Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 5892–5896, July 1992
Biochemistry

Fumarase C, the stable fumarase of *Escherichia coli*, is controlled by the *soxRS* regulon

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Contributed by Irwin Fridovich, March 26, 1992

ABSTRACT **Fumarase C was strongly induced by paraquat in a parental strain of *Escherichia coli* but was not induced in a strain lacking the *soxRS* response. Moreover, a strain that constitutively expresses the *soxRS* regulon contained more fumarase C than did the parental strain. The Mn-containing superoxide dismutase and glucose-6-phosphate dehydrogenase, members of the *soxRS* regulon, were similarly induced by paraquat. Mutational defects in glucose-6-phosphate dehydrogenase increased the induction of fumarase C by paraquat. For Mn-containing superoxide dismutase, responsiveness to paraquat was also enhanced in the glucose-6-phosphate dehydrogenase-defective strains. Overproduction of the Mn-containing superoxide dismutase, elicited by isopropyl β-D-thiogalactoside in a *tac-sodA* fusion strain, did not diminish induction of fumarase C or of glucose-6-phosphate dehydrogenase by paraquat, and induction of these enzymes was more sensitive to paraquat when the cells were growing on succinate rather than on LB medium. These results indicate that fumarase C is a member of the *soxRS* regulon and that this regulon does not respond to changes in O_2^- concentration but perhaps does respond to some consequence of a decrease in the ratio of NADPH to $NADP^+$.**

soxR (superoxide response) was defined as a genetic locus mediating positive transcriptional regulation of the biosynthesis of nine proteins that are among those induced in *Escherichia coli* in response to oxidative stress imposed by viologens or quinones (1, 2). Among the identified members of the *soxRS* response are the Mn-containing superoxide dismutase (MnSOD), glucose-6-phosphate dehydrogenase (G6PD), and endonuclease IV, all of which provide a defense against oxidative stress. Indeed, mutants constitutive in the *soxRS* response were relatively resistant to such stress, whereas nonresponding mutants were relatively more sensitive (1, 2). Further analysis of *sox* has revealed two adjacent divergently transcribed genes named *soxR* and *soxS* (3).

The *soxRS* regulon is thought to modulate a global response to O_2^-. However, the observation that overproduction of SOD did not prevent induction of G6PD in logarithmic-phase cells exposed to paraquat suggests that some aspect of redox status other than O_2^- was the signal for induction of this regulon (4). This view is strengthened by the observations that a variety of oxidants can induce expression of the *sodA* gene under anaerobic conditions that preclude O_2^- production (5–10). Since *in vitro* transcription of the MnSOD gene (*sodA*) was suppressed by NADPH (11), it seems possible that *soxRS* responds to the ratio of NADPH/$NADP^+$. We now report that fumarase C is controlled by *soxRS* and we provide additional data indicating that NADPH/$NADP^+$ is the signal to which this regulon responds.

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MATERIALS AND METHODS

Paraquat, Tris, cytochrome *c* (type III), xanthine, glucose 6-phosphate, and $NADP^+$ were from Sigma; yeast extract and Bacto tryptone were from Difco; malate was from ICN; and $MgCl_2$, EDTA, and NaCl were from Mallinckrodt. Xanthine oxidase was generously provided by K. V. Rajagopalan (Duke University).

Bacterial Strains. DJ901, with a deletion in *soxRS*; JTG936, constitutive with respect to the *soxRS* response; and GC4468, a parental strain for DJ901 and for JTG936, as described (1), were provided by B. Demple (Harvard University). DF102, a G6PD overproducer; DF100, the parental strain for DF102; DF2001, defective in G6PD (*zwf*); K10, the parental strain for DF2001; DF1671-DZ1, with a deletion defect in G6PD; and DF1671, the parental strain for DF1671-DZ1, were prepared in the laboratory of D. G. Fraenkel (12) and were supplied by B. Demple. AB1157 was provided by J. Imlay (Duke University); and plasmids pBR322 and pDT1-16 were donated by D. Touati (Institut Jacques Monod, Paris) and were inserted into AB1157 by using conventional procedures (16). Table 1 lists and describes the strains and plasmids used in these studies.

Growth Conditions. Unless otherwise specified cells were grown in LB medium (16) at 37°C. Overnight cultures were diluted 1:8 with fresh medium and incubated for 1 hr before being diluted 1:10 with fresh medium or fresh medium containing paraquat. These experimental cultures were then incubated for 3 hr and then harvested, lysed, and clarified as described (4). The SOD-overproducing strain AB1157(pDT1-16) and the corresponding control strain AB1157(pBR322) were grown overnight and then diluted 1:100 into a medium containing 60 mM K_2HPO_4, 33 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM sodium citrate, 1.0 mM $MgSO_4$, 0.4% sodium succinate, thiamine hydrochloride (10 μg/ml), 100 μM $MnSO_4$, isopropyl β-D-thiogalactoside (100 μg/ml), ampicillin (50 μg/ml), and all 20 naturally occurring amino acids (each at 40 μg/ml), with L-cystine used in place of L-cysteine. When A_{550} had reached 0.65, paraquat was added to 2.5 μM and cells were harvested at intervals thereafter for preparation of extracts.

Assays. Extracts were assayed for SOD (17), G6PD (18), fumarase (19), and protein (20, 21).

RESULTS

Induction of Fumarase by Paraquat. The *E. coli* genome contains three genes known to code for fumarase. Two of these, *fumA* and *fumC*, are at 35.5 min on the *E. coli* map (22, 23) and the third, *fumB*, is at 93.5 min (23). Fumarases A and B are dimeric labile iron–sulfur proteins, whereas fumarase C is a tetrameric stable enzyme that has homology to aspartase and to argininosuccinase (24) and that does not contain an

Abbreviations: SOD, superoxide dismutase; G6PD, glucose-6-phosphate dehydrogenase.

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Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or characteristics	Source or ref.
GC4468	F⁻ Δ*lac U169 rpsL*	1
DJ901	GC4468 plus Δ(*soxR-zjc-2205*) *zjc-2204*::Tn*10*km	1
JTG936	*soxR105* plus *zjc-2204*::Tn*10*km	1
DF102*	*zwfL2*(con)	13
DF100	Parental strain for DF102	13
K10	*fadL701 relA1 pit-1 spoT1 rrnB2 mcrB2 phoH510*	13
DF2001	asK10 plus *zwfA2*	12
DF1671	*eda-1 hisG1 rpsL115* (str^R) *pgi-2* [could also carry *pit-10 spoT1 relA1 omF627 garB10 mglP50 galP63 umg^c metB1 thi-1 azi fluA tsx rfbD1*]	12
DF1671-DZ1	As DF1671 plus Δ(*eda-zwf*)*15*	12
AB1157	F⁻ *thr-1 leuB6 proA2 his-4 thi-1 argE2 lacY1 galK2 rpsL supE44 ara-14 xyl-15 mtl-1 tsx-33*	14
pBR322	Vector, Ap^R, Tc^R	15
pDT1-16	pBR322 carrying an operon fusion between the *tac* promoter and the *sodA* gene; Ap^R *sodA*⁺	15

Str^R, streptomycin resistant; Ap^R, ampicillin resistant; Tc^R, tetracycline resistant.
*This strain exhibited a 20-fold elevation of G6PD above the level seen in DF100.

iron–sulfur cluster. These fumarases are readily differentiated since incubation overnight at 4°C, and/or incubation at 37°C for 45 min, inactivates fumarases A and B but leaves fumarase C active.

Fig. 1*A* shows that exposure of a parental strain of *E. coli* to paraquat caused a dose-dependent increase in the fumarase activity measured in freshly prepared extracts (curve 1). A Δ*sox* strain, in contrast, exhibited a decline in net fumarase (curve 2). A *soxRS* response-constitutive strain contained 4-fold more fumarase in the absence of paraquat and retained the ability to respond to paraquat by further induction of fumarase (curve 3). When these extracts were incubated at 4°C overnight and then warmed to 37°C for 45 min, before being reassayed for fumarase activity (Fig. 1*B*), paraquat strongly induced the stable fumarase C (curves 1 and 3) and caused a loss of the unstable fumarases A and B. The observation that the Δ*sox* mutant did not induce fumarase C in response to paraquat indicates that fumarase C is regulated by *soxRS*. These results were bolstered by assaying for G6PD, a known member of the *soxRS* regulon. As shown in Fig. 1*C*, curve 1, the parental strain exhibited marked induction of G6PD by paraquat, whereas the Δ*sox* mutant did not (curve 2). The *soxRS* constitutive strain exhibited elevated G6PD and responded to paraquat by further induction (data not shown).

These results indicate that fumarase C is a member of the *soxRS* regulon and fumarases A and B are not. The paraquat-induced decrease in fumarases A and B may reflect the sensitivity to oxidative inactivation characteristic of a number of iron–sulfur-containing acid dehydratases (25–28).

Effects of G6PD Deficiency on Induction of Fumarase. If the *soxRS* regulon responds to a decrease in NADPH/NADP⁺, then a mutational defect in *zwf* that eliminates an important site of NADP⁺ reduction should enhance induction of the *soxRS* response by paraquat. This is expected because the NADPH oxidized by the soluble *E. coli* NADPH:paraquat diaphorase (29) would not be as rapidly reduced at the expense of glucose 6-phosphate and the steady-state ratio of NADPH/NADP⁺ would fall more in the *zwf*-deficient strains than in the parental strain.

Fig. 2*A* demonstrates that low levels of paraquat (1–10 μM) increased the total fumarase of fresh extracts more strongly in the Δ*zwf* strain (curve 1) than in the control (curve 2). When these extracts were reassayed after an 18-hr incubation at 4°C plus a 45-min incubation at 37°C (Fig. 2*B*), it was apparent that the stable fumarase C was induced and that the Δ*zwf* mutant (curve 1) was more responsive to paraquat than was the control strain (curve 2). We again note (Fig. 2*C*) that paraquat caused a loss of the labile fumarases. It should be

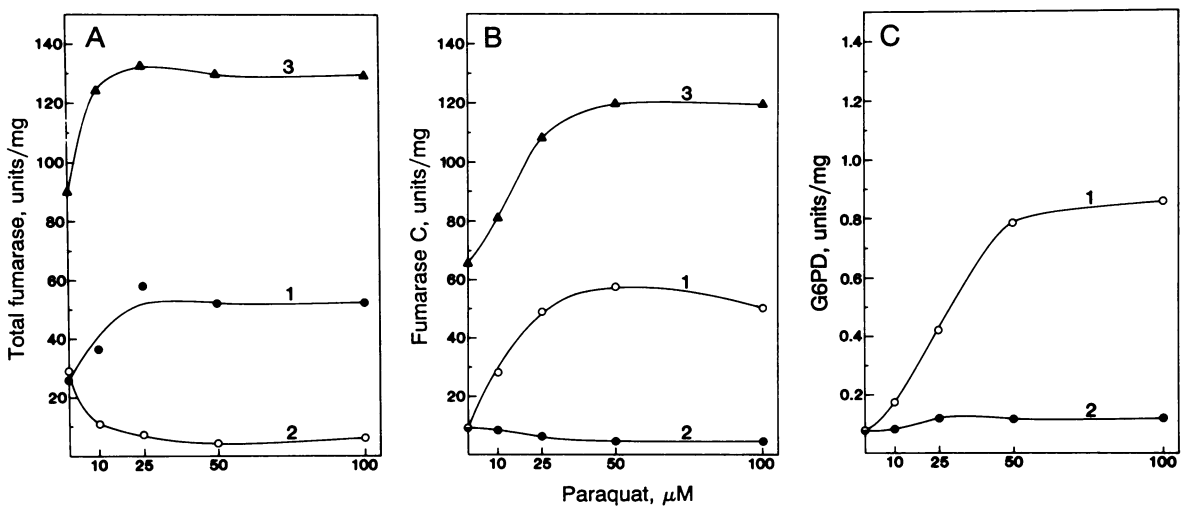

FIG. 1. Induction of fumarase and of G6PD by paraquat. *E. coli* were grown in LB medium containing the indicated concentrations of paraquat for 3 hr at 37°C. The cells were then collected by centrifugation and lysed in a French press, and the extracts were clarified by centrifugation, as described (4). The soluble extracts were assayed for protein, total fumarase (*A*), fumarase C (*B*), and G6PD (*C*). Curves: 1, parental strain (GC4468); 2, Δ*soxRS* (DJ901); 3, *soxRS* response constitutive (JTG936).

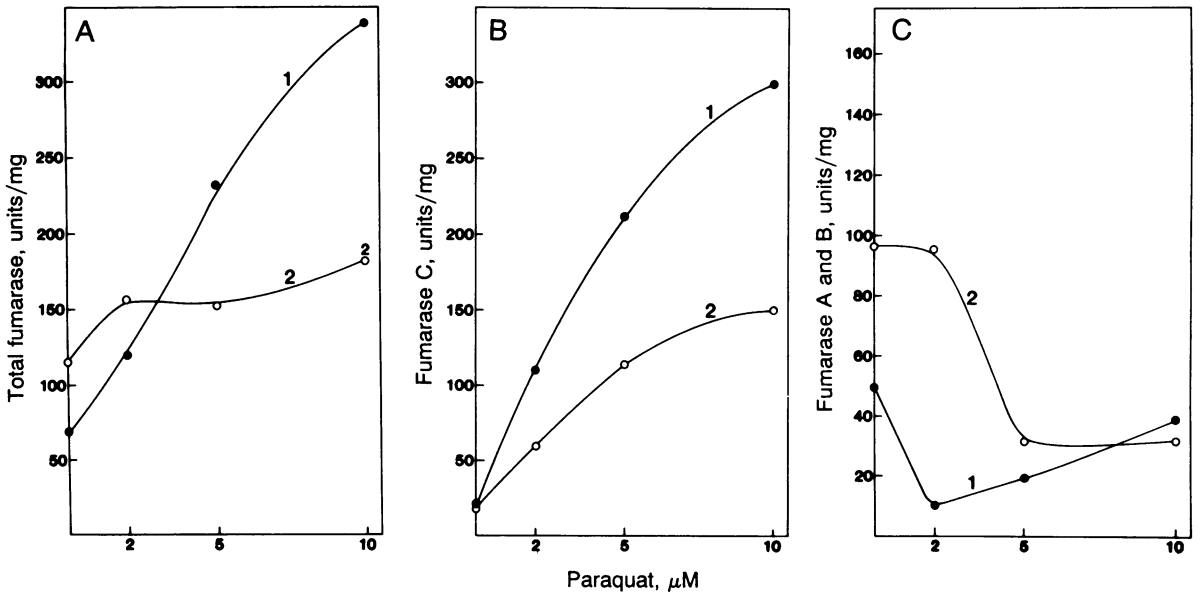

FIG. 2. Effect of a defect in G6PD on induction of fumarase. Cells were grown and extracts were prepared and assayed as in Fig. 1. Curves: 1, the mutant Δ*zwf*(DF1671-DZ1); 2, parental strain (DF1671). (*A*) Total fumarase as measured in the fresh extracts. (*B*) Fumarase C as measured after incubation to inactivate the unstable fumarases A and B. (*C*) Fumarases A plus B calculated as the difference between total fumarase and stable fumarase.

noted that the range of paraquat concentrations examined was 10-fold greater in Fig. 1 than in Fig. 2.

The effect of a defect in G6PD (*zwf*) was also examined in a different genetic background. Fig. 3*A*, curve 2, shows that the fumarase activity of the *zwf* strain is greater than the parental strain (curve 1). Elimination of the unstable fumarases by incubation (Fig. 3*B*) revealed that the *zwf* strain contained more fumarase C and accumulated more fumarase C in response to paraquat (curve 2) than did the parental strain (curve 1). Data in Fig. 3*C* again show that paraquat caused a loss of the unstable fumarases.

Induction of fumarase C by paraquat was thus greater in the *zwf*-deficient strains than in the parental strains. Comparison of the inducibility of fumarase by paraquat in the parental strains of the *zwf* and the Δ*zwf* mutants reveals that the Δ*zwf* parent was the more responsive to paraquat (compare Fig. 3, curves 1, with Fig. 2, curves 2). This may reflect their different genetic backgrounds. Thus DF1671, unlike K10, carried defects in *eda*, *pgi*, and several other sites. The *eda* and *pgi* defects would have caused impairment of carbohydrate catabolism and thus decreased generation of NAD(P)H. These results are in accord with the supposition that NADPH/$NADP^+$ may be the signal to which *soxRS* responds.

Effects of G6PD Deficiency on Induction of MnSOD. MnSOD is a member of the *soxRS* regulon (1). If NADPH/$NADP^+$ is the variable to which this regulon responds, then defects in *zwf* should increase the paraquat-mediated induction of MnSOD, as they did with fumarase C. The data in Fig. 4 demonstrate these effects. Thus the *zwf* strain was more responsive to low levels of paraquat than was its parental strain (compare Fig. 4, curves 1 and 2) and the same was true for the Δ*zwf* and its parental strain (compare curves 3 and 4).

Responses of G6PD to Paraquat. The effects of paraquat on G6PD activity in the two G6PD-deficient strains, their parental strains, and in a G6PD overproducer were also examined (Fig. 5). As expected the strains defective in *zwf* lacked G6PD activity, whether exposed to paraquat or not (curves 1 and 2), and a G6PD overproducer did contain much more G6PD activity than did the parental strains (compare curve 5 with curves 3 and 4). The observation that DF1671 was more responsive to induction by paraquat (curve 4) than was K10 (curve 3) suggests that genetic defects other than those in *zwf*

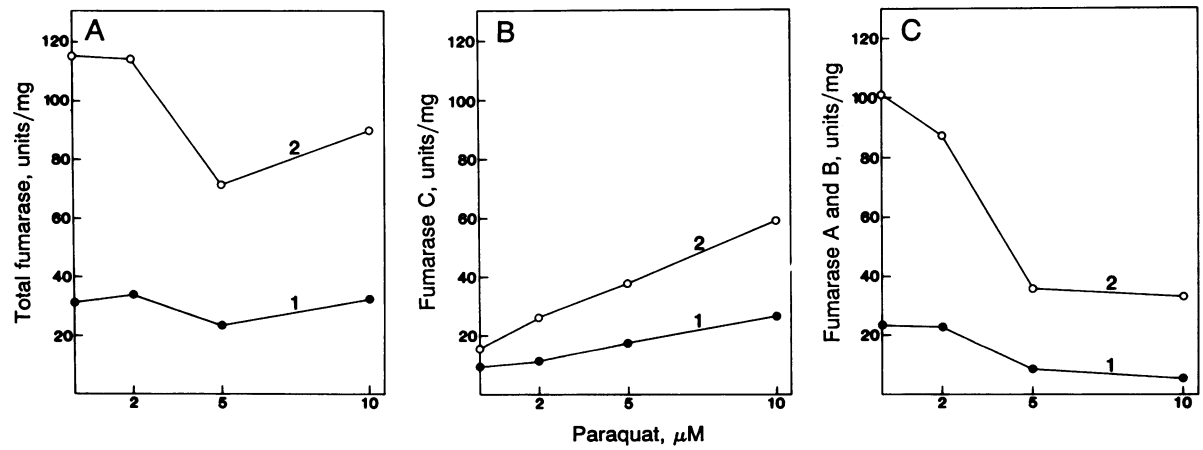

FIG. 3. Effect of another defect in G6PD on induction of fumarase. Conditions are as in Fig. 1. Curves: 1, parental strain (K10); 2, *zwf*$^-$ (DF2001). (*A*) Total fumarase. (*B*) Fumarase C. (*C*) Fumarases A and B, calculated as the difference between total fumarase and fumarase C.

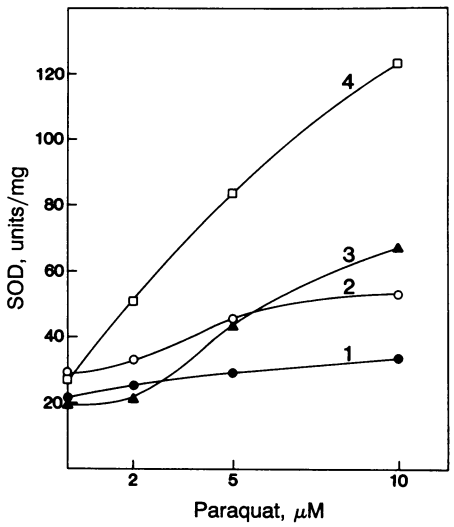

FIG. 4. Effects of defects in G6PD on induction of SOD. Cells were grown and extracts were prepared as in Fig. 1. These extracts were assayed for protein and for SOD. Curves: 1, parental strain (K10); 2, *zwf*$^-$ (DF2001); 3, DF1671; 4, DF1671-DZ1 (Δ*zwf*).

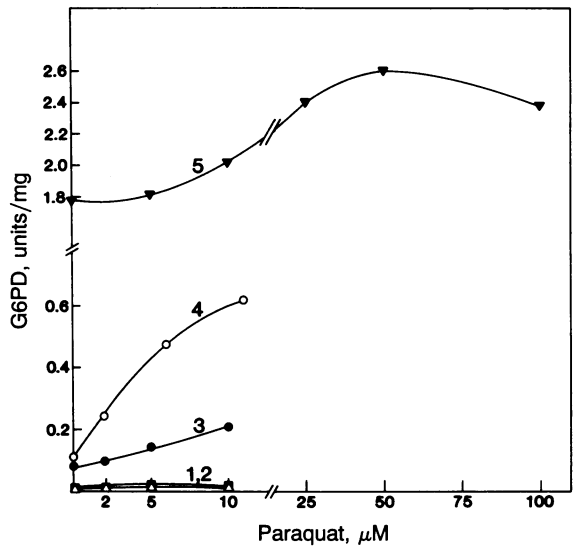

FIG. 5. Effects of defects in, or overproduction of, G6PD on induction of G6PD. Conditions of growth and preparation of extracts as in Fig. 1. Extracts were assayed for G6PD. Curves: 1, DF200 (*zwf*$^-$); 2, DF1671-DZ1 (Δ*zwf*); 3, parental strain (K10); 4, DF1671; 5, G6PD-overproducing strain (DF102).

can make the *soxRS* regulon more responsive to induction by paraquat.

Responses of G6PD Overproducer. Whereas a lack of active G6PD will almost certainly compromise the supply of NADPH, overproduction of G6PD may or may not do so because the supply of glucose 6-phosphate, by way of glucose uptake, phosphorylation, or gluconeogenesis, may become rate-limiting such that the increase in G6PD could be without effect on the rate of reduction of $NADP^+$. The ability of a G6PD-overproducing strain to induce fumarase C and SOD in response to paraquat was examined and was found to be as responsive as a parental strain (data not shown).

Effect of Overproduction of SOD. Paraquat at 2.5 μM caused a time-dependent induction of G6PD (Fig. 6, curves 1 and 2) and of fumarase C (Fig. 6, curves 3 and 4); eliciting overproduction of MnSOD with isopropyl β-D-thiogalactoside in a *tac–sodA* fusion strain did not affect these inductions (Fig. 6, compare curve 1 with 2 and curve 3 with 4). The concentration of O_2^- is thus not the signal to which *soxRS* responds. These experiments were done in a succinate medium, and it should be noted that the cells were much more responsive to paraquat in this medium than they were in LB medium. This can be explained in terms of the constraint on $NADP^+$ reduction to be expected on a nonfermentable carbon source. The SOD content of the fusion strain grown in the presence of isopropyl β-D-thiogalactoside (Fig. 6, curve 6) was much greater than that of the parental strain (curve 5); yet, both exhibited a time-dependent increase in SOD activity after exposure to paraquat.

DISCUSSION

The *soxRS* regulon provides a defense against oxidative stress imposed by dioxygen and by redox-cycling agents such as viologens and quinones. This defensive system has several facets. Thus the MnSOD, encoded by the *sodA* gene, protects against damage caused, directly or indirectly, by O_2^-.

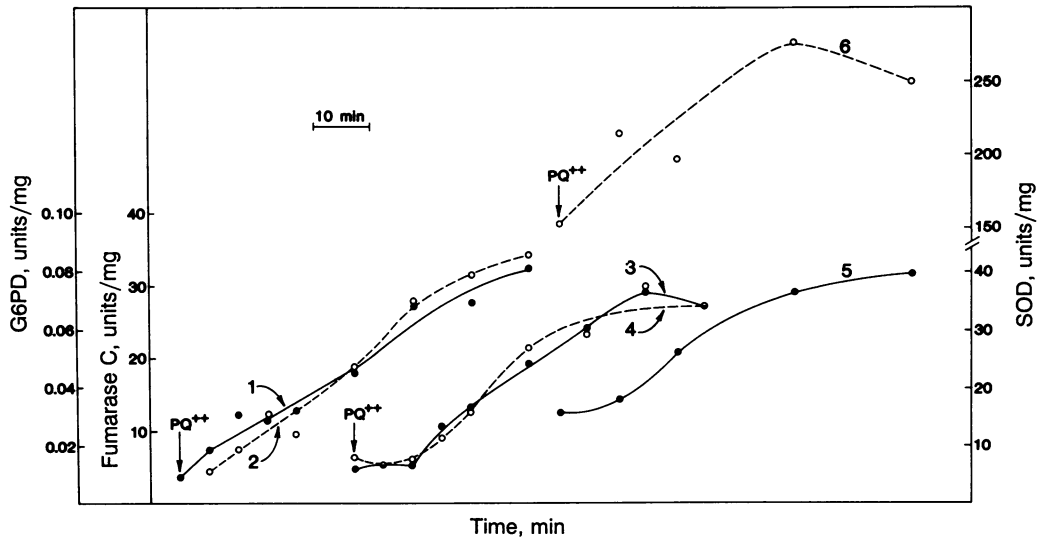

FIG. 6. Rates of induction of G6PD, fumarase, and SOD. Paraquat (PQ^{++}) was added to 2.5 μM at the arrow and at intervals thereafter cells were removed to assay protein and enzyme activities. Curves: 1, control strain [AB1157(pBR322)] assayed for G6PD; 2, SOD overproducer [AB1157(pDT1-16)] assayed for G6PD; 3, control strain assayed for fumarase C; 4, SOD overproducer assayed for fumarase C; 5, control strain assayed for SOD; 6, SOD overproducer assayed for SOD.

Endonuclease IV (30–32), the product of the *nfo* gene, repairs oxidatively damaged DNA. G6PD, the product of the *zwf* gene, maintains a high ratio of NADPH/$NADP^+$ and thus provides a reducing environment and is needed for the actions of glutathione reductase and of the alkylhydroperoxide reductase (33–35), the latter of which is itself inducible by oxidative stress (35).

Another aspect of this defensive system now appears to entail replacement of enzymes that are very susceptible to oxidative attack by isozymes that are stable to such attack. This is the category into which the stable fumarase C fits. It is not clear what advantages accrue to *E. coli* from the production of the oxidatively unstable fumarases A and B when not under oxidative stress. It is, however, obvious that replacement of fumarases A and B by the stable fumarase C is an effective adaptive response to oxidative stress. The same reasoning may apply to the regulation of MnSOD (*sodA*) by *soxRS* since MnSOD is resistant toward H_2O_2 whereas the Fe-containing SOD (FeSOD) is not. Induction of MnSOD by imposition of oxidative stress is accompanied by some decrease in FeSOD.

There also appears to be a relationship between carbohydrate metabolism and the *soxRS* regulon. Thus, defects in G6PD (*zwf*) increased the inducibility of this regulon. The ability of glucose to suppress production of MnSOD by a cAMP-independent route (36) has been noted (37).

Although *soxRS* was originally thought to be responsive to O_2^- (1, 2), this view now requires modification. Thus, two members of this regulon, MnSOD and G6PD, can be induced under anaerobic conditions by an oxidant such as diamide (38). Moreover, NADPH was seen to suppress *in vitro* transcription of *sodA* (11). The present observation that interference with the metabolism of glucose, imposed by defects in *zwf*, increased the paraquat-mediated inducibility of the *soxRS* response also argues that the *soxRS* regulon is responsive to the NADPH/$NADP^+$ ratio. It has been reported (39) that overproduction of FeSOD did not prevent induction of MnSOD by dioxygen and, therefore, that O_2^- could not be the signal for induction of MnSOD.

O_2^- is known to oxidatively inactivate several [4Fe–4S] cluster-containing acid dehydratases (25–28). It can also inactivate catalase (40) and initiate free radical chain oxidations of NAD(P)H in the presence of Mn(II) (41), V(V) (42), or lactic dehydrogenase (43). O_2^- also oxidizes catecholamines (44), tetrahydropterins (45), leukoflavins (46), and sulfite (47). It is clear that O_2^- could impose an oxidative stress that would result in depletion of NADPH. SOD, by keeping the steady-state level of O_2^- of <0.1 nM (14), minimizes these consequences of O_2^- production. In *E. coli*, which contain the wild-type level of SOD or which overproduce SOD, the steady-state level of O_2^- will be very low and it will be unable to significantly deplete NADPH. In such cells, induction of the *soxRS* response must depend upon mechanisms that deplete NADPH independent of O_2^-. However, in *sodA sodB* cells, the steady-state level of O_2^- can rise dramatically (14) and could then serve as a major factor in the depletion of NADPH. Under such conditions increasing O_2^- production could indeed cause induction of the *soxRS* response. This explains the greater responsiveness of endonuclease IV (30) and of G6PD (4) to induction by paraquat as seen with the *sodA sodB* strain compared with the parental strain.

When viewed broadly, the function of the *soxRS* regulon is to maintain redox balance and normal metabolism under conditions of oxidative stress that tend to deplete NADPH, inactivate specific enzymes, and damage DNA and membranes. This is accomplished by minimizing depletion of NADPH by O_2^- (MnSOD), increasing production of NADPH (G6PD), replacing unstable enzymes by stable isoforms (fumarase C), and augmenting repair capabilities (endonuclease IV). It will be interesting to see how well this rubric serves to rationalize the functions of yet to be identified members of the *soxRS* regulon.

We are indebted to Dr. Paul G. Gardner for his help in performing the experiments with the MnSOD overproducer and to Dr. B. Demple for providing many of the strains used in these studies. This work was supported by research grants from the Council for Tobacco Research-U.S.A., Inc., the Johnson and Johnson Focused Giving Program, and the National Science Foundation.

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