

NADPH:ferredoxin oxidoreductase acts as a paraquat diaphorase and is a member of the *soxRS* regulon

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ABSTRACT Soluble extracts of *Escherichia coli* contain four NADPH:paraquat diaphorases that were separable by anion-exchange HPLC over Mono Q. One of these was induced when the cells were exposed to paraquat. This was the case in a *soxRS*-competent strain but not in a *soxRS*-null strain, while a *soxRS*-constitutive strain overexpressed this diaphorase without the stimulus of exposure to paraquat. This NADPH:paraquat diaphorase could use cytochrome *c* or nitroblue tetrazolium as an electron acceptor, whereas O₂ was a relatively poor acceptor. This diaphorase was identified as the NADPH:ferredoxin reductase. A role for reduced ferredoxin and flavodoxin in the adaptive *soxRS* response to oxidative stress and in the regulation of the redox status of SoxR is discussed.

The dioxygen-dependent toxicity of paraquat (PQ²⁺) depends upon its entry into cells, followed by repeated cycles of univalent reduction and autooxidation, which divert the electron flow from normal pathways and simultaneously increase intracellular production of O₂⁻. Part of the toxicity of PQ²⁺ is due to this generation of O₂⁻, as demonstrated by the hypersensitivity of mutants with defects in superoxide dismutase (SOD) (1) and by the elimination of this phenotype by restoration of SOD (2). Another aspect of the toxicity of PQ²⁺ must relate to the consequences of the diversion of electron flow and this can be explored by identifying the enzymes capable of catalyzing the reduction of PQ²⁺. We have noted (3) that there are at least three NADPH:PQ²⁺ diaphorases in extracts of *Escherichia coli* and that one of these is the NADPH:thioredoxin oxidoreductase (EC 1.6.4.5).

We now identify another one of the NADPH:PQ²⁺ diaphorases of *E. coli* as the NADPH:ferredoxin oxidoreductase (EC 1.18.1.2) and demonstrate that it is regulated as a member of the *soxRS* regulon. This regulon was originally seen as an adaptive response to intracellular production of O₂⁻ (4–7). However, it is now apparent that the *soxRS* regulon can be induced by, and can provide a defense against, oxidants other than O₂⁻ (8–13). The manner in which the NADPH:ferredoxin reductase may contribute to the concerted defense against oxidative stress is considered.

MATERIALS AND METHODS

PQ²⁺ (methyl viologen), cytochrome *c* (type III), NADPH, nitroblue tetrazolium (NBT), and ferredoxin from a marine red alga were obtained from Sigma. Tris (ultrapure) was from ICN (Schwarz/Mann) and NaCl was from Mallinckrodt; yeast extract and Bacto tryptone were from Difco. Sephadex was from Pharmacia.

Culture of Cells. The strains used are listed in Table 1. Cells were grown aerobically at 37°C in a water bath shaker at 200 rpm. Overnight cultures were diluted 1:20 with fresh LB, adjusted to pH 7.5, and were incubated for 1 hr prior to the

addition of PQ²⁺ and then for an additional 2 hr. The cells were then collected by centrifugation and soluble extracts were prepared as described (3) except that 50 mM Tris-HCl at pH 7.8 was used for washing and resuspending the cells prior to lysis in the French press. Extracts were fractionated by HPLC as described below.

Assays. PQ²⁺ diaphorase was measured as the PQ²⁺-dependent oxidation of NADPH followed at 340 nm. NADPH was added to 0.1 mM to the cell extract diluted into the Tris-HCl buffer at pH 7.8 and PQ²⁺ was then added to 4 mM. In most cases NADPH oxidation was negligible until PQ²⁺ was added. When PQ²⁺-independent NADPH oxidase activity was noted, it was subtracted from the rate seen in the presence of PQ²⁺. When NBT reduction was to be followed, PQ²⁺ was replaced by 0.1 mM NBT and accumulation of the formazan was followed at 560 nm. When cytochrome *c* reduction was to be followed PQ²⁺ was replaced by 0.025 mM cytochrome *c* and the accumulation of ferrocytochrome *c* was followed at 550 nm. Ferredoxin reductase activity was measured in terms of the ferredoxin-dependent reduction of cytochrome *c* (15). The concentration of ferredoxin was then 10 μg/ml. The millimolar extinction coefficients used were 6.2 for NADPH oxidation and 21 for cytochrome *c* reduction. Ferredoxin augmented the rate of reduction of cytochrome *c* by NADPH, under the catalytic influence of the ferredoxin reductase, and the rate of reduction of ferredoxin was calculated from the magnitude of this augmentation, which was ≈3-fold in routine assays. Protein concentration was determined by the Lowry method.

Electrophoresis. Both native and SDS/polyacrylamide gel electrophoreses were done with 8–25% gradient gels using the Pharmacia Phast system. When larger slabs (14 × 14 × 0.15 cm) were used, the procedure was essentially that of Laemmli (16). Native gels were stained for the NADPH:PQ²⁺ diaphorase by soaking in 0.2 mM NADPH/4 mM PQ²⁺/1 mM NBT in 50 mM Tris-HCl (pH 7.5).

Isolation of NADPH:PQ²⁺ Diaphorase. *E. coli* GC4468 was grown in 2 liters of LB medium containing 0.1 mM PQ²⁺, collected, and extracted into 20 ml of 20 mM Tris-HCl (pH 7.5), as described above. The extract was desalted by gel filtration over a 4.5 × 45 cm column of Sephadex G-25 and was then applied to a 1.5 × 35 cm column of Matrex Gel Blue A (Amicon) equilibrated with the 20 mM Tris-HCl (pH 7.5). The column was then washed with this buffer until the A₂₈₀ of the eluate had returned to baseline, and material was eluted with 50 ml of 10 mM NADP⁺ in the Tris buffer, then with the Tris buffer, and finally with 1.0 M NaCl in the Tris buffer. The fraction eluted with NaCl was concentrated, desalted by ultrafiltration over a PM10 membrane (Amicon), and then applied to a Mono Q HR 5/5 column (Pharmacia) at a flow rate of 1 ml/min; material was eluted first with 10 ml of the 20 mM Tris buffer followed by 20 ml of a linear gradient of NaCl (0–0.4 M) in this buffer. Peaks of NADPH:PQ²⁺

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Abbreviations: PQ²⁺, paraquat; SOD, superoxide dismutase; NBT, nitroblue tetrazolium; PTH, phenylthiohydantoin.
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Table 1. Strains used

Strain	Genotype or characteristic	Ref.
GC4468	F ⁻ Δ <i>lac</i> U169 <i>rpsL</i>	13
DJ901	GC4468 plus Δ(<i>soxR-zjc-2205</i>) <i>zjc-2204::Tn10km</i>	13
JTG936	<i>soxR105</i> plus <i>zjc-2204::Tn10km</i>	13
c-1a + pEE1010	F ⁻ protrophic	14

diaphorase appeared at 0.20 and at 0.39 M NaCl. The fraction collected at 0.2 M NaCl was concentrated and desalted in Centricon-10 microconcentrators and was applied to native polyacrylamide gels at 125 μg of protein per lane. After electrophoresis, guide lanes were cut from the slab and stained either for protein or for NADPH:PQ²⁺ diaphorase activity. While the protein stain revealed ≈10 bands, the activity stain showed only one. The region of the slab gel corresponding to the band with activity was excised and soaked ≈15 hr in 0.1% SDS/5% (vol/vol) 2-mercaptoethanol at 25°C. It was then placed on top of a fresh SDS/15% polyacrylamide gel and electrophoresed. The protein was then transferred onto an Immobilon membrane (Millipore) with a semi-dry blotting apparatus (LKB). The transfer and subsequent staining were performed according to Matsudaira (17).

N-Terminal Sequence Analysis. The N-terminal sequence of the Immobilon-electroblotted sample was determined using a Porton 2090 gas-phase sequencer interfaced to a Beckman System Gold HPLC for online detection of the phenylthiohydantoin (PTH) amino acid derivatives. The excised Immobilon membrane was cut to size, overlaid with a protein support disk, and subjected to 13 cycles of Edman chemistry using standard procedure 1 on the sequencer. PTH amino acid derivatives were detected at 269 nm. A linear regression analysis of the plot of logarithm of pmol of PTH amino acid derivatives versus cycle indicated that the initial amount of sequenceable material was 7.6 pmol (≈230 ng at 30 kDa/mol). The overall repetitive yield determined from the slope of the regression line was 85%. The N-terminal sequence obtained was identified using the Swiss Protein 35 data base in the IntelliGenetics Suite VAX software package.

HPLC Fractionation. In experiments where NADPH:PQ²⁺ diaphorases were separated for the purpose of following induction of PQ²⁺ diaphorases, soluble extracts (10–20 mg of protein) were applied to a Mono Q HR 5/5 column (Pharmacia) at a flow rate of 1 ml/min. The column was washed for 10 min with 20 mM Tris·HCl (pH 7.5). Bound protein was eluted with a linear gradient of 0–1.0 M NaCl in the same buffer, and 1-ml fractions were collected. For Fig. 1, fractions 1–10 were buffer alone and fractions 10–60 represented a gradient of 0–1.0 M NaCl.

RESULTS

E. coli has been reported to contain a soluble NADPH:PQ²⁺ diaphorase (18) that was inducible by PQ²⁺ (19). We have recently realized that there are at least three NADPH:PQ²⁺ diaphorases in *E. coli* that could be separated by HPLC (3) and have found that only one of these is inducible by exposure of the cells to 0.1 mM PQ²⁺ for 2 hr (data not shown). It appeared possible that this inducible diaphorase might be a member of the *soxRS* regulon. Support for this view was provided by comparing extracts from control and from *soxRS*-constitutive strains. Fig. 1 demonstrates that only one of the NADPH:PQ²⁺ diaphorases (i.e., that eluting to fraction 21) was elevated in the *soxRS*-constitutive strain. In some HPLC runs, this diaphorase eluted in fraction 20 or was split between fractions 20 and 21. In any case, it eluted at 0.20–0.22 M NaCl. Fig. 1 shows four clearly separated NADPH:PQ²⁺ diaphorases. In some runs the two closely

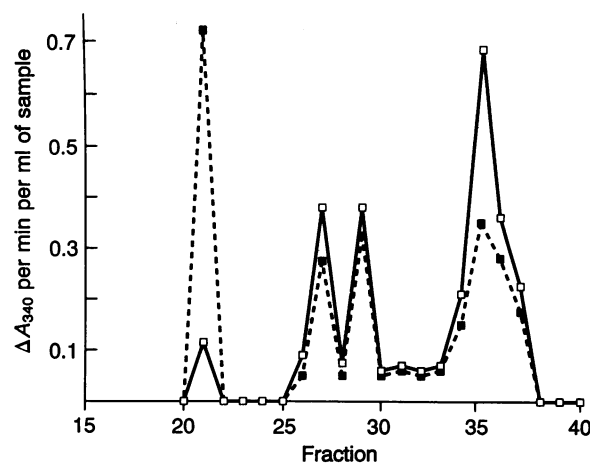


FIG. 1. Separation of NADPH:PQ²⁺ diaphorases by HPLC over Mono Q. Cells were grown as described in *Materials and Methods* except that the 1:10 dilution with fresh LB medium was not accompanied by exposure to PQ²⁺ and the cultures were incubated for 2.5 hr after this dilution. Cell lysates were subjected to HPLC and fractions were assayed for PQ²⁺-dependent oxidation of NADPH. Solid line, control cells; dashed line, *soxRS*-constitutive strain.

spaced peaks of activity were not fully resolved and appeared as a single broader peak.

It could be further shown that the fraction 21 diaphorase was strongly induced by PQ²⁺ in a parental strain but not in a *sox*-null strain. The *soxRS*-constitutive strain overproduced this diaphorase while retaining the ability to induce it further when exposed to PQ²⁺. These results, shown in Fig. 2, establish that the NADPH:PQ²⁺ diaphorase eluting in fraction 21 is a member of the *soxRS* regulon. The activity of this diaphorase could also be measured in terms of the NADPH-dependent reduction of NBT or of cytochrome *c*. When measured in these ways, data very similar to that shown in Fig. 2 were obtained (data not shown).

The reduction of cytochrome *c* by this diaphorase was only weakly inhibitable (i.e., 10–20%) by SOD, suggesting that most of the reduction of cytochrome *c*, and by extension of NBT, was due to direct electron transfer from the diaphorase. In full agreement with this conclusion was the observation that this diaphorase exhibited negligible NADPH oxidase activity. It follows that this diaphorase is not a significant source of O₂⁻ production in *E. coli* in the absence of PQ²⁺.

Identification of the Inducible Diaphorase. The partial N-terminal amino acid sequence, obtained from the inducible

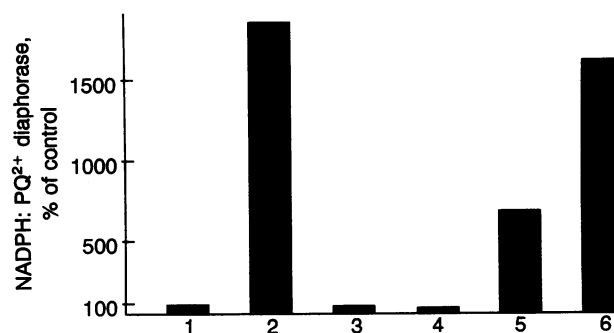


FIG. 2. NADPH:PQ²⁺ diaphorase activities of fraction 21. Cells were incubated with or without PQ²⁺, harvested, extracted, and assayed. The control rate was 2.3 nmol per min per mg of protein in the soluble extract. Bars: 1, control strain; 2, control strain exposed to 0.125 mM PQ²⁺; 3, *soxRS*-null strain; 4, *soxRS*-null exposed to 0.125 mM PQ²⁺; 5, *soxRS*-constitutive strain; 6, *soxRS*-constitutive strain exposed to 0.125 mM PQ²⁺.

PQD	ADWVTGKVTQMKN
<u>mvr A</u>	ADWVTGKVTKVQN
<u>fpr</u>	ADWVTGKVTKVQN

FIG. 3. Amino acid sequence homologies. N-terminal amino acid sequence of the *E. coli* PQ²⁺ NADPH diaphorase (PQD), the *E. coli* gene *mvrA* (residues 146–158; ref. 20), and *E. coli* ferredoxin NADPH reductase (*fpr*; ref. 14). Homologies are indicated by vertical lines.

NADPH:PQ²⁺ diaphorase, proved to be virtually identical to sequences reported for the *E. coli* NADPH:ferredoxin reductase (14) and for the *E. coli mvrA* gene (20) as shown in Fig. 3. Since the PTH derivatives of methionine and valine are usually eluted very close together, the single apparent lack of complete homology (i.e., methionine in place of valine) is probably an error. These sequence homologies strongly suggest that the inducible NADPH:PQ²⁺ diaphorase is NADPH:ferredoxin reductase.

NADPH:ferredoxin Reductase and *soxRS*. The results in Fig. 4 show that PQ²⁺ elicited a profound induction of NADPH:ferredoxin reductase in a parental strain, but not in a *soxRS*-null strain. Moreover, a *soxRS*-constitutive strain overproduced the ferredoxin reductase, while retaining the ability to further induce this enzyme in response to PQ²⁺. When an extract from a strain of *E. coli* that markedly overproduces the NADPH:ferredoxin reductase was separated by Mono Q chromatography, a major peak of protein was eluted in fractions 20 plus 21 (i.e., in the same fraction that previously corresponded to the inducible NADPH:PQ²⁺ diaphorase). SDS/PAGE of this fraction demonstrated a major component of 29 kDa (Fig. 5, lanes 1 and 4). This is the molecular mass expected for the NADPH:ferredoxin reductase (14). The protein eluting in fraction 21 exhibited a flavin-type absorption spectrum, which was virtually identical to that reported for the ferredoxin reductase (14) (data not shown). Furthermore, the strain that overproduced the ferredoxin reductase was found also to contain ≈20 times more NADPH:PQ²⁺ diaphorase activity than did the control strain. This refers to the activity in crude soluble extracts of the cells. When the activities in fractions 21 were compared, the overproducer contained >100 times more activity than did the control strain. All of this establishes that the inducible NADPH:PQ²⁺ diaphorase is NADPH:ferredoxin reductase.

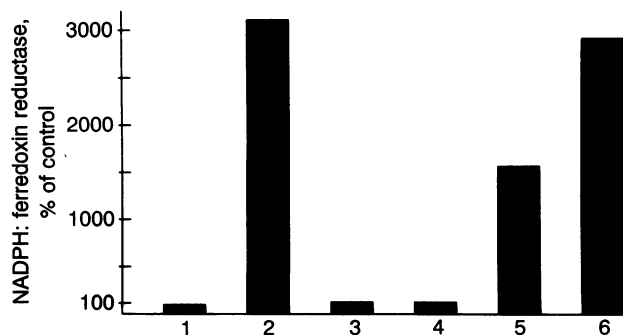


FIG. 4. NADPH:ferredoxin reductase activities of fraction 21. Conditions were as in Fig. 2 except that NADPH:ferredoxin reductase activity was measured. Bars are as described in Fig. 2. The control rate was 0.6 nmol per min per mg of protein in the soluble extract. The results are expressed as the rate of reduction of cytochrome *c* in the presence of ferredoxin, corrected for the rate in the absence of ferredoxin.

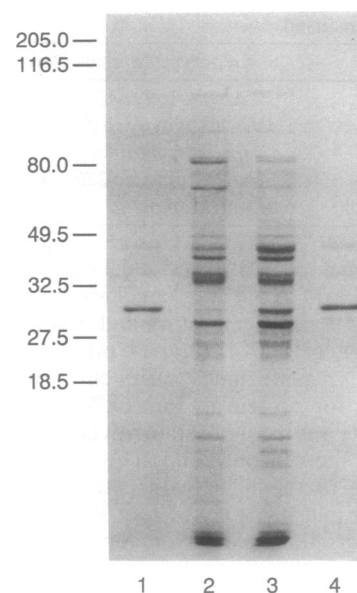


FIG. 5. SDS/PAGE (12% gels) of Mono Q fractions 20 and 21. Extracts from cells were separated by chromatography on Mono Q HR 5/5. Fraction 20 (5 μ g of protein) from the NADPH:ferredoxin reductase-overproducing strain C-1a:pEE1010 (lanes 1 and 4), combined fractions 20 plus 21 (20 μ g of protein) from *E. coli* GC4468 grown in the absence of PQ²⁺ (lane 2) or for 2 hr in the presence of 0.1 mM PQ²⁺ (lane 3). Molecular masses in kDa are indicated on the left.

Extracts from untreated and PQ²⁺-treated *E. coli* GC4468 and from the NADPH:ferredoxin reductase-overproducing strain were separated by Mono Q chromatography. Fraction 20 of the overproducer and the combined fractions 20 and 21 of *E. coli* GC4468 were subjected to SDS/PAGE.

Fig. 5 shows that, at the expected molecular mass of NADPH:ferredoxin reductase (lanes 1 and 4), a protein that is only faintly visible in untreated *E. coli* GC4468 (lane 2) is present in severalfold higher amounts in cells treated with 0.1 mM PQ²⁺ (lane 3). This establishes that the increased activity of NADPH:ferredoxin reductase after PQ²⁺ treatment is due to *de novo* protein synthesis.

DISCUSSION

It is clear that NADPH:ferredoxin reductase is the inducible NADPH:PQ²⁺ diaphorase and that it is regulated by *soxRS*. PQ²⁺ exerts its ill effects by at least two means. One of these involves increased production of O₂⁻, which can be largely countered by the presence of FeSOD plus the induction of MnSOD. The other involves diversion of electron flow from constructive pathways. Elucidation of these constructive pathways, which would be starved for electrons by the interactions with PQ²⁺, can be approached by identifying the PQ²⁺ diaphorases.

NADPH:ferredoxin reductase, NADPH:thioredoxin reductase (3), and sulfite reductase (21) are all in this category. There are undoubtedly others.

A full accounting for the actions of PQ²⁺ must include its effects on such enzymes. Thus, PQ²⁺ imposes auxotrophies for sulfur-containing amino acids and does so even in SOD-proficient *E. coli* (22). This can be explained by the inhibition of the reduction of sulfite to sulfide by the diversion of electrons from sulfite reductase to PQ²⁺.

Identification of NADPH:ferredoxin reductase, as a member of the *soxRS* regulon, allows us to speculate about its defensive role. Thus, reduced ferredoxin and flavodoxin undoubtedly play an important role in the reductive activation of a number of enzymes, among which are ribonucleotide

reductase (14), methionine synthase (23, 24), and pyruvate:formate lyase (25). There are two enzymic routes for the reduction of ferredoxin/ flavodoxin. One of these is the NADPH:ferredoxin reductase and the other is the pyruvate:ferredoxin reductase (24). Both of these are capable of transferring electrons to PQ^{2+} , but the K_m for PQ^{2+} is ≈ 7 mM for the NADPH enzyme (results not shown) and is only 0.05 mM for the pyruvate enzyme (24). At any level of PQ^{2+} below 10 mM, the constructive action of the pyruvate enzyme will be more compromised by the presence of PQ^{2+} than that of the NADPH enzyme.

SoxR is the sensor of the *soxRS* regulon. Its oxidation converts it into an activator of the *soxS* gene and SoxS in turn activates transcription of the members of this regulon (26, 27). It now appears that SoxR is an iron-containing protein and may even contain an iron-sulfur cluster.[§] Its redox status must depend upon the balance between oxidation, perhaps by O_2^- and other oxidants, and reduction. Reduction of SoxR could be caused by reduced flavodoxin and ferredoxin. In that case the entire system is self-regulating, because the NADPH:ferredoxin reductase is a member of the regulon. Thus net oxidation of SoxR activates the regulon, among whose consequences is increased reduction of flavodoxin/ferredoxin, which results in reduction of SoxR and reversal of the activation. Regulation by the ratio of NADPH/NADP⁺ (10, 11) can now also be clarified since the reduction of ferredoxin/ flavodoxin may depend upon this ratio. It should also be noted that the pyruvate:ferredoxin reductase is subject to oxidative inactivation and can be reactivated by dithiothreitol plus Fe(II) (24). It has indeed been shown to contain an iron-sulfur cluster (28, 29). Induction of the NADPH:ferredoxin reductase as part of the *soxRS* regulon can thus also be viewed as an instance of replacement of oxidation-sensitive by oxidation-stable isoenzymes; similar to fumarase C replacing fumarases A + B (11).

Electron transfer between flavodoxin and ferredoxin on one hand and the iron-sulfur clusters of enzymes on the other has been demonstrated (14, 29, 30). Because PQ^{2+} diverts the electron flow from both the pyruvate:ferredoxin reductase and NADPH:ferredoxin reductase, it will diminish the ratio of reduced/oxidized "doxins." This, in turn, will decrease the balance between reduced and oxidized iron-sulfur clusters in enzymes and proteins. O_2^- can exert the same final effect by directly oxidizing susceptible [4Fe-4S] clusters, such as those in aconitase (31), 6-phosphogluconate dehydratase (32), and dihydroxy acid dehydratase (33), which are probably also able to accept electrons from the "doxins." Indeed O_2^- can directly oxidize flavodoxin as well (34).

Since SoxR is believed to contain an iron-sulfur cluster,[§] it is reasonable to suppose that its redox balance also responds to the ratio of the reduced/oxidized "doxins." What the *soxRS* regulon responds to may thus be the balance between reduced and oxidized "doxins." This view can explain the inductions caused by O_2 (35) and by aerobic PQ^{2+} , as well as the anaerobic inductions by diamide (9, 36), NO ,[§] PQ^{2+} plus NO_3^- (9), and even by iron-chelating agents such as *o*-phenanthroline (36).

[§]Demple, B., Hidalgo, E., Nunoshiba, T. & Li, Z., Program and Abstracts of the Fortieth Harden Conference of the Biochemical Society, Aug. 28-Sept. 4, 1993, Nethy Bridge, Scotland, p. 22 (abstr.).

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