Glucose-6-phosphate dehydrogenase and ferredoxin-NADP(H) reductase contribute to damage repair during the *soxRS* response of *Escherichia coli*

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The NADP(H)-dependent enzymes glucose-6-phosphate dehydrogenase (G6PDH) and ferredoxin(flavodoxin)-NADP(H) reductase (FPR), encoded by the *zwf* and *fpr* genes, respectively, are committed members of the *soxRS* regulatory system involved in superoxide resistance in *Escherichia coli*. Exposure of *E. coli* cells to the superoxide propagator methyl viologen (MV) led to rapid accumulation of G6PDH, while FPR was induced after a lag period of several minutes. Bacteria expressing G6PDH from a multicopy plasmid accumulated higher NADPH levels and displayed a protracted *soxRS* response, whereas FPR build-up had the opposite effects. Inactivation of either of the two genes resulted in enhanced sensitivity to MV killing, while further increases in the cellular content of FPR led to higher survival rates under oxidative conditions. In contrast, G6PDH accumulation over wild-type levels of expression failed to increase MV tolerance. G6PDH and FPR could act concertedly to deliver reducing equivalents from carbohydrates, via NADP⁺, to the FPR acceptors ferredoxin and/or flavodoxin. To evaluate whether this electron-transport system could mediate reductive repair reactions, the pathway was reconstituted *in vitro* from purified components; the reconstituted system was found to be functional in reactivation of oxidatively damaged iron–sulfur clusters of hydro-lyases such as aconitase and 6-phosphogluconate dehydratase. Recovery of these activities after oxidative challenge was faster and more extensive in transformed bacteria overexpressing FPR than in wild-type cells, indicating that the reductase could sustain hydro-lyase repair *in vivo*. However, FPR-deficient mutants were still able to fix iron–sulfur clusters at significant rates, suggesting that back-up routes for ferredoxin and/or flavodoxin reduction might be called into action to rescue inactivated enzymes when FPR is absent.

Received 20 October 2005

Revised 29 December 2005

Accepted 4 January 2006

INTRODUCTION

Reactive oxygen species (ROS), such as the superoxide anion radical and its derivatives hydrogen peroxide and the hydroxyl radical, represent a serious threat to aerobic life. Along the path of evolution, organisms thriving in air have developed a number of adaptive devices to cope with this menace. In *Escherichia coli* and other enterobacteria, the induction of a suite of unlinked genes by the *soxRS* regulatory system confers tolerance to superoxide and nitric oxide (Gaudu *et al.*, 1997; Greenberg *et al.*, 1990; Nunoshiba *et al.*, 1992; Pomposiello & Demple, 2001; Pomposiello *et al.*, 2001; Tsaneva & Weiss, 1990; Wu & Weiss, 1992). The sensor of

this regulon is the SoxR protein, a dimeric transcription factor that contains [2Fe–2S] centres (Nunoshiba *et al.*, 1992; Wu & Weiss, 1992). When *E. coli* cells are exposed to superoxide-propagating compounds such as the redox-cycling herbicide methyl viologen (MV), the iron–sulfur cluster of SoxR undergoes univalent oxidation to yield the oxidized, active form of the protein, which subsequently stimulates the synthesis of SoxS, a transcriptional activator of the AraC/XylS family, by productive interaction with its promoter (Ding & Demple, 1997; Gaudu *et al.*, 1997; Pomposiello & Demple, 2001). Increased SoxS levels, in turn, induce expression of the various genes of the regulon via σ^{70} RNA polymerase (Gaudu *et al.*, 1997; Pomposiello & Demple, 2001). Components of the *soxRS* system are recruited to combat the toxic effects of oxidants at various levels, including ROS scavenging, replacement of sensitive targets by resistant counterparts and damage repair (Gaudu *et al.*, 1997; Pomposiello & Demple, 2001). Most *soxRS* members

Abbreviations: Fd, ferredoxin; Fld, flavodoxin; FPR, ferredoxin(flavodoxin)-NADP(H) reductase; G6PDH, glucose-6-phosphate dehydrogenase; MV, methyl viologen; 6PGD, 6-phosphogluconate dehydratase; ROS, reactive oxygen species.

are also subject to transcriptional modulation by the SoxS homologues MarA and Rob, which activate the closely related regulons *marRAB* and *rob*, involved in multiple antibiotic resistance and tolerance to organic solvents, respectively (Aleksun & Levy, 1997).

Among the SoxS targets present in *E. coli*, there are a number of genes encoding enzymes and proteins engaged in oxido-reductive processes. They include glucose-6-phosphate dehydrogenase (G6PDH), ferredoxin(flavodoxin)-NADP(H) reductase (FPR) and its electron acceptor substrates flavodoxin (Fld) I and II (Griffith & Wolf, 2001; Pomposiello & Demple, 2001; Zheng *et al.*, 1999). G6PDH, encoded by the *zwf* gene, catalyses the first step in the oxidative branch of the pentose phosphate pathway, which generates ribose for nucleoside synthesis and NADPH for reductive pathways and repair reactions (Csonka & Fraenkel, 1977; Fraenkel, 1987). Besides its induction by the *soxRS/marRAB/rob* systems, G6PDH expression undergoes growth-rate-dependent regulation on different carbon sources (Rowley *et al.*, 1991; Wolf *et al.*, 1979). *E. coli* and *Salmonella* strains devoid of G6PDH activity are still able to grow on glucose (Fraenkel, 1968), but display increased susceptibility to oxidants and killing by murine macrophages (Greenberg *et al.*, 1990; Lundberg *et al.*, 1999; Nunoshiba *et al.*, 1995). Yeast G6PDH mutants are also abnormally sensitive to oxidative stress (Nogae & Johnston, 1990), and in humans, the G6PDH deficiency responsible for haemolytic anaemia is characterized by enhanced oxidant sensitivity and decline of NADPH levels in erythrocytes (Scott *et al.*, 1991).

The physiological role played by FPR during normal growth is poorly understood. In nonphotosynthetic organisms and tissues, this FAD-containing enzyme mediates electron transfer from NADPH to ferredoxin (Fd) or Fld, providing low-potential electron carriers required for a plethora of oxido-reductive pathways (reviewed by Carrillo & Ceccarelli, 2003; Ceccarelli *et al.*, 2004). Fld is employed in *E. coli* for the reductive activation of several anaerobic enzymes (Blaschkowski *et al.*, 1982; Wan & Jarrett, 2002), and Fd for the assembly of iron-sulfur clusters (Djaman *et al.*, 2004). Insertional mutants lacking FPR display no obvious growth penalty, the only phenotype being, once again, reduced tolerance to oxidative damage (Bianchi *et al.*, 1995; Krapp *et al.*, 1997, 2002).

While most components of the *soxRS* regulon play distinct and well-recognized protective roles, little is known of the actual contributions of G6PDH and FPR to the concerted cell response against oxidative stress. The beneficial effects of G6PDH have been attributed to the provision of NADPH for scavenging and repair reactions (Lundberg *et al.*, 1999), but the identities of the pathways that benefit from increased provision of reducing equivalents remain conjectural. Among the early targets of superoxide toxicity there is a family of metal-dependent hydro-lyases that includes fumarase A, aconitase B, 6-phosphogluconate dehydratase (6PGD) and hydroxyacid dehydratase. These enzymes employ a solvent-exposed $[4\text{Fe-4S}]^{2+}$ cluster as a Lewis

acid to bind the leaving hydroxyl group during substrate dehydration (Djaman *et al.*, 2004; Imlay, 2003). Their susceptibility to superoxide stems from the ability of this ROS to oxidize the catalytic iron to generate $[4\text{Fe-4S}]^{3+}$, an unstable intermediate that rapidly decomposes into $[3\text{Fe-4S}]^{1+}$ and Fe^{2+} (Imlay, 2003), thereby inactivating the corresponding enzymes and disabling the metabolic pathways to which they belong (Flint *et al.*, 1993; Gardner & Fridovich, 1991a, b; Kennedy *et al.*, 1983; Varghese *et al.*, 2003). Conversion of the $[3\text{Fe-4S}]^{1+}$ cluster back to the active form requires reduction and metallation, taking place within a few minutes after the oxidative condition has subsided (Djaman *et al.*, 2004). *E. coli* mutants lacking Fd are still able to repair iron-sulfur centres, albeit at lower rates (Djaman *et al.*, 2004), suggesting that this carrier may be a physiological electron donor for the process. A comparable contribution of iso-functional Fld has not been evaluated so far. G6PDH and FPR could act concertedly in the provision of reducing equivalents for dehydratase reactivation by establishing a short electron-transport chain in which G6PDH supplies NADPH, which could be later used by FPR as a substrate in the reduction of Fd and/or Fld.

Build-up of NADPH levels during episodes of oxidative stress may also have unwanted consequences. Accumulation of the reduced nucleotide is expected to downregulate the *soxRS* system by keeping the SoxR sensor in a reduced state (Gardner & Fridovich, 1993; Koo *et al.*, 2003), thus slowing down or even switching off the entire response. In addition, NADPH may favour the propagation of deadly hydroxyl radicals through the Fenton reaction by redox-cycling the free iron leached from iron-sulfur clusters, either directly (Brumaghim *et al.*, 2003) or as a substrate of flavin reductase (Woodmansee & Imlay, 2002). This enzyme generates reduced flavins, which are the preferred physiological Fenton reductants during hydroxyl radical formation *in vivo* (Woodmansee & Imlay, 2002). The coordinated induction of G6PDH and FPR during the *soxRS* response could exert counteracting effects on the NADPH pool that might be important for the maintenance of redox homeostasis in the stressed cell (Krapp *et al.*, 2002).

To gain insights into the roles of G6PDH and FPR in the protection against oxidative stress, we investigated several aspects of their function as members of the *soxRS* regulon. We report herein that G6PDH and FPR could establish a minimal electron-transport system that provides reducing equivalents for scavenging and repair reactions, including reductive reactivation of oxidized hydro-lyases. FPR participates in this pathway by supplying reduced Fd and/or Fld, but its contribution did not determine survival *in vivo*, presumably due to the existence of alternative routes for reduction of these low-potential electron carriers.

METHODS

Bacterial strains and culture media. *E. coli* cells used in this work (Table 1) were routinely grown at 37 °C in Luria-Bertani (LB) or M9 minimal media (Sambrook *et al.*, 1989), supplemented with

Table 1. *E. coli* strains and plasmids

Strain or plasmid	Genotype or relevant characteristics*	Source or reference
Strains		
HB301	W3110 $\Delta(\text{argF-lac})U169$	Rowley <i>et al.</i> (1991)
HB351	W3110 $\Delta(\text{argF-lac})U169$ <i>zeb-1::Tn10</i> $\Delta(\text{edd-zwf})22$	Rowley <i>et al.</i> (1991)
DF100	HfrC <i>tonA22 garB10 ompF627 relA1 pit-10 spoT1T2^r</i>	Rowley <i>et al.</i> (1992)
DF102	DF100 <i>zwfL2</i>	Rowley <i>et al.</i> (1992)
B247	MC4100 (λ JW2), Amp ^r Kan ^r [λ JW2: $\Phi(\text{soxS}'::\text{lacZ}')$]	Wu & Weiss (1992)
BL21(DE3)pLysS	F ⁻ <i>ompT hsdS_B (r_B⁻ m_B⁻) dcm gal</i> (DE3), pLysS, Cam ^r	Novagen
GC4468	F ⁻ Δ lac U169 <i>rpsL</i>	Greenberg <i>et al.</i> (1990)
Plasmids		
pSU18	pACYC184 carrying the multiple cloning site of pUC18, Cam ^r	Martinez <i>et al.</i> (1988)
pDR17	pBR322 carrying the <i>E. coli zwf</i> gene, Amp ^r	Rowley <i>et al.</i> (1991)
pSUG6PDH	pSU18 carrying the <i>E. coli zwf</i> gene, Cam ^r	This study
pETG6PDH	pET-28b(+) carrying <i>E. coli zwf</i> gene, Kan ^r	This study
pSUFPR	pSU18 carrying the <i>E. coli fpr</i> gene, Cam ^r	Krapp <i>et al.</i> (2002)

*Amp^r, ampicillin resistance; Cam^r, chloramphenicol resistance; Kan^r, kanamycin resistance.

0.4% (w/v) glucose and the corresponding antibiotics. IPTG was added at a final concentration of 0.5 mM when expression of plasmid-borne genes (Table 1) was desired. Cells used for determination of aconitase and 6PGD activities were cultured in a gluconate medium containing 60 mM K₂HPO₄, 33 mM KH₂PO₄, 7.6 mM (NH₄)₂SO₄, 1.7 mM sodium citrate, 1 mM MgSO₄, 10 µg thiamine hydrochloride ml⁻¹, 0.25% (w/v) Casamino acids and 0.4% (w/v) potassium gluconate, adjusted to pH 7.

Bacterial viability. Cells were grown in LB broth to OD₆₀₀ 0.3, serially diluted and spotted on M9 agar plates containing 100 µg chloramphenicol ml⁻¹ and 0.5 mM IPTG, when required, and the indicated concentrations of MV. In the disk diffusion method, 100 µl of a bacterial suspension containing ~10⁹ cells ml⁻¹ was mixed with 3 ml 0.7% (w/v) molten agar at 42 °C and poured onto M9 agar plates. After hardening, MV solutions of various concentrations were added in 5 µl aliquots to paper disks (5 mm diameter) placed on the agar surface. The zones of growth inhibition were measured after incubation for 30 h at 37 °C. Statistical analysis was conducted using a two-sided *t*-test.

Enzymic assays. For the preparation of *E. coli* extracts, cells were disrupted by sonic oscillation, and the resulting lysates cleared by centrifugation at 15 000 g for 15 min. Protein concentration was estimated in the supernatants by a dye-binding assay (Sedmak & Grossberg, 1977), using bovine serum albumin as standard. G6PDH, FPR and β -galactosidase activities were determined according to Kao & Hassan (1985), Krapp *et al.* (2002) and Miller (1992), respectively. Aconitase was measured at 25 °C in 90 mM Tris/HCl, pH 8, 20 mM sodium isocitrate, by following the formation of *cis*-aconitate at 240 nm (ϵ_{240} = 3.6 mM⁻¹ cm⁻¹). 6PGD activity was determined in 50 mM Tris/HCl, pH 7.6, 10 mM MgCl₂, 8 mM 6-phosphogluconate. After 5 min at 25 °C, the reaction was stopped by dilution with 1 ml of 50 mM Tris/HCl, pH 7.6, and heated in a boiling water bath for 2 min. Samples were centrifuged and the amounts of pyruvate were determined in the supernatants by reaction with NADH and lactate dehydrogenase (Gardner & Fridovich, 1991a).

In all cases, one activity unit is defined as the amount of enzyme that catalyses the transformation of 1 µmol substrate per minute under the conditions of the assay.

Preparation of recombinant G6PDH and antisera. The *zwf* gene present in plasmid pDR17 (Rowley *et al.*, 1991) was amplified by PCR using two primers with specific restriction sites incorporated (*Bam*HI and *Hind*III). The forward and reverse oligonucleotides were designed to hybridize with the +68 to +81 and the +1542 to +1559 regions of the *zwf* gene, respectively, counting from the transcription initiation site. The PCR product was cloned in pGEM-T-easy (Promega), digested with *Bam*HI and *Hind*III, and finally ligated to compatible sites of pET-28b(+) (Novagen). The resulting plasmid, pETG6PDH, contained the entire *zwf* coding region fused in-frame to an N-terminal hexahistidine tag. After expression in *E. coli* BL21(DE3)pLysS, the fusion protein was isolated by passage through a Ni-NTA agarose column (Qiagen). Purified G6PDH displayed a specific activity of about 100 units mg⁻¹ and migrated as a single 55 kDa polypeptide in SDS-PAGE. Antisera directed against both G6PDH and FPR were prepared by rabbit immunization.

MV-dependent induction of G6PDH and FPR. Exposure of *E. coli* GC4468 cells to MV was carried out by diluting 10 ml of an overnight culture in 1 litre of fresh LB medium. The resulting suspension was incubated for 30 min at 37 °C with vigorous shaking and MV was added to a final concentration of 100 µM. Fractions were transferred at various times to prechilled tubes, centrifuged and resuspended in 1 ml of a solution containing 50 mM phosphate buffer, pH 7.6, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and 200 µg chloramphenicol ml⁻¹ to prevent *de novo* protein translation. Cells were lysed and the contents of G6PDH and FPR analysed by SDS-PAGE and immunoblotting with specific antisera. Secondary antibodies conjugated to alkaline phosphatase were employed for detection. To estimate the half-life of the enzymes upon removal of the inducer, cultures were exposed to 100 µM MV for 4 h at 37 °C, harvested, washed in LB broth to eliminate MV, and processed as indicated above. Immunoreactive bands were integrated using the Multi-Analyst Package 1.1 from Bio-Rad, and the amounts of G6PDH and FPR were estimated by comparison with blotted pure enzymes of known concentration.

Time-course of *soxRS* induction by MV. Overnight cultures of B247 cells carrying a chromosomal *soxS'*::*lacZ* fusion (Wu & Weiss, 1992) and transformed with either pSU18, pSUFPR (Krapp *et al.*, 2002) or pSUG6PDH (containing the *zwf* gene cloned in the *Bam*HI/*Hind*III sites of pSU18) were diluted 1/100 in fresh LB broth

supplemented with 25 µg ml⁻¹ chloramphenicol. Cells were cultured at 37 °C, and 0.5 mM IPTG and 100 µM MV were successively added at 30 min and 150 min of incubation, respectively. Samples were removed at various times after MV challenge to assay for β -galactosidase and G6PDH activities.

The NADP(H) levels were estimated by a redox cycling assay, after alkaline extraction of the pyridine nucleotides (Krapp *et al.*, 2002).

Oxidative damage and repair of dehydratases containing [4Fe-4S]²⁺ centres. To measure the MV-dependent inactivation and *in vivo* recovery of aconitase and 6PGD, GC4468 cells transformed with either pSU18 or pSUFPR were cultured at 37 °C in 500 ml gluconate medium to OD₆₀₀ 0.6–0.8. MV was then added to a final concentration of 100 µM, and incubation was continued for 30 min in the presence of 200 µg kanamycin ml⁻¹ to block new protein synthesis. Cells were collected by centrifugation, rinsed to remove MV, and resuspended in 60 ml of the same medium containing kanamycin. The bacterial suspension was incubated at 37 °C without agitation. Aliquots were removed at intervals and centrifuged at 4 °C. The collected cells were lysed by sonic oscillation in 1 ml ice-cold 50 mM Tris/HCl, pH 7.6, containing 0.6 mM MnCl₂ and 20 µM barium DL-fluorocitrate to stabilize aconitase (Gardner & Fridovich, 1991b). Lysates were clarified by centrifugation at 15 000 g for 15 min, and the supernatants were immediately assayed for the corresponding enzymic activities. The same procedure was used to estimate inactivation and repair of hydro-lyases in FPR-deficient mutants, except that chloramphenicol (200 µg ml⁻¹) was employed instead of kanamycin to inhibit translation.

To evaluate dehydratase reactivation *in vitro*, GC4468 cells transformed with pSU18 were grown in gluconate medium to OD₆₀₀ 0.8, harvested and ruptured as described above. Lysates were stirred for 30 min at 30 °C to inactivate oxygen-sensitive dehydratases. Reactivation experiments were carried out in a reconstituted system made up of 50 mM Tris/HCl, pH 7.6, 0.3 mM NADP⁺, 3 mM glucose 6-phosphate, 1 unit G6PDH, 0.3 µM FPR, 5 µM of either Fd or Fld, 1 mM L-cysteine and bacterial extracts corresponding to 150 µg soluble protein (complete system). Fractions were taken at various times and assayed for aconitase and 6PGD activities. Recombinant FPR, Fd and Fld were prepared by published procedures (Wan & Jarrett, 2002). Statistical analysis was conducted using a two-sided *t*-test.

RESULTS AND DISCUSSION

Time-course of G6PDH and FPR induction during the *soxRS* response of *E. coli*

All members of the *soxRS*/*marRAB*/*rob* regulons contain recognition sequences for the corresponding transcription factors, termed the 'soxbox' or 'marbox' sites (Griffith & Wolf, 2001; Martin *et al.*, 2000; Wood *et al.*, 1999). Each regulatory protein differs in the extent to which it activates particular genes, with the consequence that regulon members are expressed to different levels during, for instance, the *soxRS* response. In a number of experiments using transcriptional fusions between the promoters and *lacZ*, maximal induction of *fpr* (and *fldA*) by both MV and SoxS was more than twofold higher than that of *zwf* (Martin *et al.*, 2000; Wood *et al.*, 1999). However, these assays only addressed the transcriptional component of expression and did not investigate the induction kinetics.

We compared the time-courses of G6PDH and FPR accumulation in cells exposed to 100 µM MV, as well as the kinetics of disappearance after removal of the reagent (Fig. 1). This concentration of MV provided for almost maximal induction of the *soxRS* response (see below), with negligible effects on growth rate. The levels of the two enzymes were estimated by immunoblotting with specific antisera, instead of using promoter fusions as is common practice, to incorporate post-transcriptional and translational regulation, if any, and to measure actual accumulation rates. The results confirmed that FPR is a minor protein in unstressed *E. coli* cells (~0.01 % of the total soluble

Fig. 1. Induction and decay of FPR and G6PDH during the *soxRS* response of *E. coli*. The upper parts of the figure show typical immunoblots obtained during induction (a) and decay (b) of the *soxRS* response, and the lower parts illustrate the G6PDH (○) and FPR (●) time-courses. *E. coli* ribosomal protein S1 was used as a control to detect any lane-to-lane differences in total protein loading. (a) Time-courses of FPR and G6PDH build-up during MV challenge. Aliquots were withdrawn at the times indicated, cells were lysed and supernatants corresponding to 7 (G6PDH) or 20 (FPR) µg soluble protein were separated by SDS-PAGE and immunoblotting. G6PDH and FPR amounted to 3.21 ± 0.55 (0.32 %) and 0.12 ± 0.02 (0.01 %) µg per mg of total soluble protein, respectively, in uninduced *E. coli* cells. Further increases in both enzymes with respect to total protein were normalized to these concentrations. (b) Half-lives of FPR and G6PDH after MV removal. Cells that had been incubated for 4 h at 37 °C with 100 µM MV were washed with fresh LB broth and processed as indicated above. The relative amounts of each enzyme accumulated after 4 h of MV induction (~3.9 % and ~0.3 % of total soluble protein for G6PDH and FPR, respectively) were taken as 1, and subsequent declines expressed as a fraction of these values. The inset in (b) shows a semilogarithmic representation of the decline in G6PDH and FPR. Data points represent the means of three independent experiments with SE ≤ 15 % of the mean. Curves were fit to the experimental data using Sigma Plot 8.02.

protein), whereas basal G6PDH levels were comparatively high ($\sim 0.3\%$ of the total soluble protein), in accordance with its involvement in the pentose phosphate pathway. Expression of this dehydrogenase was rapidly induced by MV, while FPR levels showed little change during the first 30 min of exposure. After that lag period, the reductase contents increased steeply and overtook the maximal induction reached by G6PDH (Fig. 1a), in agreement with the data obtained with fused promoters (Martin *et al.*, 2000; Wood *et al.*, 1999). G6PDH and FPR accumulation correlated with concomitant increases in specific activity (data not shown). When the cells were returned to normal growth conditions, the levels of the two enzymes declined to a stable value within 2 h of MV removal (Fig. 1b).

G6PDH is considered the main source of NADPH in *E. coli* and related bacteria (Csonka & Fraenkel, 1977). Hence, the cellular nucleotide pool may be reduced immediately after superoxide challenge due to rapid induction of this dehydrogenase, while NADPH-consuming FPR only began to accumulate at later stages of the *soxRS* response.

Effect of G6PDH and FPR contents on MV-driven induction of the *soxRS* regulon

The size and redox state of the NADP(H) pool are expected to influence the progress of the *soxRS* response because NADPH is the source of reducing equivalents for the SoxR reductase, which maintains the sensor protein reduced and inactive (Koo *et al.*, 2003). Indeed, activation of the *soxRS* regulon by redox-cycling agents has been directly attributed to faulty SoxR reductase function due to NADPH depletion (Gaudu *et al.*, 2000; Koo *et al.*, 2003; Liochev & Fridovich, 1992). As already indicated, changes in G6PDH expression might affect the onset of the *soxRS* response by modifying the redox status of the pyridine nucleotide pool. To test this contention, we monitored the MV-dependent induction of a reporter *soxS'::lacZ* gene fusion in *E. coli* strains expressing various levels of the dehydrogenase. A survey of the existing literature indicates that induction of the *soxRS* response has been studied under a wide range of MV concentrations, from less than $10\ \mu\text{M}$ (Gaudu *et al.*, 2000) up to $500\ \mu\text{M}$ (Griffith *et al.*, 2004). In a detailed study of dose dependency, Gort & Imlay (1998) showed that SoxS induction saturated above $150\ \mu\text{M}$ MV in a 45 min assay. We therefore used $100\ \mu\text{M}$ MV, a concentration employed by several authors (Chander *et al.*, 2003; Liochev *et al.*, 1994).

Cell-free extracts obtained from transformants overexpressing a plasmid-borne G6PDH displayed specific activities of $1.62\ \text{units (mg total soluble protein)}^{-1}$, as compared to $0.09\ \text{units mg}^{-1}$ in siblings carrying the supporting vector pSU18. Higher G6PDH activities correlated with an approximately twofold increase in the NADPH/NADP⁺ ratio (from ~ 2.3 to ~ 4.3). Both ratios declined to about 50% after a 90 min challenge with $100\ \mu\text{M}$ MV, but the difference between cells transformed with pSUG6PDH or pSU18 was maintained. The *soxRS* response proceeded

normally in the overexpressing bacteria, although they accumulated 40% less β -galactosidase than the corresponding controls at all times assayed (Fig. 2). G6PDH activities increased further on exposure to MV (data not shown), indicating that SoxS-dependent induction was still functional to a significant extent. As already reported (Gaudu *et al.*, 2000), *zwf* mutants displayed a stronger response to MV with a maximum induction of the *soxS* promoter that was approximately twofold over that of wild-type cells (data not shown), but kinetic characterization of the time-course proved difficult due to the extreme MV sensitivity of the deficient bacteria.

The behaviour of the *zwf* strains contrasted with that of cells in which the FPR contents were modified. Overexpression of the flavoprotein resulted in higher *soxRS* induction in the presence of MV, about threefold above the level reached by cells transformed with pSU18 (Fig. 2, inset). Time-courses of *soxRS* induction in the transformants expressing FPR were similar to those previously reported (data not shown, but see Krapp *et al.*, 2002). The inducing effect of FPR is similar to that of other NADPH consumers that stimulate the *soxRS* response, such as Fld I (Zheng *et al.*, 1999) or desulfoferrodoxin (Gaudu *et al.*, 2000).

Fig. 2. Induction of single-copy *soxS'::lacZ* by MV in *E. coli* cells expressing G6PDH. Bacteria containing a chromosomal *soxS'::lacZ* fusion (strain B247), and transformed with either pSU18 (●) or pSUG6PDH (○), were incubated at 37°C with $100\ \mu\text{M}$ MV in the presence of $0.5\ \text{mM}$ IPTG. Samples were taken at intervals to assay for β -galactosidase activity. Each data point represents the mean of determinations from four independent experiments with $\text{SE} \leq 10\%$ of the mean. Inset, maximal levels of *soxS'::lacZ* induction in bacteria transformed with pSU18 (black bars), pSUG6PDH or pSUFPR (grey bars). Experimental conditions were as above, and β -galactosidase activities were determined at 90 min after MV challenge. Induction levels of the overexpressing strains were normalized to those attained by cells transformed with the supporting plasmid, which were taken as 1.

Thus, G6PDH and FPR did exert opposite effects on *soxRS* induction. It is conceivable that rapid accumulation of the *zwf* product after exposure to superoxide could eventually slow down the protective response to suboptimal levels, while the subsequent expression of FPR (and Fld) will counterbalance this effect by reoxidizing the pyridine nucleotide pool and preventing further increases in NADPH level.

Overexpression of G6PDH does not increase the MV tolerance of *E. coli*

E. coli cells deficient in either G6PDH or FPR displayed enhanced sensitivity to various sources of oxidative stress, when compared to isogenic strains harbouring functional versions of the corresponding genes (Bianchi *et al.*, 1995; Greenberg *et al.*, 1990; Krapp *et al.*, 1997, 2002; Lundberg *et al.*, 1999). On the other hand, FPR build-up in an otherwise wild-type background led to increased cell survival, indicating that the protective effects of this reductase were dose-dependent even beyond endogenous levels of expression and induction (Bianchi *et al.*, 1995; Krapp *et al.*, 1997, 2002).

In contrast, *E. coli* cells transformed with pSUG6PDH were as resistant as their wild-type siblings when spotted on either minimal or rich media supplemented with MV, as described in Methods (data not shown). To rule out the possibility that the lack of G6PDH effect could be caused by deficiencies in the expression system, the use of a plasmid-borne gene or other shortcomings of the experimental set-up, we also evaluated tolerance by a disk diffusion method, and included an *E. coli* strain that overproduces G6PDH from a chromosomal *zwf* gene with activating mutations in the promoter region (Fraenkel & Banerjee, 1971; Fraenkel & Parola, 1972). The G6PDH-deficient mutant was still abnormally susceptible to MV toxicity in this system, but the overproducers failed to display increased tolerance, irrespective of whether the enzyme was expressed from the plasmid or the chromosome (data not shown). These results suggest that the maximum protective effects of G6PDH (or its relevant products, i.e. NADPH) are attained during the *soxRS* response in stressed *E. coli* cells, and that any further increase is inconsequential in terms of tolerance.

G6PDH and FPR can activate oxidant-sensitive dehydratases *in vitro*

The delayed appearance of FPR and the distinctive features of its contribution to the defensive system against oxidative stress argue against involvement of this reductase in ROS avoidance and detoxification, and suggest that its protective role might be related to repair activities required at later stages of the adaptive response, once the oxidative damage has occurred. Among the oxido-reductive pathways that could benefit from the surplus of low-potential electron carriers generated during the *soxRS* response, reactivation of oxidatively damaged hydro-lyases containing $[4\text{Fe-4S}]^{2+}$ clusters is a likely candidate. These enzymes are rapidly inactivated by superoxide and other ROS (Flint *et al.*, 1993;

Gardner & Fridovich, 1991a, b; Kennedy *et al.*, 1983), and since they are involved in many important metabolic pathways, prompt repair of the iron-sulfur clusters is essential if the cells are to survive. Indeed, their importance can be gauged by considering that oxidant-resistant fumarase and aconitase are induced as part of the *soxRS* regulon to compensate for the loss of the corresponding activities during the oxidative stress situation (Liochev & Fridovich, 1992; Pomposiello & Demple, 2001; Varghese *et al.*, 2003).

Assembly of iron-sulfur centres shows a strict requirement for Fd (a conspicuous FPR substrate), but the role of this protein in cluster repair is less clear (Djaman *et al.*, 2004). The possible contribution of isofunctional Fld to this process has not been evaluated. To determine if FPR could be the source of reducing power during cluster repair, we designed a reconstituted electron-transport system in which the oxidation of glucose 6-phosphate was coupled via G6PDH and FPR to reduction of Fd or Fld (Fig. 3a). Exposure of *E. coli* extracts to air caused rapid inactivation of typical hydro-lyases such as aconitase and 6PGD. Subsequent incubation of the extract with the reconstituted system in the presence of L-cysteine led to recovery of the two activities within 30 min (Fig. 3b, c). Controls lacking

Fig. 3. *In vitro* repair of iron-sulfur centres in oxidized hydro-lyases. Dehydratases present in *E. coli* extracts were inactivated by exposure to air and subsequently reactivated by a reconstituted electron-transport chain containing G6PDH and FPR (a). G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate. Aconitase (b) and 6PGD (c) activities were determined after 30 min incubation in the complete system containing either Fd or Fld, or in mixtures lacking Fd/Fld, FPR, G6PDH or cysteine (Cys). Controls ('None') were incubated in 50 mM Tris/HCl, pH 7.6, for the same time. Values reported are the mean $\pm$ SE of three independent experiments.

Fd, G6PDH, FPR or cysteine failed to significantly reactivate the enzymes in the time-frame of the experiment, while addition of Fld instead of Fd resulted in similar levels of recovery (Fig. 3b, c). Fld is not associated with the group of genes involved in assembly of iron-sulfur centres to which Fd belongs (Djaman *et al.*, 2004). Conversely, Fld (but not Fd) is induced during episodes of oxidative stress (Pomposiello & Demple, 2001; Zheng *et al.*, 1999).

Cysteine could be replaced in the reconstituted system by other thiols, including glutathione and dithiothreitol, but with lower efficiency (data not shown). The strict requirement of thiol groups is intriguing, since modified clusters that are still amenable to rapid reactivation are not expected to disintegrate beyond the $[3\text{Fe-4S}]^{1+}$ state (Djaman *et al.*, 2004). However, the dependence of hydro-lyase reactivation on the addition of thiols is well documented, and both oxidized and nitrosylated clusters are quickly repaired *in vitro* when incubated with iron and SH-containing compounds (Kennedy *et al.*, 1983; Schwarz *et al.*, 2000; Varghese *et al.*, 2003; Yang *et al.*, 2002). In our hands, however, incorporation of ferrous ions into the reactivation assay had little effect on activity recovery (data not shown), indicating that the iron present in the bacterial extracts is probably recycled for reconstitution of the functional clusters. The preference for cysteine also suggests that cysteine desulfurase encoded by the *iscS* gene (and eventually other members of the *isc* operon) could be involved in repair, but further research will be necessary to properly address this question.

Involvement of FPR in reactivation of hydro-lyases *in vivo*

To investigate if the iron-sulfur cluster repair activities displayed by FPR in the reconstituted system have physiological consequences, *E. coli* cells expressing various levels of the reductase were exposed to MV, and then shifted to an MV-free medium to monitor dehydratase reactivation. Kanamycin or chloramphenicol was used to prevent new protein synthesis during the periods of inactivation and recovery. When wild-type cells were subjected to a 30 min challenge with the superoxide propagator, aconitase activities declined to ~10 % of their initial values, while 6PGD dropped to less than 5 % of its pre-treatment activity (Fig. 4a, b). Removal of the reagent resulted in time-dependent recovery of both activities to about 50 % of their original levels (Fig. 4). Exposure of *fpr* mutants to the same treatment led to a similar inactivation, whereas recovery was slightly delayed in this strain, relative to the wild-type cells (data not shown). These results essentially reproduced those obtained by Krapp *et al.* (2002) and Djaman *et al.* (2004), who considered the observed differences between the strains of little significance, and suggested that the contribution of FPR to iron-sulfur cluster repair was thus marginal. However, overexpression of a plasmid-encoded reductase dramatically accelerated activity recovery to levels beyond those present in unstressed bacteria (Fig. 4), indicating that a significant part of the aconitase and a

Fig. 4. Oxidative damage and repair of hydro-lyases in bacteria overexpressing FPR. *E. coli* GC4468 cells, transformed with pSU18 (●) or pSUFPR (○), were exposed to 100 μM MV for 30 min, rinsed and further incubated in gluconate medium supplemented with 200 μg kanamycin ml^{-1} . Aliquots were removed at the indicated times and assayed for aconitase (a) and 6PGD (b) activities as described in Methods. Data points represent the mean of determinations from three independent experiments with $\text{SE} \leq 15\%$ of the mean. (c) FPR accumulation in cells transformed with pSU18 or pSUFPR after MV-dependent induction. Samples were collected at the times indicated as -30 or 0 min in (a) and (b), lysed and processed essentially as described in Fig. 1. Cleared extracts corresponding to 15 μg total soluble protein were loaded for immunoblot detection of FPR.

small fraction of 6PGD were inactive under aerobic growth conditions.

Concluding remarks

G6PDH is rapidly induced at the onset of the *soxRS* response (Fig. 1), leading to accumulation of NADPH that could be used by scavenging enzymes such as glutathione reductase and thioredoxin reductase. As an electron donor for SoxR reductase (Koo *et al.*, 2003) and flavin reductase

(Woodmansee & Imlay, 2002), NADPH may have a pro-oxidant effect by limiting the *soxRS* response and fostering Fenton-type reactions. Delayed expression of FPR could in principle counterbalance these effects through its NADPH-consuming activity (Krapp *et al.*, 1997, 2002). It is not clear whether the differential time-courses displayed by the two enzymes are important for the correct deployment of the *soxRS* response, or merely reflect different sensitivities of the corresponding promoters to SoxS build-up. Rapid FPR accumulation could lead to overoxidation of the NADP(H) pool (Krapp *et al.*, 2002), removing NADPH necessary for scavenging enzymes required at an early stage of the response, whereas the flavoprotein is needed later during the stress situation, once the damage has been done and repair mechanisms are invoked. Our results therefore indicate that NADPH increases rapidly during the *soxRS* response despite the activities of FPR and other reductases until the regulon is self-restrained by negative feed-back regulation, presumably through the activity of SoxR reductase (Fig. 2). G6PDH plays a dominant protective role during this stage, but further increases have little effect, probably because optimal NADPH levels have already been reached. The role of FPR appears to be more related to the reduction of low-potential electron carriers (Fd and Fld), rather than NADPH oxidation. The flavoenzyme was able to act as electron donor for Fd- or Fld-dependent reactivation of oxidatively damaged hydro-lyases *in vitro* (Fig. 3) and *in vivo* (Fig. 4). However, the contribution of FPR to this repair mechanism only becomes evident when the reductase accumulates over wild-type levels of expression and induction, whereas its participation during the normal *soxRS* response elicited by sublethal concentrations of MV appears to be relatively minor. The reasons for this behaviour are not entirely clear, but they may be related to the presence of alternative routes for Fd and Fld reduction in *E. coli*, which might partially or totally compensate the FPR deficiency. One such pathway has been already identified; it involves pyruvate-ferredoxin-(flavodoxin) reductase (Blaschkowski *et al.*, 1982). The role of FPR in the provision of low-potential electron carriers for damage repair during the *soxRS* response could therefore be masked in experiments involving FPR-deficient mutants by the partly overlapping activities of the pyruvate-dependent enzyme. Similarly, anaerobic pathways that require reduced Fld for the activity of pyruvate-formate lyase, ribonucleotide reductase and other key enzymes can proceed in *fpr* null mutants (Bianchi *et al.*, 1995; Krapp *et al.*, 1997).

Blaschkowski *et al.* (1982) determined the kinetic properties of the two reductases, and showed that they display similar catalytic efficiencies (k_{cat}/K_m values of $0.01\text{--}0.02\ \mu\text{M}^{-1}\ \text{s}^{-1}$, based on Fld affinity). However, the K_m for pyruvate is $1.6\ \text{mM}$ (Blaschkowski *et al.*, 1982), compared to less than $10\ \mu\text{M}$ for NADPH (Carrillo & Ceccarelli, 2003). The actual performance of the enzymes as providers of reduced Fld and/or Fd *in vivo* will therefore depend on the concentrations of electron donors and the enzyme levels, especially after FPR induction under oxidative stress. The results thus suggest that the electron-transport system established by

G6PDH and FPR is not a dedicated pathway, since both reduced Fld and NADPH can be supplied by alternative sources. Although pyruvate-ferredoxin(flavodoxin) reductase is apparently not regulated by oxidants, its involvement in the protection against oxidative stress deserves further investigation.

ACKNOWLEDGEMENTS

The authors wish to thank Dr Alejandro Viale (IBR, Argentina) for critical reading of the manuscript and many helpful suggestions. This work was supported by grant PICT-07853 from the National Science Agency (ANPCyT, Argentina). N. C. and A. R. K. are staff members of the National Research Council (CONICET, Argentina).

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