Antioxidant vitamins C and E affect the superoxide-mediated induction of the *soxRS* regulon of *Escherichia coli*

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The mechanism of activation of *Escherichia coli* redox sensory protein SoxR is still unclear: a [2Fe–2S] cluster contained in a SoxR dimer is potentially redox-sensitive, but the nature of the signal is unknown. Antioxidant vitamins C (ascorbate) and E (α -tocopherol) were used to explore the mechanism of activation of the SoxR protein *in vivo*. Treating *E. coli* cells with ascorbate or α -tocopherol increased their tolerance to paraquat (PQ, a redox-cycling compound), even in the absence of the *soxRS* locus, suggesting a radical-quenching activity. When using a *soxS'::lacZ* fusion, whose expression is governed by activated SoxR, ascorbate and α -tocopherol also prevented the expression of β -galactosidase after PQ treatment. A secondary activity was observed in cells carrying *soxR101*, a mutation resulting in the constitutive expression of the *sox* regulon, where the overexpression of *soxS'::lacZ* was also reduced by ascorbate or α -tocopherol treatment. Additionally, different mechanisms of action were revealed as α -tocopherol was capable of preventing both PQ and menadione (MD) lethality, whilst ascorbate prevented PQ lethality but increased MD-mediated cell death. It is proposed that α -tocopherol, positioned in membranes, can prevent superoxide-dependent membrane damage; however, water-soluble ascorbate is unable to do so and can even increase the concentration of oxygen radicals reacting with released membrane-associated Fe(II).

Keywords: superoxide radical, antioxidants, *soxRS*, *Escherichia coli*, vitamins C and E

INTRODUCTION

Escherichia coli cells cope with oxidative stress produced by extracellular nitric oxide or intracellular superoxide mainly by the induction of at least 11 genes regulated by the *soxRS* locus (Dempse, 1991). The SoxR protein is responsible for the first regulatory step of a mini-cascade. It is produced as inactive apo-SoxR and then it is dimerized and two Fe–S clusters are assembled into the dimer, possibly in cysteine residues near the C termini of each monomer (Hidalgo *et al.*, 1995). These centres are susceptible to oxidation, thus stabilizing the dimer and making for a stronger binding of RNA polymerase to the *soxS* promoter (Hidalgo & Dempse, 1994). Both reduced and oxidized forms of [2Fe–2S]SoxR, and even apo-SoxR *in vitro*, have equal affinity for the *soxS* promoter, but only [2Fe–2S]SoxR is capable of inducing the transcription of *soxS*. The likely

difference lies in the fact that oxidized [2Fe–2S]SoxR is stable, whereas reduced [2Fe–2S]SoxR is not and may spontaneously revert to the apo-SoxR form incapable of relating to σ^{70} -containing RNA polymerase (Hidalgo & Dempse, 1996; Hidalgo *et al.*, 1995). On the other hand, recent experiments have shown that reversion to the apo-SoxR form does not occur (Gaudu *et al.*, 1997), so a difference in affinity or a DNA conformational change could be responsible for transcriptional activation by the oxidized but not the reduced form. SoxS, in turn, is responsible for the transcription of around 11 defence and repair genes, while acting as an auto-repressor (Nunoshiba *et al.*, 1992, 1993b).

The nature of the signal that triggers [2Fe–2S]SoxR activation is still unclear; furthermore, most studies on the subject have been done using the purified protein *in vitro*. Agents or conditions that increase the intracellular concentration of superoxide radicals ($O_2^{\cdot-}$) specifically elicit the *soxRS* response (Nunoshiba *et al.*, 1992). Exposure to nitric oxide (Nunoshiba *et al.*, 1993a, 1995)

Abbreviations: MD, menadione; PQ, paraquat.

Table 1. Strains and plasmids

Strain or plasmid	Genotype
Strains	
GC4468	<i>E. coli</i> K-12 <i>soxRS</i> ⁺ F ⁻ Δ <i>lac rpsL</i>
DJ901	GC4468 Δ (<i>soxRS</i> – <i>zjc2205</i>) <i>zjc2204</i> ::Tn10Km
JTG1052	GC4468 <i>soxR101 zjc2206</i> ::Tn10Km
TN521	DJ901 ϕ (<i>soxR</i> ⁺ <i>soxS</i> ⁺ :: <i>lacZ</i>)
TN531	DJ901 ϕ (Δ <i>soxR soxS</i> ⁺ :: <i>lacZ</i>)
TN5315	TN531 but <i>recA56 srlC300</i> ::Tn10Km
Plasmid	
pTN101	pSE380 (Invitrogen) with an <i>EcoRI</i> – <i>HindIII</i> PCR (<i>soxR101</i>) insert

and other nitrous compounds (Nunoshiba & Demple, 1993) also activates SoxR. It has been suggested that direct oxidation of the Fe–S clusters might be the signal (Hidalgo & Demple, 1994), or that this oxidation might be the consequence of altered NAD(P)H/NAD(P)⁺ or oxidized/reduced ‘doxin’ (as in flavo-doxins and ferredoxins) ratios (Liochev *et al.*, 1994). Yet another possibility is that the [2Fe–2S] cluster in SoxR is continuously being oxidized under aerobic conditions and being actively reduced by an enzymic step; this putative enzyme could either be superoxide-sensitive or NAD(P)H-dependent (Hidalgo & Demple, 1994; Liochev & Fridovich, 1992; Gaudu *et al.*, 1997). Failure to reduce SoxR results in the accumulation of the oxidized, stable form, leading to the activation of the regulon promoters through SoxS.

In addition to the *soxRS* response, ~ 30 other genes are activated in *E. coli* cells during superoxide exposure (Demple & Amábile-Cuevas, 1991). In addition, constitutive enzymes that specifically inactivate superoxide (e.g. Fe- and Cu,Zn-superoxide dismutases) exist, and nonspecific antioxidant defence mechanisms such as glutathione and glutathione reductase. Antioxidant vitamins, which play definitive roles in higher organisms, do not seem to be important in prokaryotes.

Antioxidant vitamins have been known to act as powerful free radical quenchers in several biological systems through their ability to accept an additional electron and become radicals themselves. These vitamin radicals are more stable, and can be regenerated, avoiding in this way possible cell damage (Moslen, 1992). In higher eukaryotes, ascorbic acid (vitamin C) acts as a free radical quencher in aqueous cell compartments, becoming an ascorbyl radical and then dehydroascorbic acid. α -Tocopherol (vitamin E) exerts its antioxidant action in lipophilic cell domains such as membranes. When lipid peroxidation occurs, α -tocopherol stops the chain reaction by becoming a cromanyl radical. In this relatively stable radical form, the unpaired electron rotates in α -tocopherol's cromanyl ring. The cromanyl radical can be regenerated by two ascorbate molecules or by the glutathione–glutathione reductase system (Ames *et al.*, 1993).

In this work, we treated *E. coli* cells with ascorbate or α -tocopherol to ascertain if they can affect the superoxide-dependent activation of the *soxRS* regulon *in vivo*; we also assessed the possible protective role of these antioxidant vitamins in superoxide lethality. As ascorbate and α -tocopherol are not known to be essential for bacterial metabolism, these experiments also provided important clues as to toxicity mechanisms of redox-cycling compounds used to generate intracellular superoxide and on the physiology of the *soxRS* response itself.

METHODS

Strains, media and reagents. *E. coli* strains used are listed in Table 1 and were kind gifts from B. Demple, Harvard School of Public Health, MA, USA. They were all grown in LB broth or solid media (Maniatis *et al.*, 1982). Paraquat (PQ; Sigma) and menadione bisulfite (MD; Sigma) were used as superoxide-generating agents (Cunningham *et al.*, 1986). Fresh sodium ascorbate (Sigma) solution was added to the indicated concentrations; α -tocopherol acetate (a gift from Searle de México) was added as an homogeneous suspension, prepared in a tissue grinder just before use.

PQ, MD, ascorbate and α -tocopherol treatment in liquid media. Overnight bacterial cultures of strains TN521 and TN531 were diluted and grown to early exponential phase in LB broth at 37 °C in an orbital shaker (150 r.p.m.); ascorbate to final concentrations of 0.1, 1.0 and 10.0 mM, or α -tocopherol to 0.01, 0.1, 1.0 and 10.0 mM were added and the mixtures incubated for 15 min. Twenty-five or 50 μ M PQ or 300 μ M MD was then added, followed by further incubation for 30 min before measurement of β -galactosidase activity. Strain TN5315/pTN101 was grown to early exponential phase and then IPTG (Sigma) was added to a concentration of 1 mM. After 30 min, ascorbate or α -tocopherol (10 mM) was added and cells incubated for another 15 min, at the end of which superoxide-generating agents were added. After a 30 min incubation, β -galactosidase activity was assayed.

Measurement of β -galactosidase activity. This was done as described by Miller (1992). Briefly, the optical density (at 600 nm) of each culture was determined and cells permeated with SDS/chloroform (because of coloration produced by MD, cultures treated with this agent were washed by centrifugation and resuspension before OD₆₀₀ determination); an aliquot was added to Z buffer (Na₂HPO₄·7H₂O, 60 mM; NaH₂PO₄·H₂O, 40 mM; KCl, 12 mM; MgSO₄·7H₂O,

1 mM; β -mercaptoethanol, 38.5 mM; pH 7.0) containing ONPG (Sigma), the mixture incubated at 37 °C until a change in colour was detected and optical density then measured at 420 nm.

Preparation of PQ and MD gradient plates with and without vitamins. To assess the effect of ascorbate and α -tocopherol on PQ and MD lethality, PQ/vitamin gradient plates were prepared as reported previously (Cunningham *et al.*, 1986). LB agar plates contained PQ [14 mg (40 ml)⁻¹] or MD [90 mg (40 ml)⁻¹] on the bottom, and ascorbate or α -tocopherol (10 mM) on the top. Early-exponential-phase liquid cultures of strains GC4468 and DJ901 were suspended in liquefied soft LB agar and 'stamped' across the gradient using a glass slide. Plates were incubated at 37 °C for 18 h and the length of growth was then measured. Strain JTG1052 was assayed in the same way, but plates contained 28 mg PQ (40 ml)⁻¹ to achieve measurable killing of this oxidative-stress resistant strain; MD concentration was the same as above.

RESULTS

Ascorbate and α -tocopherol inhibit PQ-mediated induction of *soxS'::lacZ*

PQ treatment of strain TN521 induced the expression of the *soxS'::lacZ* fusion, measured as increased β -galactosidase activity compared to basal levels (1.5 to 2- and 3 to 4-fold induction by 25 or 50 μ M PQ treatment, respectively; Table 2). This induction was prevented in a dose-dependent manner by ascorbate and α -tocopherol treatment, although lower concentrations of α -tocopherol were required to achieve the same effects as for ascorbate. Basal levels were also diminished in the cells treated only with 10 mM ascorbate or α -tocopherol (Table 2). Strain TN531 (*soxR*) was unresponsive to either PQ or vitamin treatments (data not shown).

α -Tocopherol inhibits MD-mediated induction of *soxS'::lacZ* but ascorbate does not

In strain TN521, the increase in β -galactosidase activity induced by 300 μ M MD treatment was similar to that obtained with 50 μ M PQ (~3-fold; Table 2);

α -tocopherol also prevented the induction in a dose-dependent manner (Table 2) but ascorbate showed a diminished preventive activity, lower as its concentration increased. Experiments with strain TN531 also proved that these effects were SoxR-dependent.

A *soxR^c* mutant is also responsive to vitamin treatments

Ascorbate and α -tocopherol were capable of reducing β -galactosidase activity in strain TN5315/pTN101 both in IPTG-treated and non-induced cells. Plasmid pTN101 carries a copy of *soxR101*, a *soxR* allele that mediates the constitutive expression of the *sox* regulon genes (thus named *soxR^c*), even in the absence of oxidative stress. This constitutive expression was diminished by antioxidants, 27% by ascorbate and 50% by α -tocopherol. Treatment with IPTG elicited a ~2.2-fold overinduction of the *soxS'::lacZ* fusion (actual levels of SoxR^c were not assessed), which was also reduced by ascorbate (31%) and α -tocopherol (53%) treatment.

Protective effects of ascorbate and α -tocopherol on PQ-induced lethality

PQ lethality was prevented completely by the presence of 10 mM α -tocopherol, in both GC4468 (*soxRS*⁺) and DJ901 (Δ *soxRS*) strains; 10 mM ascorbate totally protected GC4468 cells but protected only partially DJ901 cells (Fig. 1). Dehydroascorbate had no effect on cell killing by PQ (data not shown). Strain JTG1052 (*soxR101*), a constitutively active SoxR mutant, was resistant to PQ concentrations used against strains GC4468 and DJ901; it was slightly protected by both vitamins against a double PQ concentration (Fig. 1).

Effects of ascorbate and α -tocopherol on MD-induced lethality

Cell killing was completely prevented with α -tocopherol in strains JTG1052 (*soxR101*), GC4468 (*soxRS*⁺) and DJ901 (Δ *soxRS*). Surprisingly, ascorbate not only did

Table 2. Effect of oxidants and antioxidants on strain TN521 (harbouring *soxS'::lacZ* fusion)

Values represent β -galactosidase activity compared to the basal level ($\equiv$ 1) and are means of at least three individual experiments. Standard deviations were no greater than 10% in any case. ND, Not determined.

Oxidant (μ M)	Antioxidant (mM)							
	None	Ascorbate			α -Tocopherol			
		0.1	1	10	0.01	0.1	1	10
None	1.0	ND	ND	0.64	ND	ND	ND	0.65
PQ 25	1.78	1.26	1.02	0.75	1.59	0.90	0.73	0.69
PQ 50	3.0	1.02	0.85	0.55	1.60	0.89	0.69	0.52
MD 300	2.98	1.02	1.29	1.93	1.73	1.62	1.09	0.81

Fig. 1. Effect of antioxidant vitamins on PQ-mediated cell death. Gradient plates containing PQ [14 or 28 mg (40 ml)⁻¹] were prepared and inoculated as described in Methods. Growth was scored as the percentage of the maximum length possible (80 mm). α -Tocopherol (vitE) or ascorbate (vitC) were added to the top layer at a final concentration of 10 mM. Strain DJ901 was not assayed on PQ 28 plates. Bars represent results from a typical experiment; at least three independent experiments were performed, with similar results.

Fig. 2. Effect of antioxidant vitamins on MD-mediated cell death. Gradient plates containing MD [90 mg (40 ml)⁻¹] were prepared and inoculated as described in Methods. α -Tocopherol (vitE), ascorbate (vitC) or dehydroascorbate (DvitC) were added to the top layer at a final concentration of 10 mM. Bars represent typical results, from one among at least three experiments.

not protect as effectively as α -tocopherol against MD toxicity, but also acted synergically with MD, killing the three strains completely. In contrast, dehydroascorbate did not have such a potent effect on MD-mediated cell killing (Fig. 2).

DISCUSSION

A large amount of biochemical and molecular evidence on the mechanisms of redox-sensing and activation of the *E. coli* SoxR protein have accumulated recently (Gaudu & Weiss, 1996; Hidalgo & Demple, 1994, 1996; Hidalgo *et al.*, 1995; Wu *et al.*, 1995). However, very few *in vivo* experiments have been done on this subject.

Ascorbate and α -tocopherol are known free-radical scavengers (Ames *et al.*, 1993). We observed an increased tolerance to PQ, a water-soluble intracellular superoxide generator, in the presence of both ascorbate and α -tocopherol, and a reduction in basal and PQ-induced β -galactosidase activity in cells bearing the *soxS'::lacZ* fusion when treated with either antioxidant vitamins. However, when using MD, which diverts electrons from the respiratory chain within the cell membrane (Cadenas, 1989), as redox-cycling agent a different effect was observed. Whilst liposoluble α -tocopherol prevented both MD toxicity and *soxS'::lacZ* induction, hydrosoluble ascorbate increased MD toxicity and poorly prevented β -galactosidase overproduction in an inversely dose-dependent manner. We propose that α -tocopherol quenches radicals generated close to the cell membrane and/or stabilizes the membrane itself, therefore reducing the deleterious effects of both PQ and MD. On the other hand, ascorbate is capable of scavenging PQ-generated superoxide in the cytosol, but is less capable of protecting against the effects of MD within lipophilic environments. Furthermore, as iron availability within the cell seems to be partially regulated by several membrane-bound systems (Hassan & Schrum, 1994), MD-mediated membrane damage, such as lipoperoxidation, may cause iron release, allowing for the generation of additional oxygen radicals by ascorbate-mediated Fenton reactions (Shamberger, 1984). Fe(II) pretreatment increased by $\sim 10\%$ the toxicity of PQ in the presence of ascorbate in gradient plates (unpublished results), suggesting Fe-ascorbate interactions leading to toxic by-products. If these hypotheses are correct, the impact of membrane-related events upon intracellular superoxide toxicity (e.g. lipoperoxide generation, Fe(II) release, etc.) and *soxRS* regulatory mechanisms should be examined.

Wild-type SoxR protein has a DNA-binding domain, possibly responsible for binding to the *soxS* promoter, and a cysteine cluster at the C terminus (AmáBILE-Cuevas & Demple, 1991), possibly involved in the anchoring of the [2Fe-2S] complex (Hidalgo *et al.*, 1995). Mutations or deletions at the end of the *soxR* gene (not involving the cysteine cluster), such as those found in *soxR101* and JTG1052, result in the readily oxidized but still superoxide-inducible expression of *soxS* (Nunoshiba & Demple, 1994; Tsaneva & Weiss, 1990). Surprisingly, both ascorbate and α -tocopherol were able to diminish the β -galactosidase activity in *soxR101*, *soxS'::lacZ* cells, induced only by SoxR101 overproduction. It is possible that this reflects the radical-quenching capabilities of both antioxidants, since SoxR101 still retains some of its redox-respon-

siveness. However, we would expect this effect to be equimolar, not proportional (i.e. the reduction in β -galactosidase Miller units should be the same if antioxidants are just quenching oxygen radicals); but, while the β -galactosidase activity reduction was of ~ 12000 Miller units in TN5315/pTN101-treated cells, it only amounted to ~ 3000 units in strain TN521. We surmise that vitamins are acting on SoxR-activating steps apart from the mere quenching of superoxide radicals.

The SoxR protein is obviously generated as a metal-free monomer and may become dimerized even in the absence of iron. A first regulatory step on SoxR activation would be iron availability and, when the [2Fe-2S]-containing dimer is formed, it is readily oxidized to an active, stable form under normal aerobic conditions (Hidalgo *et al.*, 1995). A second regulatory step may involve the active reduction of [2Fe-2S]SoxR back to its unstable form, perhaps through a NAD(P)H-mediated reaction, catalysed by an unknown enzyme or even by the C-terminal region of SoxR (Hidalgo & Demple, 1996). NAD(P)H cell pools might be depleted as a consequence of redox stress, preventing SoxR reduction (Liochev & Fridovich, 1992); also, mutant protein SoxR101 may not be susceptible to reduction due to the mutation near the C-terminus and therefore accumulates in its oxidized form, continuously activating the *soxS* promoter (Hidalgo *et al.*, 1997). Ferredoxin oxidoreductase, a recently discovered member of the *soxRS* regulon, acts as a diaphorase during PQ induction but when superoxide production ceases, it acts as a doxin reducer and could be responsible for SoxR inactivation, acting as a reduction-dependent feedback mechanism of the regulon (Liochev *et al.*, 1994).

α -Tocopherol may be capable of preventing SoxR and SoxR101 activation by quenching radicals and protecting membranes from oxidation, therefore diminishing the release of membrane-bound iron. Ascorbate is also capable of scavenging radicals in hydrophilic compartments and may be able to directly reduce oxidized SoxR proteins, returning them to their inactive form. Ascorbate-mediated reduction may be non-specific, affecting both SoxR and SoxR101 proteins. Glutathione, another water-soluble antioxidant agent, is also capable of affecting SoxR activation *in vivo* (although it activates SoxR in an oxygen-dependent manner *in vitro*; Din & Demple, 1996).

Nitric oxide is another known inducer of the *soxRS* response and is capable of destroying Fe-S clusters in many proteins other than SoxR. NO $^{\cdot}$ is also known to participate in the regulation of iron availability in mammals during infectious processes, although the regulatory effect involves iron sequestering instead of liberation (Sellers *et al.*, 1996). Iron availability affects the expression of at least one member of the *soxRS* regulon, *fumC*, in a *soxR*-dependent way (Park & Gunsalus, 1995). Since iron ions seem to play a key role in the regulation of the *soxRS* system, it is likely that antioxidant vitamins are affecting the availability of this

metal as well, resulting in the effects described here. Along with the involvement of *soxRS* in multiple antibiotic resistance, which may even be triggered by environmental pollutants (Fuentes & Amábile-Cuevas, 1997), its response to iron levels underscores the notion that *soxRS* may be substantially important in Gram-negative bacterial virulence.

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