucose 6-phosphate; lane 5, 3.0 mM NADH; lane 6, 3.0 mM ammonium formate; lane 7, 3.0 mM NADH plus 3.0 mM ammonium formate. Reactions were pre-equilibrated for 10 min at 37°C before initiating transcription and translation by adding 0.25 μg of template DNA. Reactions were stopped after 20 min of synthesis at 37°C, and mRNA were isolated, electrophoresed, blotted, probed, and radioautographed as described under "Materials and Methods." S-30 extracts prepared from E.coli K12 as described under "Materials and Methods" were supplied with formate dehydrogenase at a concentration of 0.10 units/ml of extract protein.

β-lac

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at 3.0 mM did not noticeably decrease sodA mRNA levels as shown by the results in Fig. 1, lanes 1 and 5, nor did a NADH regenerating system utilizing added formate dehydrogenase plus formate (lane 7) cause an observable suppression. These results suggest that the cellular reductant NADPH, but not NADH, is capable of modulating the redox-sensitive factor affecting sodA gene transcription.

Table I shows the results of the analysis of the effect of NADPH and glucose 6-phosphate on the absolute levels of sodA (MnSOD) mRNA and β-lactamase (β-lac) mRNA under reaction conditions similar to those used in Fig. 1, except that in these reactions S-30 extracts were prepared from E. coli B as in our earlier report (21). The results with E. coli B and K12 were grossly similar. NADPH at 3.0 mM slightly increased the overall transcription rate as indicated by an increase in β-lactamase mRNA, while not altering the level of sodA mRNA. The relative effect was a decrease in sodA transcripts by approximately 3-fold. Adding 3.0 mM glucose 6-phosphate to the reaction mixture for regeneration of ATP.

The effect of increasing glucose 6-phosphate concentrations in the presence of 3.0 mM NADPH on sodA transcription (top of Fig. 2, lanes 2–7) and the effect of increasing NADPH concentrations in the presence of 3.0 mM glucose 6-phosphate (top of Fig. 2, lanes 8–15) were also examined. The results of the quantitation of mRNA by scanning radioautographs are presented in the bottom panel of Fig. 2. As shown by the data, glucose 6-phosphate and NADPH caused half-maximal suppression of sodA mRNA levels at concentrations of approximately 250 and 30 μM, respectively. While these data do not reveal the steady-state concentrations of glucose 6-phosphate, NADPH, or NADP*, the effective concentrations are within range of the published Kₗ values of E. coli glucose-6-phosphate dehydrogenase for glucose 6-phosphate (145 μM) and for NADP* (15 μM) (32) and are below the respective cellular concentrations of 0.5–1.5 mM, and 170 μM and 220 μM for glucose 6-phosphate, NADP*, and NADPH in E. coli (33, 34).

Modulation of sodA Transcription by a Glutathione Redox Buffer—It seemed probable that GSH, DTT, and β-mercaptoethanol suppressed in vitro MnSOD mRNA levels by reducing a regulator of sodA transcription in the S-30 extract. Thus, we supposed that oxidants would oppose this effect. The effect of redox status on in vitro transcription of sodA (MnSOD) was examined by using a redox buffer containing GSSG and GSH. The levels of both sodA transcripts and control β-lactamase (0-lac) transcripts were measured by scanning radioautographs of mRNA blots. The results of this analysis presented in Fig. 3A demonstrate that increasing concentrations of the oxidant GSSG increased the level of transcription of the MnSOD gene relative to that of the control β-lactamase gene. We noted, however, that more oxidizing conditions had an adverse effect on general transcription as evidenced by a decrease in the transcription of the β-lactamase gene. Fig. 3B more clearly illustrates the effect of various GSSG/GSH redox ratios on the relative levels of sodA transcripts. The relative level of transcription of sodA showed a sigmoidal dependence on the redox ratio of GSSG to GSH at several levels of GSH thus indicating that the redox status of the reaction mixture, rather than the absolute level of GSH or GSSG, was modulating transcription. Moreover, the data show maximal as well as basal levels of sodA transcription relative to β-lactamase transcription in this system. Half-maximal transcription of sodA was observed at a GSSG/GSH ratio of ≈1.5. These experiments are in accord with the observation that the absolute [GSH] has no effect on MnSOD expression in E. coli (24), and moreover, these results suggest that a GSSG-GSH couple would be an unlikely signal for the normal redox regulation of sodA transcription given that the GSSG/GSH ratio in E. coli has been reported to be less than 1:100 (35).

SI Nuclease Analysis of In Vitro sodA Transcripts—The results of the analysis of mRNA transcripts indicated that a single major manganese superoxide dismutase gene sodA transcript was produced from pDT1-5 by the in vitro system and that this transcript was suppressed by reduced glutathione, dithiothreitol, and β-mercaptoethanol (21). Further analysis of these in vitro transcripts was performed in order to identify the transcription start site. Hybridization of transcripts with the 5'-end-labeled 220 nucleotide EcoRI/NcoI fragment of pD11c, containing 5' sequences of sodA, followed by digestion with the single-stranded nuclease, S1, and separation on a 6% DNA-sequencing gel produced a protected fragment with the expected size (123 bases) for transcripts accurately initiated from the sodA promoter (36) as shown by the data in lanes 3–5 in Fig. 4. Run-through transcripts were also detected as evidenced by some protection of the full-length 220-nucleotide probe shown in lanes 3–5. We conclude that the transcription start site, which is affected by thiols and NADPH in vitro, corresponds to the sodA start site in E. coli (36).

**Table I**

Effects of NADPH and glucose 6-phosphate on sodA transcription

Transcription plus translation reactions were performed with plasmid template pDT1-5 in the presence or absence of NADPH, NADP*, and glucose 6-phosphate (G-6-P), and the effects on MnSOD gene (sodA) and β-lactamase gene mRNA transcripts were quantitated by Northern blot analysis and linear scanning densitometry as described under "Materials and Methods." Reactions were pre-equilibrated for 10 min at 37 °C with the indicated additions, and transcription and translation was initiated by adding 25 ng/μl of template DNA. Reactions were terminated after 20 min of incubation at 37 °C. S-30 extracts prepared from E. coli B were used for these reactions.

| Addition | [β-lac mRNA] | [MnSOD mRNA] | [β-lac mRNA] | [MnSOD mRNA] |
|----------|--------------|--------------|--------------|--------------|
| None     | 6.9*         | 32.3         | 4.7          | 1.5          |
| NADPH (3 mM) | 18.7        | 28.3         | 1.0          | 0.19         |
| NADPH (3 mM) and G-6-P (3 mM) | 31.3        | 6.0         | 1.5          | 3.3          |
| G-6-P (3 mM) | 26.1        | 38.8         | 1.5          | 3.3          |
| NADP* (30 μM) | 11.3        | 37.2         | 1.5          | 3.3          |

* Concentrations are in arbitrary absorption units.
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Dithiothreitol Suppresses a sodA-galK Promoter Fusion—The effect of reductants was postulated to be due to a decreased initiation of transcription from the sodA promoter rather than to a decreased mRNA stability. Fusion of the 120-bp EcoRI/XmaIII DNA fragment of pD11c, which contains 90 base pairs upstream of the sodA transcription start site (36), to the promoterless galactokinase gene (galK) in the promoter fusion vector pK01 (27) resulted in the DTT-sensitive synthesis of the 40-kDa galactokinase protein (GalK), as is evident by comparison of the GalK bands in lanes 1 and 2 or in lanes 3 and 4 in Fig. 5B. With 0.034 pmol (850 pm) of pPG4.1, DTT decreased GalK synthesis ~2.5-fold (Fig. 5B, lanes 1 and 2), but at higher levels of template, the effect of DTT was diminished as shown in lanes 3 and 4 of Fig. 5B. This decrease presumably reflects a limited abundance of the redox-sensitive regulatory factor in the S-30 extract.

In the absence of the 120-bp sodA operator-promoter fragment, low levels of GalK synthesis were observed as shown by the autoradiographs in Fig. 5A. This low level of synthesis is probably due to a low background level of nonspecific transcription from upstream plasmid promoters and may account for a part of the DTT-insensitive GalK synthesis in the sodA-galK promoter fusion observed in Fig. 5B. In addition, a small stimulation of overall protein synthesis was also apparent with DTT as evidenced by increased β-lactamase synthesis. These results indicate that part of the information necessary for the redox-sensitive control of MnSOD synthesis is present in the 120-bp 5' sequence of sodA, and furthermore, the results indicate that the specific reductant suppression of sodA transcripts in vitro is unlikely to be due to mRNA degradation by an endonuclease or 3'-exonuclease.

DISCUSSION

We have investigated the effects of the cellular reductants glutathione, NADPH, and NADH on the transcription of sodA in an in vitro transcription plus translation system using the plasmid template pDT1-5. The results demonstrate that NADPH, but not NADH, selectively inhibits sodA transcription. Furthermore, suppression of sodA transcription was greatest when glucose 6-phosphate was supplied to the reaction to allow NADPH regeneration by the endogenous glucose-6-phosphate dehydrogenase. The synergistic effect of glucose 6-phosphate and NADPH suggests that the [NADPH]/([NADPH] + [NADP⁺]) ratio rather than the absolute

\[ \text{NADPH} \text{Inhibits sodA Transcription} \]

**FIG. 2. Effects of NADPH and glucose 6-phosphate on sodA transcription.** Top, in vitro transcription and translation directed by supercoiled plasmid DNA template pDT1-5 was performed in the presence of 3.0 mM NADPH with increasing concentrations of glucose 6-phosphate in lanes 2-7 or in the presence of 3.0 mM glucose 6-phosphate with increasing concentrations of NADPH in lanes 8-15. Reactions (40 μl) were pre-equilibrated for 10 min at 37°C before initiating synthesis with 25 ng/pl of pDT1-5. RNA was isolated, electrophoresed, blotted, probed, and blots were radioautographed as described under “Materials and Methods.” The concentrations of glucose 6-phosphate present in the reactions analyzed in lanes 2-7 were 0, 0.25, 0.50, 1.0, 2.0, and 3.0 mM, respectively, and the concentrations of NADPH in the reactions probed in lanes 8-15 were 0, 0.03, 0.10, 0.25, 0.50, 1.0, 2.0, and 3.0 mM, respectively. Lane 1 shows the products of the reaction performed in the absence of added NADPH and glucose 6-phosphate while lane 16 was performed with the 30 μM NADP⁺ normally included in the synthesis reactions. Bottom, the relative levels of sodA (MnSOD) and β-lactamase (β-lac) transcripts are presented in the presence of 3.0 mM NADPH as a function of glucose 6-phosphate (panel A) and 3.0 mM glucose 6-phosphate as a function of NADPH (panel B) were quantitated by linear scanning densitometry. The reductant dithiothreitol was absent in these reactions. S-30 extracts were prepared from E. coli B as described under “Materials and Methods.”
[NADPH] is important for the modulation of sodA transcription. Moreover, the ability of a redox buffer, containing oxidized and reduced glutathione, to also modulate sodA mRNA levels, as shown by the data in Fig. 3, indicates that the redox state of the reaction mixture is the relevant factor, rather than 5-phosphogluconate levels for example.

Redox signals have been proposed for the sodA transcription regulators SoxRS (37-39), Fnr (40, 41), and ArcAB (41, 42), thus making it difficult to ascribe the redox effects observed in vitro to a single one of these global transcription regulators. However, the inability of reductants such as dithiothreitol and 2-mercaptoethanol to affect Fnr transcription activation or repression in vitro (43) make it unlikely that thiols or NADPH modulate sodA transcription via Fnr. Also, the ArcAB system requires both the redox sensor, ArcB, and the transcription regulator, ArcA, for redox control (41, 42) and the membrane-anchored component, ArcB, would be significantly removed with the membrane fraction during the

Fig. 3. Effect of GSSG/GSH ratio on sodA transcription. Transcription and translation directed by plasmid pDT1-5 was performed in the presence of reduced (GSH) and oxidized (GSSG) glutathione. Reactions were pre-equilibrated with glutathione (GSH and GSSG) for 10 min at 37 °C before adding template DNA. After 20 min of coupled transcription plus translation at 37 °C, reactions were terminated and mRNA was isolated, electrophoresed, blotted, and probed. Blots were then autoradiographed, and mRNAs were quantitated by scanning densitometry. Reactions were performed and the relative ratios of mRNAs produced were measured with increasing concentrations of GSSG in the presence of 4.0, 2.0, and 1.0 mM GSH in panel A, lines 1-3, respectively. In panel B, the relative ratio of mRNAs produced are plotted with respect to the GSSG/GSH ratio.

Fig. 4. Transcription start site mapping. Messenger RNA isolated from the in vitro transcription plus translation reaction, performed in the absence of reductant, was hybridized with a 5'-kinased 220-base DNA probe (EcoRI/NcoI pDT11c) containing 123 bases complementary to the 5' sequences of the known sodA transcript. Hybridization reactions were treated with S1 nuclease, and the products of the nuclease reaction were separated on a 6% polyacrylamide-urea sequencing gel; gels were then fixed, dried, and radioautographed. End-labeled αX174/HaeIII fragments were separated in the outside lanes and used as size markers (M). Lane 1, 220-base probe only; lane 2, control S1 nuclease digestion of 220-base probe only; lane 3, 220-base probe hybridized with in vitro mRNA and treated with S1 nuclease; lane 4, as in lane 3 except mRNA was increased 3-fold; lane 5, as in lane 3 except mRNA was 9-fold greater. The amount of mRNA used in the hybridization in lane 3 corresponded to ~1/175 of the total mRNA from a 40-μl synthesis reaction. The asterisk marks the protected fragment having the expected size of 123 bases.

Fig. 5. Effect of dithiothreitol on a sodA-galK promoter fusion. In vitro transcription plus translation was directed by the promoterless-galactokinase (galK) vector pK01 (panel A) or the sodA-galK promoter fusion vector pPG4.1 (panel B) in the absence (lanes 1 and 3) or presence of 2.0 mM dithiothreitol (lanes 2 and 4). Synthesis reactions (40 μl total volume) were directed by 0.034 pmol of plasmid DNAs (0.1 μg pK01 and 0.1 μg pPG4.1) in lanes 1 and 2 and by 0.068 pmol of templates in lanes 3 and 4. [3H]Leucine-labeled polypeptides from 20-min synthesis reactions, using S-30 extracts prepared from E. coli K12, were separated by SDS-polyacrylamide gel electrophoresis, and gels were fixed, fluorographed, dried, and radioautographed as described under "Materials and Methods."
The operator-promoter sequences of sodA, zwf, and nfo were compared. The arrow-ended single underlined bases are the 19-bp palindrome in sodA (36) while the double underlined sequences are the identified -35-bp RNA polymerase contact sites. Bold lettered base codes show the recognized homology between sodA, zwf (44), and nfo (45). The data presented and published results (20) indicate that the anabolic reduction charge as well as the oxygen tension. Such a mechanism would provide a useful way for the cell to detect changes in the anabolic reduction charge as well as the oxygen tension. Future studies using the in vitro system and mutant E. coli S-30 extracts should help further define the regulators as well as the mechanism(s) of redox control of sodA transcription.

The sodA-galK promoter fusion results presented in Fig. 5 indicate that the sequence information for the redox-sensitive regulation of sodA is contained in the 120 base pairs of 5′ operator-promoter DNA. This sodA operator-promoter region contains 90 base pairs of DNA upstream from the sodA transcription start site and encompasses several overlapping regulatory sites including the Fur-binding site (12), the Fnr-binding site (14), and a 19-bp palindromic sequence (36). The near perfect 19-bp palindromic homology with 5′ sequences of the SoxRS-regulated genes zwf (glucose 6-phosphate dehydrogenase) (44) and nfo (endomucinase IV) (45) may locate the binding site for SoxRS-dependent regulation. This homology is greatest between sodA and zwf as depicted in Fig. 6; however the palindromicity is not as obvious in either zwf or nfo. To our knowledge, the ArcAB-binding site on sodA remains to be identified and may also be contained in this 120-bp sodA operator-promoter DNA.

Maintenance of the anabolic reduction charge is important for the proper functioning of NADPH-dependent biosynthetic (25) and oxidant detoxification pathways (46). Moreover the [NADPH] /[NADP+] ratio has been found to be a specific signal for mediating the light regulation of chloroplast malate dehydrogenase and fructose-1,6-bisphosphatase (47-49) and for regulating initiation of protein synthesis in eukaryotes (50-56).