Induction of the soxRS Regulon of *Escherichia coli* by Superoxide*

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The soxRS regulon orchestrates a multifaceted defense against oxidative stress, by inducing the transcription of ~15 genes. The induction of this regulon by redox agents, known to mediate O$_2^-$ production, led to the view that O$_2^-$ is one signal to which it responds. However, redox cycling agents deplete cellular redoxants while producing O$_2^-$ and one may question whether the regulon responds to the depletion of some cytoplasmic redoxant or to O$_2^-$ or both. We demonstrate that raising [O$_2^-$] by mutational deletion of superoxide dismutases and/or by addition of paraquat, both under aerobic conditions, causes induction of a member of the soxRS regulon and that a mutational defect in soxRS eliminates that induction. This establishes that O$_2^-$ directly or indirectly, can cause induction of this defensive regulon.

The soxRS (supersoxide response) regulon positively controls ~15 genes in *Escherichia coli*. The inductions of this regulon by redox cycling agents, such as paraquat, plumbagin, and phenazine methosulfate, which are capable of mediating O$_2^-$ production, led to the view that this regulon is capable of responding to O$_2^-$ (1–3). This conclusion was strengthened by the observation that H$_2$O$_2$, heat shock, or ionizing irradiation did not induce the soxRS regulon (1–4). Moreover FumC was induced by paraquat more strongly in a sodA sodB strain than in its sodA-replete parent (5), and its induction in the parental strain was eliminated by mutational deletion of the soxRS response (6), thus indicating that O$_2^-$ could cause induction of soxRS. However it was also noted (6) that marked overproduction of sodA did not diminish induction of members of this regulon such as fumarase C and glucose-6-phosphate dehydrogenase, an indication that O$_2^-$-independent induction was also a reality. In accord with this view was the finding that NADPH could diminish in vitro transcription/translation of the sodA gene (7). Nitric oxide has also been shown to induce soxRS and to do so in the absence of dioxygen (8).

There is strong evidence that the SoxR protein, which is the sensor of the soxRS regulon (4, 9, 10), occurs in oxidized and reduced forms and that the oxidized form is the activator of soxRS transcription (11–14). The balance between the oxidized and reduced forms of SoxR within *E. coli* can undoubtedly be influenced in multiple ways. For example either by oxidation of reduced SoxR or by reduction of oxidized SoxR. O$_2^-$ could accelerate the former process and, by inhibition of the oxidized SoxR reducing systems, the latter. Yet there seems to be disagreement about whether O$_2^-$ is one of the factors that influences the redox status of SoxR. Thus, Nunoshiba et al. (4) used an operon fusion, of the soxS promoter to the lacZ gene, to show that redox cycling agents induced this system in an O$_2^-$-dependent manner and that dioxygen itself was a stronger inducer in a SOD-deficient (sodA sodB) strain than in a sod-replete strain. Thus supporting the view that O$_2^-$ could, directly or indirectly, induce soxRS. Wu and Weiss (10) also presented evidence supporting this view. Gort and Imlay (15), using a soxS::lacZ fusion strain, reported that lack of SOD did not cause induction of soxS under aerobic conditions. Thus we have several groups reporting that elevating O$_2^-$ by elimination or diminution of SOD was sufficient to cause this induction, and another group (15) reporting that this was not the case.

Fumarase C is a member of the soxRS regulon (6), and we have previously noted that it was induced under aerobic conditions by mutational deletion of SodA + SodB (5). Furthermore the induction of FumC by paraquat was greater in the sodA sodB than in the SOD-replete parental strain. These results support the view that O$_2^-$ can induce the soxRS regulon or alternatively that the induction of FumC by O$_2^-$ was mediated by some other regulon. We explore this further by investigating the effect of deleting soxRS upon the induction of FumC. Our finding is that FumC induction by O$_2^-$ is ablated by mutational elimination of the soxRS response. It follows that O$_2^-$ can induce the soxRS regulon and that the soxRS regulon is the sole mediator of the induction of FumC by O$_2^-$.

MATERIALS AND METHODS

Paraquat was obtained from Sigma and malate from ICN. Bacto-tryptone, casamino acids, and yeast extract were from Difco. The strains of *E. coli* used were as follows: GC4468 = parent (16); DJ 901 = GC4468 ∆(soxR::Zeo2204) Zep2205:Trn10 (provided by B. Demple) (2); QC1799 = GC4468 ∆sodA3, ∆ sodB-kan (16); and QC1817 = GC4468 ∆sodA3, ∆ sodB-kan, ∆ sox8::cat (obtained by transduction of the ∆ sox 8::cat mutation into QC1799). (The soxRS deletion was provided by B. Weiss (3).) Strains were grown overnight at 37 °C, with shaking in air, in LB, or in M9CA media containing 50 μg/ml kanamycin and/or 30 μg/ml chloramphenicol where required. These cultures were diluted as described in the figure legends into media not containing antibiotics, and paraquat was added after 1 h, and incubation was continued for 75 min. Cells were then harvested, washed in 50 mM potassium phosphate, 0.1 mM EDTA at pH 7.8, and then resuspended in this buffer and lysed in a French press. The extracts were clarified by centrifugation, and protein (17) and fumarase C (5, 6) were assayed. One unit of fumarase was taken to be the activity that converted 1 mM malate to fumarate using ΔEP/Δt = 1.62 mV/s/cm. The total concentration of l-malate was 50 mM, and the assay buffer was 50 mM sodium phosphate, pH 7.3, at 25 °C.

RESULTS

Induction of FumC by Dioxygen and Paraquat—Paraquat can be univalently reduced, at the expense of NADPH, by a number of diaphorases present in *E. coli* (18), and the paraquat monoca-
The production of O$_2^-$ by SOX is thus increased within aerobic E. coli by paraquat. The net effect of paraquat on the steady state concentration of O$_2^-$ and on the redox status of the cell will be greater in an sodA sodB mutant than in its SOD-replete parent. Therefore, we should expect that paraquat should induce a member of the soxRS regulon such as FumC more strongly in an sodA sodB strain than in the parental strain. Bars 1, 2, and 3 in Fig. 1 show that 10 and 25 $\mu$M paraquat caused a dose-dependent induction of FumC in the parental strain, whereas bars 4-6 show the greater response to paraquat exhibited by an sodA sodB strain. It is also noted that the lack of SOD, in the absence of paraquat, caused a 3-fold induction of FumC (compare bars 1 and 4). It follows that raising the steady state concentration of O$_2^-$, whether by introducing paraquat or by removing SOD, was sufficient to cause induction of FumC. Bars 7 and 8 show that the sodA sodB soxRS triple mutant was unresponsive to O$_2^-$ in that it could not elevate FumC in response to aerobic paraquat. This establishes that O$_2^-$ induced FumC and did so via the soxRS regulon. Hence the soxRS regulon is responsive to O$_2^-$ No induction by paraquat was seen in the soxRS-deficient but otherwise SOD-proficient strain DJ901 (results not shown). This confirms our previous conclusion (6) that the induction of FumC in wild type strains of E. coli is entirely soxRS dependent.

The induction of FumC caused by deletion of SODA and sodB was greater in cells that had been grown in M9CA rather than in the richer LB medium. This is made apparent by comparison of bars 1 and 2 in Fig. 2 with bars 1 and 4 in Fig. 1. Thus there was a 3-fold induction, caused by the deletion of SOD activity, in the LB-grown cells and a 7-fold induction in the M9CA-grown cells. Bar 3 in Fig. 2 shows that soxRS was as essential for the induction of FumC in the M9CA-grown cells as it was in the LB-grown cells. The experiment shown in Fig. 2 was repeated under dioxygen-depleted conditions. This was done by placing 0.2% inocula, in fresh M9CA medium in a BBL gas pack jar, which was then incubated for 5.5 h before the cells were harvested and extracts prepared for FumC assay. The gas pack jars were not evacuated before incubation so hypoxic, rather than anoxic, conditions prevailed. The sodA sodB extracts were found to have 0.026 units/mg protein of FumC activity, whereas the parental extracts had 0.014 units/mg. Thus dioxygen depletion diminished the

**Discussion**

Because the induction of FumC, whether by addition of paraquat or by deletion of SODA and sodB, was dependent on soxRS and dioxygen, it follows that O$_2^-$ can induce soxRS. The induction of the soxRS regulon depends upon the oxidation of the reduced form of SoxR, because the oxidized SoxR is the transcriptional activator of soxS. There must be a pathway for the reduction of oxidized SoxR, and the steady state will depend on the balance between the rates of oxidation of reduced SoxR and of reduction of the oxidized SoxR. O$_2^-$ or some product thereof, might effect this steady state by directly oxidizing reduced SoxR and/or inhibiting the reduction of oxidized SoxR. Although the mechanism remains unknown, it is clear that the soxRS regulon can be induced by O$_2^-$.

Although we are in agreement with Gort and Imlay (15) concerning the importance of SOD as a defense against O$_2^-$ some exception must be taken to their conclusion that induction of FumC by O$_2^-$ is not adequate to compensate for the inactivation of FumA by O$_2^-$. They used an sodA sodB Ptc-soxA strain which could not induce Mn-SOD in response to increased [O$_2^-$]. In an SOD-competent wild type strain, in contrast, the inactivation of FumA would be lessened by the induction of Mn-SOD, which combined with the induction of FumC, should then be adequate to balance the decrease in FumA.

Inductions caused by O$_2^-$-generating compounds such as paraquat cannot be unequivocally attributed to O$_2^-$ because redox cycling agents deplete cellular reductants while producing O$_2^-$ and that depletion will interfere with the reduction of oxidized SoxR. An indication that depletion of cellular reductants can induce soxRS, independent of O$_2^-$, was the anaerobic induction seen with paraquat plus the electron sink nitrate (20, 21). No such ambiguity is encountered when O$_2^-$ is raised by deletion of SOD. In that case, if cellular reductants are also diminished, O$_2^-$ is the cause of that diminution. Thus O$_2^-$ can induce the soxRS regulon whose members provide manifold defenses against the oxidative damage imposed by O$_2^-$ and its progeny.

An estimation of the O$_2^-$-dependent and O$_2^-$-independent routes of induction of soxRS can be attempted. Thus the level of [O$_2^-$] in the sodA sodB strain is 20-fold higher than in the parental strain (22), and this caused an 3-fold induction of FumC. Gort and Imlay (15), by using a strain in which the level of SOD could be modulated, reported that a 10-fold diminution of [SOD] was a threshold for induction of FumC and resulted in
modest induction. A 10-fold decrease in [SOD] would correlate with a more than 5-fold increase in $[O_2^-]$ as discussed below, because SOD is the major sink for $O_2^-$ in the parental strain. In the sodA sodB strain paraquat can cause much more than a 20-fold increase in $[O_2^-]$ as compared with $[O_2^-]$ in the wild type and this allows dramatic induction of FumC, as shown in Fig. 1. In the SOD-replete parental strain, in contrast, the increase in $[O_2^-]$ because of paraquat is strongly limited by the action of SOD and by the further induction of SodA elicited by paraquat. Thus the induction of the soxRS regulon by paraquat in the parental strain must largely be because of the depletion of cellular reductants by paraquat rather than to $O_2^-$. Of course, this is even more emphatically the case in strains overproducing SOD and explains why overproduction of SOD does not prevent induction of the soxRS regulon by paraquat (6). The induction of SodA is finely tuned so as to minimize both the toxic effects of $O_2^-$ and the induction of the soxRS regulon by $O_2^-$.

The degree of protection provided by the wild type level of SOD to all $O_2^-$-sensitive targets in E. coli has been estimated (22) and that leads to a number of interesting deductions. Thus the rate of formation of $O_2^-$ ($V_T$) must be equal to the sum of its rates of consumption by SOD ($V_{SOD}$) and by all other targets ($V_f$), i.e.,

$$V_T = V_{SOD} + V_f$$  \hspace{1cm} (Eq. 1)

and

$$V_{SOD} = k_{SOD}[SOD][O_2^-]$$  \hspace{1cm} (Eq. 2)

and

$$V_f = k_f[T][O_2^-]$$  \hspace{1cm} (Eq. 3)

therefore

$$[O_2^-] = \frac{V_T}{k_{SOD}[SOD] + k_f[T]}$$  \hspace{1cm} (Eq. 4)

Application of Eq. 4 would require several difficult measurements and/or estimations so another approach is useful, from Eq. 1, as follows.

$$\frac{V_f}{V_T} = \frac{V_{SOD}}{V_T} + 1$$  \hspace{1cm} (Eq. 5)

and

$$\frac{V_f}{V_T} = \frac{V_{SOD}}{V_T} + 1$$  \hspace{1cm} (Eq. 6)

When $V_{SOD} = V_T$, one-half of all the $O_2^-$ flux is being scavenged by SOD and in analogy to the classical assay for SOD activity (23) in which SOD competes with cytochrome c for the flux of $O_2^-$, we can define this amount of SOD activity as 1 biological unit. We have previously found that wild type E. coli contains 19 biological units of SOD on the basis of its inhibition of lucigenin luminescence (22). Hence in these cells $V_T = 0.05 V_f$, whereas in sodA sodB cells $V_f = V_T$.

Fig. 3 presents (100) $V_f/V_T$ as a function of the number of biological units, which is the ratio $V_{SOD}/V_T$. This plot ignores changes in biological units because of enzyme inductions and changes in $V_T$ because of consumption of targets. A 10-fold decrease in [SOD] leaves 1.9 biological units and then $V_T$ is increased 7-fold and, because $[O_2^-]$ is directly proportional to $V_T$ (Eq. 3), so is $[O_2^-]$. Thus we see that $[O_2^-]$ is less than inversely proportional to [SOD] because of the effect of the multiple targets for $O_2^-$ in E. coli. It also follows that $O_2^-$ is both more deleterious and a better inducer of the soxRS response than

would be concluded on the basis of a simple inverse relationship between $[O_2^-]$ and [SOD]. Moreover the wild type level of [SOD] is seen as providing 95% protection rather than the 99% protection deduced by Gort and Imlay (15). This 5-fold difference in amount of $O_2^-$ damage to targets is certainly explicable on the basis of the existence of targets in addition to the $[4Fe-4S]$ containing dehydratases considered by Gort and Imlay (15).

In wild type E. coli has been estimated to be $-1 \times 10^{-10}$ M (15). SOD-null E. coli will contain 20 times more, or $2 \times 10^{-9}$ M $O_2^-$ and the threshold for induction of FumC via the soxRS regulon by $O_2^-$ will be at $-7 \times 10^{-10}$ M. Variation of these numbers will, of course, occur as growth conditions change. Thus the ratio of $V_{SOD}/V_f$ appeared to be approximately 40/1 when the cells were suspended in 0.25% glucose but was much less when they were suspended in LB or in succinate (22); we therefore used 19/1 as an average approximation. Several papers (15, 24, 25) allow estimation that $V_{SOD}/V_f$ lies in the range 10–20, in agreement with our present estimate.

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