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A Combination of Two Antioxidants (An SOD Mimic and Ascorbate) Produces a Pro-Oxidative Effect Forcing *Escherichia coli* to Adapt *Via* Induction of *oxyR* Regulon

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Abstract

Cationic Mn(III) *N*-alkylpyridyl (MnTalkyl-2(or 3)-PyP^{5+}) and *N, N′*-dialkylimidazolylporphyrins (MnTDalkyl-2-ImP^{5+}) have been regarded as the most powerful SOD mimics/peroxynitrite scavengers – *i. e.* antioxidants. The ethyl-, MnTE-2-PyP^{5+} (AEOL10113), and hexylpyridyl-, MnTnHex-2-PyP^{5+} and diethylimidazolylporphyrin, MnTDE-2-ImP^{5+} (AEOL10150) have been mostly studied *in vitro* and *in vivo*. Given the *in vivo* abundance of cellular reductants, MnPs can couple with them in removing superoxide. Thus, they could be readily reduced from Mn^{III}P to Mn^{II}P with ascorbate and glutathione, and in a subsequent step reduce either $O_2^{\cdot-}$ (while acting as superoxide reductase) or oxygen (while exerting pro-oxidative action). Moreover, MnPs can catalyze ascorbate oxidation and in turn hydrogen peroxide production. The *in vivo* type of MnP action (anti- or pro-oxidative) will depend upon the cellular levels of reactive species, endogenous antioxidants, availability of oxygen, ratio of $O_2^{\cdot-}$- to peroxide-removing systems, redox ability of MnPs and their cellular localization/bioavailibility. To exemplify the switch from an anti- to pro-oxidative action we have explored a very simple and straightforward system – the superoxide-specific aerobic growth of SOD-deficient *E. coli*. In such a system, cationic MnPs, *ortho* and *meta* MnTE-2-(or 3)-PyP^{5+} act as powerful SOD mimics. Yet, in the presence of exogenous ascorbate, the SOD mimics catalyze the H_2O_2 production, causing oxidative damage to both wild and SOD-deficient strains and inhibiting their growth. Catalase added to the medium reversed the effect indicating that H_2O_2 is a major damaging/signaling species involved in cell growth suppression. The experiments with *oxyR*- and *soxRS*-deficient *E. coli* were conducted to show that *E. coli* responds to increased oxidative stress exerted by MnP/ascorbate system by induction of *oxyR* regulon and thus upregulation of antioxidative defenses such as catalases and peroxidases. As anticipated, when catalase was added into medium to remove H_2O_2, *E. coli* did not respond with upregulation of its own antioxidant systems.

Keywords

Mn porphyrins; antioxidants; pro-oxidants; MnTE-2-PyP^{5+}; AEOL10113; MnTE-3-PyP^{5+}; *E. coli*; adaptive response; *oxyR* regulon

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1. INTRODUCTION

1.1. Mn Porphyrins

Cationic Mn(III) *ortho N*-substituted pyridylporphyrins are among the most powerful mimics of superoxide dismutases [1,2]. Their potency depends on the presence of cationic charges close to the metal site which exerts strong electron-withdrawing effects and affords electrostatic attractions for the reaction with anionic $O_2^{\cdot -}$ and $ONOO^-$. The metal centered reduction potential for Mn(III)P/Mn(II)P redox couple, $E_{1/2}$ ~ +300 mV *vs* NHE is at the midway between the potential for the oxidation and reduction of $O_2^{\cdot -}$ [1,2]. Thus, such *ortho* cationic MnPs reduce and oxidize $O_2^{\cdot -}$ with similar rate constants [1,2]. The $O_2^{\cdot -}$ dismuting ability, as described with $k_{cat}(O_2^{\cdot -})$, parallels the peroxynitrite scavenging [3]. They are further able to scavenge other reactive species such as $CO_3^{\cdot -}$ [3] and HClO [4, Ferrer-Sueta unpublished]. They also stoichiometrically bind $^{\cdot}NO$, and release it depending upon the oxygen levels [5]. The possible biological consequences of $^{\cdot}NO$ binding and release have not yet been explored. MnPs likely react with peroxyl and alkoxyl radicals also [6,7]. MnPs also inhibit activation of those transcription factors that have been tested thus far: HIF-1α, NF-κB, AP-1, and SP-1 [8–18]. Consequently they modulate the expression of proteins involved in proliferative and apoptotic pathways [8–18]. Strong experimental evidence indicates that such actions upon cellular transcriptional activity contribute to the beneficial effects of these compounds observed with radiation injury [8–11], skin cancerogenesis [12], breast cancer [13–15], stroke, hemorrhage [16,17], and diabetes [18 and refs therein]. Data indicate that those MnPs with high $k_{cat}(O_2^{\cdot -})$ are the most potent inhibitors of NF-κB transcriptional activity [18]. *The k_{cat} can thus be considered a good predictor of the MnP efficacy in vivo.* The *in vitro* and *in vivo* efficacy of Mn porphyrins as well as their chemistry and biology were recently reviewed [1,2,18–20].

The lipophilic cationic *N*-alkylpyridylporphyrins have been developed aiming at high cellular and mitochondrial accumulation and transport across the blood brain barrier [21–29]. Increased bioavailability of more lipophilic *meta* isomers was anticipated to compensate to some extent for somewhat inferior antioxidant potency in comparison to more hydrophilic, but also more SOD-active *ortho* analogues [21]. Indeed, *meta* MnTE-3-PyP^{5+} and *ortho* MnTE-2-PyP^{5+} proved equally efficacious in allowing SOD-deficient *E. coli* to grow to a similar extent as a wild type [1,2]. Both compounds were chosen for this study.

1.2. Ascorbate, Cancer and Metalloporphyrins

Ascorbic acid, the infamous antioxidant and cofactor of many enzymes [30], is present *in vivo* as a monovalent ascorbate anion (HA^-) because the hydroxyl group at position 3 has pK_a 4.17 [31,32]. The cellular concentration of ascorbate is millimolar, while plasma and extracellular fluid concentrations are micromolar [30,33,34]. The highest levels of ascorbate are found in the brain, adrenal glands, white blood cells and skeletal muscle [30].

Ascorbate is transported into the cell *via* two sodium-dependent transporters - SVCT1 and SVCT2. The diffusion-based transport of two-electronically oxidized ascorbate, dehydroascorbate (A), occurs *via* glucose transporters of the GLUT family (GLUT1, GLUT3 and GLUT4) [30,35,36]. A is trapped inside the cell *via* reduction to ascorbate [30,35]. Under physiological conditions, A transport may not be a major route of ascorbate transport into the cell because: (1) plasma concentration of A (2 μM) is much lower than that of ascorbate (40–60 μM); (2) glucose may easily compete for the cellular uptake with low levels of A; (3) concentration of ascorbate in the cells which lack SVCT transporters is low [30]. However, under pathological conditions of increased oxidative stress, more ascorbate may be oxidized to A [37,38] which would favor its transport *via* GLUT transporters [39]. Further, due to the high glycolytic metabolism and increased glucose

supply, cancer cells overexpress GLUT transporters on the surface, which may enable them to accumulate A as well [40].

1.2.1. Spatial Effects of Ascorbate – Intracellular Ascorbate—Tumor utilizes its "own" oxidative stress to signal the proliferation. When that is excessive, tumor undergoes apoptosis. Cytotoxic effects of ascorbate, enhanced *via* catalysis with redox able biological molecules such as iron porphyrins, quinones and metalloproteins, may possibly be selective to cancer cells due to: (1) the endogenous tumor oxidative stress, *i.e.* poor antioxidative status with often insufficient levels of antioxidant enzymes and in particular unfavorable ratio of $O_2^{\cdot-}$- to H_2O_2-removing systems which diminishes tumor ability to fight sudden increased burden of reactive species; and (2) increased ascorbate accumulation in tumors *via* GLUT transporters. Large differences among cancer types with respect to redox status must be accounted for. Importantly, there is still no definite consensus about the ascorbate levels in tumors; data showing both increased [40, 41] and decreased [42] levels have been reported. Kuiper et al for the first time indicated recently that high-grade tumors are ascorbate-deficient [43]. Ascorbate is a cofactor of prolylhydroxylases whose action destabilizes HIF-1α. The authors found that low ascorbate content was associated with elevated HIF-1α, VEGF, and GLUT-1 which would favor tumor progression [43]. In contrast, tumors with high levels of ascorbate had lower levels of HIF-1α [43].

1.2.2. Extracellular Ascorbate—Levine et al showed that millimolar ascorbate levels suppressed tumor growth *via* extracellular production of H_2O_2 (Fig. 3) [44,45]. The reaction is reportedly catalyzed by metal containing proteins [44], such as those bearing iron porphyrins as active sites [46]. *In vivo*, millimolar extracellular concentrations can be achieved only by *iv* or *ip* administration of ascorbate, since there is a tight control of intestinal absorption/tissue accumulation/tubular reabsorption after oral administration of ascorbate [47,48]. Verrax and Calderon [49] confirmed cytotoxicity of *ip* and *iv,* but not oral ascorbate in diminishing tumor growth. Synergistic effects of ascorbate and several cytotoxic drugs have been reported also [40,49–64].

1.3. Mn Porphyrins and Cellular Reductants – Antioxidative Action

Cationic Mn(III) *N*-alkylpyridylporphyrins of positive metal-centered reduction potential $E_{1/2}$, being in the range of +50 and +500 mV *vs* NHE for Mn(III)P/Mn(II)P redox couple, can be readily reduced with endogenous reductants such as ascorbate and glutathione [1,2,6,7,18,65–67], as well as with flavoproteins [68]. Once reduced from $Mn^{III}P$ to $Mn^{II}P$ they can reduce $O_2^{\cdot-}$, acting as superoxide reductase (Fig. (1B)). Given the abundance of cellular reductants such scenario is more likely than their action as SOD mimics (Fig. (1A)). Further, they can reduce $ONOO^-$ one-electronically, while cycling from $Mn^{III}P$ to $O{=}Mn^{IV}P$, whereby powerful oxidants $O{=}Mn^{IV}P$ and $^{\cdot}NO_2$ radical are formed (Fig. (2A)). The $O{=}Mn^{IV}P$ could be reduced back to $Mn^{III}P$ with ascorbate, uric acid or glutathione [67]. However, the reduction of $ONOO^-$ two-electronically employing $Mn^{II}P/O{=}Mn^{IV}P$ redox couple is more probable *in vivo* and would result in a formation of benign nitrite, NO_2^- (Fig. (2B)). The removal of superoxide, $CO_3^{\cdot-}$, and $ONOO^-$ can thus occur *via* coupling with endogenous reductants also (Figs. (1B and 2B)). The antioxidative action of Mn porphyrins has been witnessed and implicated in numerous animal models of oxidative stress, such as radiation, central nervous system injuries, diabetes etc [1,2,18]. Recently our efforts were directed towards understanding the role of MnPs in cancer either alone or when combined with cellular reductants. The existing data point to antioxidative mode of action. MnTE-2-PyP^{5+} reduces tumor angiogenesis in mouse 4T1 breast cancer study *via* suppression of HIF-1α and its genes, VEGF and bFGF as well as suppression of oxidative stress [15]. MnTE-2-PyP^{5+} also suppresses oxidative stress and the incidence and multiplicity of skin tumors in a mouse skin cancerogenesis model *via* inhibition of AP-1 activation and down-

regulation of proliferating cellular nuclear antigen, PCNA [12]. In a recent glioma study with mice bearing intracranial xenografts, lipophilic MnTnHex-2-PyP^{5+} produced a statistically significant ($P \leq 0.001$) increase in median mouse survival for 33% with glioblastoma multiforme, D-256 MG, and 173% with pediatric medulloblastoma, D-341 MED xenografts [69]. While therapeutic potency has been evaluated, the mechanistic studies are in progress.

1.4. Mn Porphyrins and Cellular Reductants – Pro-Oxidative Action

The ability of the most potent *ortho N*-alkylpyridylporphyrin-based SOD mimics to reduce and oxidize $O_2^{\cdot -}$ with nearly identical rate constants, the high intracellular levels of reductants, and the existing data justify further efforts to understand the diverse *in vitro* and *in vivo* mechanisms of MnPs actions.

Cellular reductants are undoubtedly involved in antioxidative action of Mn porphyrins (Figs. (1 and 2)). However, the coupling with ascorbate can promote the pro-oxidative action of Mn porphyrins also, *via* scheme shown in Fig. (3). Cationic *ortho* Mn(III) *N*-alkylpyridylporphyrins undergo oxidative degradation with ascorbate; uv/vis spectroscopy indicates the same spectral change of MnPs exposed to either ascorbate or to H_2O_2 [70–72]. Such data clearly show that metalloporphyrins produce H_2O_2 when coupled with ascorbate, which eventually destroys them. Further, both Fe and Mn porphyrins of different charge and reducibility are able to catalyze hydroxylation of an anticancer drug cyclophosphamide in the presence of ascorbate, whereby mimicking cyt P450 [73]. Thus, in the presence of ascorbate, MnP could act as a catalyst of ascorbate-driven oxygen consumption leading to the superoxide, hydrogen peroxide and hydroxyl radical production as shown in Fig. (3). Finally, given the abundance of oxygen, $Mn^{II}P$ itself may react with O_2 rather than with $O_2^{\cdot -}$ producing superoxide and eventually peroxide (Fig. (3)). Such pro-oxidant action of MnP is a viable option *in vivo* due to: (1) the high cellular levels of ascorbate and glutathione as well as other redox able proteins; and (2) high levels of O_2 relative to $O_2^{\cdot -}$. In such scenario cells may be killed *via* increased oxidative stress. Alternatively, and depending upon the H_2O_2 levels, H_2O_2 may signal cells to upregulate anti-apoptotic survival pathways [74] inducing adaptive responses *via* upregulation of endogenous antioxidant defenses [75].

Several *in vitro* and *in vivo* studies pointed to the pro-oxidative action of Mn porphyrins. Identical efficacy of either MnTnHex-2-PyP^{5+} or Gd texaphyrin in amyotrophic lateral sclerosis G93A model were thus far attributed to opposing, anti- and pro-oxidative mechanisms, respectively [76–79]. Yet, such data could imply that a common, possibly pro-oxidative mechanism might be operative with both compounds [76–79].

Pro-oxidative action of MnPs has been suggested by Jaramilo *et al.* also [80,81]. When MnTE-2-PyP^{5+} was given to lymphoma cells along with cyclophosphamide and glucocorticoids, synergistic effects were observed and attributed to H_2O_2/GSH-based glutathionylation of p65 unit of NF-κB which deprived cells of glutathione. Levels of GSSG were unchanged [80,81]. It is possible that inhibition of AP-1 and HIF-1α by MnTE-2-PyP^{5+} may also result from S-glutathionylation.

The pro-oxidative action of MnP/ascorbate system has been already shown *in vitro* in 5 different cancer cell lines [82, Aird et al unpublished]. Also Tian et al [83] showed the pro-oxidative action of MnTM-2-PyP^{5+}/ascorbate in cellular studies. Similar *in vivo* and *in vitro* cancer studies, where endogenous Fe porphyrins acted as catalysts of ascorbate–driven production of reactive species, was also reported by Chen et al [46].

To further increase our insight into the pro-oxidant action of cationic *N*-alkylpyridyl Mn porphyrins, we tested the effect of MnP/ascorbate in a superoxide-specific system, aerobic growth of SOD-deficient *E. coli*. In such a system, only those MnPs that are potent SOD mimics would allow SOD-deficient *E. coli* mutant to grow aerobically as well as the wild type. Yet when cellular reductant, ascorbate was added to the medium containing MnPs, the effects were reverted: rather than removing $O_2^{\cdot -}$, its production and subsequent H_2O_2 formation was increased, which suppressed the growth of *E. coli*. The pro-oxidative action of MnPs was prevented with the addition of catalase.

In summary, the most powerful MnPs-based SOD mimics are able to equally effectively give and accept electrons, and can thus act as pro- and antioxidants, depending upon the levels of reactive species and endogenous antioxidants, ratio of superoxide dismutases- to peroxide-removing enzymes, reducibility of MnPs, their antioxidant potential and their cellular localization. *Therapeutic effects can thus be achieved via two scenarios where Mn porphyrin could perturb the fragile redox balance between reactive species and antioxidants: (1) the removal of reactive species that would prevent cell proliferation; (2) or increased production of such species that would result in apoptosis/necrosis.*

2. EXPERIMENTAL

2.1. General

Sodium L-ascorbate was from Sigma (>98% purity), catalase was from Boehringer Mannheim. Chloramphenicol, spectinomycin and kanamycin were from Sigma.

2.2. Mn Porphyrins

MnTE-2-PyP^{5+} and MnTE-3-PyP^{5+} were prepared as describer earlier [21,70]. Initial *E. coli* studies were performed with both Mn porphyrins. As the effects were more pronounced with MnTE-3-PyP^{5+}, this compound was used in subsequent studies.

2.3. *E. Coli* Strains

The strains of *E. coli* used in this study were as follows: GC4468 = parental strain; SOD-deficient, *sodA*$^{-}$ *sodB*$^{-}$, QC1799 = GC4468 Δ*sod*A3, Δ*sod*B-kan; and *soxRS*-deficient, DJ 901 = GC4468 Δ(*soxRS-zjc-2204*)*901zjc-2205*::Tn*10*Km [86] (D. Touati, Institute Jacques Monod, CNRS, Universite Paris, France). The *soxRS* deletion was provided by B. Weiss [87]. AB1157 = parental strain (*F-thr-1; leuB6; proA2; his-4; thi-1; argE2; lacY1; galK2; rpsL; supE44; ara-14; xyl-15; mtl-1; tsx-33*); JI132, SOD-deficient, (same as AB1157 plus (*sodA::mudPR13)25 (sodB-kan)1-Δ2*); and *oxyR*-deficient, AS430 = GC4468 ΔoxyR::spec, were provided by J. Imlay (Department of Microbiology, University of Illinois, Urbana, IL, USA). Two sets of parental and SOD-deficient strains were used in order to test the impact of the genetic background. No significant differences between the strains from different origin were observed.

2.4. Growth Media

LB medium contained 10 g Bacto-tryptone, 5 g yeast extract, and 10 g NaCl per liter and was adjusted to pH 7.0 with ~ 1.5 g of K_2HPO_4. Casamino acid (M9CA) medium consisted of minimal A salts (6 g Na_2HPO_4, 3 g K_2HPO_4, 1 g NH_4Cl, and 0.5 g NaCl per liter [88]; $MgSO_4$ and $CaSO_4$ were autoclaved separately and added to the cooled A salts to a final concentration of 20 mM and 100 μM respectively), 0.2 % casamino acids, 0.2 % glucose, 3 mg pantothenate and 5 mg of thiamine per liter. Minimal (5 amino acids, 5AA) medium was prepared as M9CA medium except that casamino acids were replaced by L-leucine, L-threonine, L-proline, L-arginine, L-histidine, each at final concentration of 0.5 mM [89].

2.5. *E. Coli* Growth

Strains were grown overnight, aerobically at 37 ± 0.1 °C, with shaking at 200 rpm, in LB medium containing 350 μg/mL kanamycin, 30 μg/mL chloramphenicol, or 120 μg/mL spectinomycin as required. The overnight cultures were diluted 500-fold into M9CA or 5AA medium not containing antibiotics. Deionized water was used throughout the study. Spectinomycin at a concentration of 500 μg/mL was used to inhibit protein synthesis.

2.6. The Effect of Mn Porphyrins on the Growth of JI and AB Strains of *E. Coli* in the Presence and Absence of Ascorbate

The experiments were carried out in triplicates. Briefly, cultures were grown aerobically in either M9CA or 5AA medium in 96-well plates. The effect of Mn porphyrins (in the range of 1 to 10 μM), ascorbate (in the range of 1–10 mM), or combination of MnP + ascorbate on the growth was followed turbidimetrically at 600 nm. Controls without additions of compounds to the growth media were run in parallel. In the experiments with catalase, the enzyme was first purified from preservatives and was added to the growth medium at a final concentration of 1,000 units/mL, five minutes prior to the addition of MnPs and ascorbate.

2.7. The Adaptive Response of *E. Coli Via* Induction of *oxyR* and *soxRS* Regulon

For these studies parental (GC4468), *oxyR*-deficient (AS430), and *soxRS*-deficient (DJ901) strains were used.

2.8. Peroxidase and Catalase Activities

Cultures were grown in M9CA medium to A_{600nm} of 0.5 – 0.6. At that point either MnTE-3-PyP^{5+} or ascorbate alone, or both combined were added to the medium. After 2 hours of growth, the cultures were chilled on ice, and cells were harvested by centrifugation at 4 °C. The cell pellet was washed three times, resuspended in 0.8 mL of 50 mM K-phosphate buffer (pH 7.0), and then lysed by sonication. Debris was removed by centrifugation at 14,000 × g for 10 minutes. The cell-free extracts thus obtained were assayed for superoxide dismutase [90], nitroreductase A [91], fumarase C [92], catalase and peroxidase activities [93], and proteins were determined by the method of Lowry [94].

Student t-test was used to determine the statistical significance. (*) presents $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Mn Porphyrins Allow SOD-Deficient *E. Coli* to Grow Aerobically Substituting for the SOD Enzymes

Two Mn porphyrins were studied here: the *ortho* and *meta* isomers of Mn(III) *meso*-tetrakis(*N*-ethylpyridinium-2(or 3)-yl) porphyrin (Fig. (4)).

The effects of both compounds on the growth of parental and SOD-deficient strains were examined in two different media: nutrient-rich M9CA medium (Fig. (5)), and minimal five amino acids medium (Fig. (6)). The lack of SOD imposes phenotypic deficiencies on *E. coli* (slow aerobic growth and inability to synthesize branched-chain, aromatic and sulfur-containing amino acids), which do not allow the SOD-deficient mutants ($sodA^{-}sodB^{-}$) to grow aerobically in minimal medium. Growth, however, can be restored by compounds acting as SOD mimics [99]. Since under our experimental conditions SOD-deficient overnight LB cultures were directly diluted into five amino acids medium, nutrients and metabolites were inevitably transferred from the LB medium, which allowed $sodA^{-}sodB^{-}$ slow aerobic growth in minimal medium.

MnTE-2-PyP^{5+} and MnTE-3-PyP^{5+} differ with respect to lipophilicity, metal-centered reduction potential and the degree of electrostatic facilitation [100] for the reaction with $O_2^{\cdot-}$ (Table 1) and $ONOO^-$. MnTE-3-PyP^{5+} is ~10-fold less potent SOD mimic, but is ~10-fold more lipophilic compound than MnTE-2-PyP^{5+}. The bioavailability compensates for the inferior SOD-activity and reducibility of MnTE-3-PyP^{5+} (Table 1). In turn, they appear of similar ability to protect SOD-deficient *E. coli.* MnTE-3-PyP^{5+} crosses cell wall easier, which can be an explanation for its slightly higher efficiency compared to MnTE-2-PyP^{5+} when used at lower concentrations (5 and 10 μM) (Figs. (5 and 6)) [21]; both have been essentially of identical efficacy at 20 μM levels [21].

3.2. Ascorbate Alone at High Levels Suppresses *E. Coli* Growth

With 1 mM ascorbate, no significant toxicity was exerted to both wild type AB1157 and SOD-deficient *E. coli* strain, JI132 growing either in M9CA Fig. (5) or in five amino acids medium Fig. (6). Higher, 5 and 10 mM levels of ascorbate (alone, without MnP) suppress *E. coli* growth significantly. The similar effects were reported by Campos et al [101]; slight differences in magnitude observed are due to the differences in the composition of the growth medium. Campos et al reported that the peroxide levels increased from 3.2 to 7.4 μM, when the ascorbate concentration in medium increased from 1 to 10 mM [101].

3.3. Mn Porphyrins Catalyze Ascorbate-Driven Peroxide Production Imposing Oxidative Stress upon *E. Coli*

As noted above, both strains AB1157 and JI132 grew in both media similarly with and without 1 mM ascorbate Figs. (5 and 6). When MnP was added as a catalyst of ascorbate-driven peroxide formation, the growth of both types of *E. coli* and in both media was suppressed. Fig. (5) relates to the growth in nutrient-rich M9CA medium, and Fig. (6) to the growth in minimal five amino acids medium.

As the cells grew further beyond 6 hours, the ascorbate got consumed, while at the same time more cells were present in the medium to degrade the exogenous peroxide. Also, the wild type *E. coli* overcame the peroxide-mediated damage *via* adaptive response inducing *oxyR* regulon as showed below. The SOD-deficient strain in minimal medium however, lacking superoxide dismutase presumably underwent vast oxidative damage during ascorbate/MnP-mediated peroxide production that prevented it to recuperate (Fig. (7)). Damage is in part due to the high levels of "free iron" in SOD-deficient *E. coli*; along with increased H_2O_2 production, that would eventually lead to Fenton-chemistry driven $^{\cdot}OH$ production [102–104]. Once again, Fig. (7B) stresses the point that when combined with ascorbate, Mn porphyrins do not act (at least not predominantly) as SOD mimics. Even though they can react with both superoxide (antioxidative mechanism) or oxygen (pro-oxidative mechanism) the pro-oxidative action obviously prevails due to the abundance of oxygen and ascorbate.

3.4. Involvement of H_2O_2 in Suppression of *E. Coli* Growth – Effect of Catalase

To test if the inhibitory effect of MnTE-3-PyP^{5+} + ascorbate is indeed due to the extracellular H_2O_2 production, catalase (1,000 U/mL) was added to the growth medium five minutes before the addition of MnTE-3-PyP^{5+} + ascorbate. To account for their SOD like activity, 5 and 10 μM Mn porphyrins were used in experiments presented in Figs. (5–7). The 1 μM Mn porphyrin was enough to catalyze ascorbate oxidation. The results obtained with the parental strain AB1157 are shown in Fig. (8). Addition of catalase to the complete M9CA (Fig (8, Panel **A**)), or to five amino acids medium (Fig. (8, Panel **B**)) prevents growth inhibition caused by MnTE-3-PyP^{5+} + ascorbate. Similar results were obtained with SOD-deficient strain (not shown).

3.5. Adaptive Response of *E. Coli* to the Increased Peroxide Levels

E. coli responds to oxidative insult by inducing a battery of genes aiming at preventing and repairing oxidative damage. Two main antioxidant regulons have been well studied in *E. coli*, *soxRS*, controlling the induction of MnSOD among other genes, and *oxyR*, inducing peroxidases and catalases [101,105]. It is therefore reasonable to expect that adaptation of *E. coli* to oxidative stress caused by ascorbate, or MnTE-2(or 3)-PyP^{5+} + ascorbate will depend on those regulons. To test this possibility, mutants unable to activate either *soxRS* (DJ901) or *oxyR* regulons (AS340) were used. Fig. (9) compares the effect of ascorbate alone (1 mM) or in combination with MnTE-2-PyP^{5+} or MnTE-3-PyP^{5+} (1 μM) on the growth of the parental (GC4468) and the two mutant strains derived from it. Growth was monitored long enough to account for adaptation. At the selected concentrations of ascorbate and MnTE-2(or 3)-PyP^{5+}, the growth of the parental and the *soxRS*$^{-}$ mutant strains was not affected (panels A and B respectively), but the growth of *oxyR*$^{-}$ mutants was suppressed (panel C).

It is important to note that in the *oxyR*-deficient cells treated with ascorbate only, no increase of A_{600nm} was detected until 12 hours of incubation, and the small growth with ascorbate + MnTE-2(or 3)-PyP^{5+} could be detected no earlier than at 15 hours of incubation. The most probable reason is that cells were able to resume the growth only at a point when ascorbate got consumed.

As mentioned above, among the adaptive responses to oxidative stress are induction of MnSOD (*soxRS*-dependent) and catalases and peroxidases (*oxyR*-dependent). It is reasonable to expect that the generation of ROS ($O_2^{\cdot -}$, H_2O_2) *via* metalloporphyrin-catalyzed oxidation of ascorbate would induce those regulons, and in turn would lead to expression of enzymes, which are under their control. Experiments where parental strains (AB1157 and GC4468) were grown in the presence of varying concentrations of either MnTE-2-PyP^{5+} or MnTE-3-PyP^{5+} and ascorbate did not show any induction of MnSOD or any other *soxRS*-regulated enzyme (fumarase C, nitroreductase A) (data not shown).

Additional investigations revealed that the presence of ascorbate suppressed the induction of the *soxRS* regulon by redox-cycling agents, for example, paraquat (data not shown). This led us to the conclusion that most probably ascorbate suppresses the oxidation of the soxR protein preventing its activation. Such an effect has already been reported [103], but it might be a consequence of suppressed paraquat uptake [106]. More plausible explanation for the lack of induction of the *soxRS* regulon is that redox-cycling and superoxide production takes place outside the cells [106], and because *E. coli* cell wall is impermeable for $O_2^{\cdot -}$, no induction of the *soxRS* can be observed.

To test the induction of *oxyR*-dependent antioxidant enzymes we incubated the parental (GC4468) and *oxyR*-deficient (AS430) cells for two hours in the presence of 1 μM MnTE-3-PyP^{5+} and 1 mM ascorbate, and determined the activities of catalases (Fig. (10, Panel **A**)) and peroxidases (Fig. (10, Panel **B**)). To show that the increase in the peroxidase and catalase activity is a consequence of the adaptive response to increased levels of hydrogen peroxide in the medium, catalase was added to the medium at 1,000 U/mL. In such scenario, H_2O_2 was removed by exogenous catalase, and no subsequent upregulation of catalases and peroxidases was therefore required, and consequently not detected (Fig. (10)).

3.5.1. Spectinomycin Suppresses Adaptive Response Via Blocking Protein Synthesis—To prove that the increase of peroxidase and catalase activities results from gene induction, spectinomycin was used to block protein synthesis [104]; consequently, no increase in peroxidase and catalase activities was detected (Fig. (11)).

In conclusion, our experiments clearly indicate that MnP/ascorbate treatment imposes oxidative stress upon *E. coli* which leads to an adaptive response *via* upregulation of H_2O_2-removing proteins.

3.6. The Relevance of *E. Coli* Data to Mammalian Systems

We showed herein, in a superoxide-specific system, that in an appropriate environment which is deficient in peroxide removing systems, compounds which remove $O_2^{\cdot -}$ may produce $O_2^{\cdot -}$ and H_2O_2. Ascorbate [3,67], glutathione [18], tetrahydrobiopterin [65] and flavoproteins [68] are all able to reduce cationic Mn(III) *N*-alkylpyridylporphyrin [1,2]. In a subsequent step $Mn^{II}P$ may reduce oxygen to produce $O_2^{\cdot -}$, rather than scavenge $O_2^{\cdot -}$. Given the orders of magnitude higher availability of oxygen than superoxide, reduction of oxygen by $Mn^{II}P$ may be preferred over $O_2^{\cdot -}$ reduction, regardless of the lower rate constant for the former. The rate constant for the reduction of oxygen to $O_2^{\cdot -}$ by Mn^{II}TM-4-PyP^{4+} is 1.1×10^6 $M^{-1}s^{-1}$ [107]. Based on that value the rate constant for the reaction of *ortho* MnTE-2-PyP^{5+} with O_2 is estimated to be 8×10^4 $M^{-1}s^{-1}$, while the rate of reduction of $O_2^{\cdot -}$ to H_2O_2 by MnTE-2-PyP^{5+} is ~ 5×10^7 - ~ 10^8 M^{-1} s^{-1} [71]. Moreover, Mn porphyrin would catalyze ascorbate oxidation with oxygen, which would result in peroxide production also Fig. (3).

E. coli does not have endogenous ascorbate. Thus our study is not of immediate relevance to *E. coli* biology. Yet *E. coli* is a simple model for proof-of-principle studies which conclusions could inspire and challenge future studies of mammalian systems. Mammalian cells are rich in ascorbate, which localizes both in mitochondria and cytosol [35,108]. Ascorbate alone was shown to exert anticancer effects, while metalloporphyrins and/or other redox able compounds such as quinones could serve as catalysts [44–46,49–51,110–112].

Several publications have already provided the evidence for the pro-oxidative actions of cationic Mn(III) *N*-alkylpyridylporphyrins in the presence of cellular reductants in mammalian systems. Our group [82], Tian et al [83] and Jaramillo et al [80,81] showed that the pro-oxidative mechanism resulted in the anticancer effects of MnPs in the presence of ascorbate [82,83] or glutathione and H_2O_2 [81] in different cancer cells such as HeLa, Caco, 4T1, HCT116 [82] and lymphoma [80,81]. Further, the inhibition of NF-κB activation by MnP-induced oxidation of its p50 subunit [18, 113], which resulted in the suppression of cytokines IL-6 and IL-8 and chemoattractant MCP-1, has been indicated by Tse et al in the protection of human pancreatic cells [1,2,18,113]. Further investigation is needed to show if glutathionylation of p65 subunit of NF-κB [80] might be involved in the NF-κB inactivation by MnPs in the case of human islet cells also. It is possible that the inhibition of HIF-1α and AP-1 may as well occur *via* glutathionylation of those proteins. With cancer cells the anticipated and preferred cytotoxic effects were observed when MnPs were administered along with ascorbate. With human islet cells the suppression of excessive inflammatory and immune pathways, thus antioxidative effects of MnPs were detected.

Tumors are frequently under continuous oxidative stress; they utilize the increased levels of ROS as a signal for their proliferation [114,115]. Assuming the ascorbate deficiency of an aggressive tumor [43], MnP may increase the tumor oxidative burden when given along with ascorbate, whereby preventing its progression; the pro-oxidative mechanism would be operative. A number of studies have reported the anticancer effects of ascorbate and some of them are listed herein [40,44–64,109–112].

The MnPs likely act differentially on the transcriptional activity of tumors *vs* normal cells. This may be a consequence of the different redox status of tumor *vs* normal cells, and different ratio of superoxide to peroxide removing enzymes. It may also be due to the different transcription factor profile in tumor *vs* non-tumor cells [116–119].

In normal cells with plenty of peroxide-removing systems, the *in vivo* antioxidative actions of MnPs would prevail. Radioprotection of normal tissue appears to be a very obvious case of MnP antioxidative actions [1,2,18]. Yet, while the effects observed are clearly antioxidative, again suppression of transcription factors might have occurred as a consequence of MnP pro-oxidative action. MnP catalysis of ascorbate oxidation giving rise to the increased H_2O_2 levels may though not be excluded as a possibility for a mode of MnP action. Increased H_2O_2 levels could lead to an adaptive response alike shown in this *E. coli* study, resulting in the upregulation of endogenous antioxidative defenses. Perhaps we need to distinguish between the effects observed and the mechanism of actions of MnPs *in vivo*. Please see also Discussion in ref 69 related to the possible types of MnP action/s *in vivo*.

In summary, the resulting type of MnP action, pro- or antioxidative, would depend upon the balance of cellular oxidants and antioxidants, oxygen levels and in particular upon the ability of cells to remove superoxide and peroxide and would thus differ between cancer and normal cells. It would also depend upon the tissue, cellular and subcellular accumulation of Mn porphyrin.

4. CONCLUSIONS

The most potent Mn porphyrins are undoubtedly able to effectively remove $O_2^{\cdot -}$ and $ONOO^-$. Due to their redox abilities to easily adopt several oxidation states, +2, +3, +4 and +5, similar abilities to reduce and oxidize superoxide and ability to couple with cellular reductants, Mn porphyrins can act both as anti- and pro-oxidants. In a superoxide-specific system, acting as antioxidants, MnPs are able to substitute for SODs, allowing SOD-deficient *E. coli* to grow aerobically equally well as the wild type. Yet, when ascorbate was added to the growth medium along with MnP, which catalyzed ascorbate-driven peroxide generation, the cell growth was suppressed; MnP acted in a pro-oxidative manner. Catalase prevented the damage indicating H_2O_2 as a key cytotoxic player. Wild type *E. coli* was able to recover in both nutrient-rich and minimal medium *via* adaptive response, while SOD-deficient strain recovered only in a nutrient-rich medium. The adaptive response *via* induction of *oxyR* regulon, which controls antioxidative genes essential for the removal of peroxides, resulted in significantly enhanced activities of endogenous catalases and peroxidases. When catalase was added to the medium to remove exogenous peroxides, the adaptive response *via* upregulation of peroxidases and catalases was not observed.

Mn porphyrins distribute *in vivo* in plasma, extracellular space, and all tissues [1,2,18–20,26]. Within cell they have been detected in cytosol, nucleus and mitochondria. Due to the ubiquitous availability of reductants and oxygen, the impaired redox balance under pathological conditions such as is tumor, and complexity of *in vivo* redox systems and redox chemistry and biology of MnPs, both actions of Mn porphyrins must be accounted for. Future work is needed to gain full insight into the nature of MnPs action/s *in vivo*, and to understand the circumstances under which pro- or antioxidative types of MnP actions prevail.

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ABBREVIATIONS

MnP	Mn porphyrin

HA^-	Singly deprotonated ascorbic acid
A^{2-}	Doubly deprotonated ascorbic acid
$HA^{\cdot}$	Protonated ascorbyl radical (one-electroniaclly oxidized ascorbic acid)
$A^{\cdot-}$	Deprotonated ascorbyl radical
A	Two-electronically oxidized ascorbic acid, dehydroascorbic acid
MnTE-2-PyP^{5+}	Mn(III) *meso*-tetrakis(*N*-ethylpyridinum-2-yl)porphyrin, E2, AEOL10113, FBC-007
MnTE-3-PyP^{5+}	Mn(III) *meso*-tetrakis(*N*-ethylpyridinium-3-yl)porphyrin, E3
MnTnHex-2-PyP^{5+}	Mn(III) *meso*-tetrakis(*N*-n-hexylpyridinium-2-yl)porphyrin
MnTDE-2-ImP^{5+}	Mn(III) m*eso*-tetrakis(*N, N′*-diethylimidazolium-2-yl)porphyrin, AEOL10150
$E_{1/2}$	Half-wave reduction potential
SOD	Superoxide dismutase
NHE	Normal hydrogen electrode
$ONOO^-$	Peroxynitrite
$O_2^{\cdot-}$	Superoxide
$^{\cdot}NO$	Nitric oxide
$CO_3^{\cdot-}$	Carbonate radical
HIF-1α	Hypoxia inducible factor 1α
AP-1	Activator protein-1
VEGF	Vascular endothelial growth factor
bFGF	Basic fibroblast growth factor
NF-κB	Nuclear factor κB
SPEC	Spectinomycin, protein synthesis inhibitor

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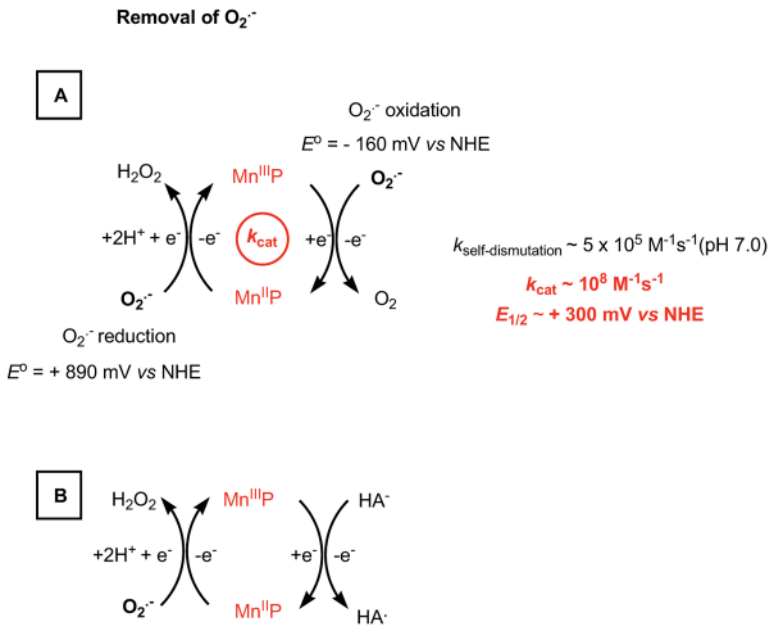

Fig. 1.

The $O_2^{\cdot-}$ dismutation (**A**) and reduction (**B**) by Mn porphyrins.

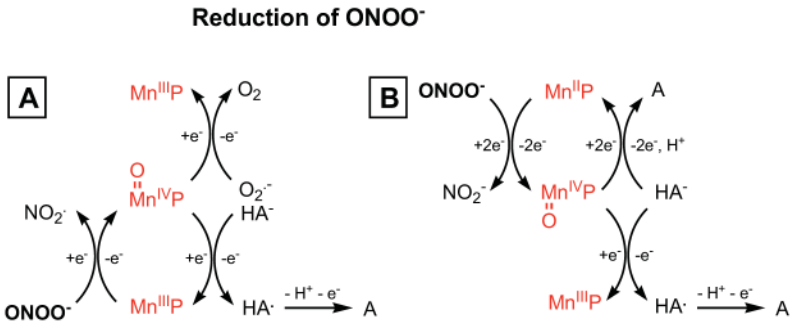

Fig. 2.

The $ONOO^-$ reduction by Mn porphyrins catalyzed by ascorbate *via* $Mn^{III}P/O{=}Mn^{IV}P$ redox couple (**A**) and $Mn^{II}P/O{=}Mn^{IV}P$ redox couple (**B**).

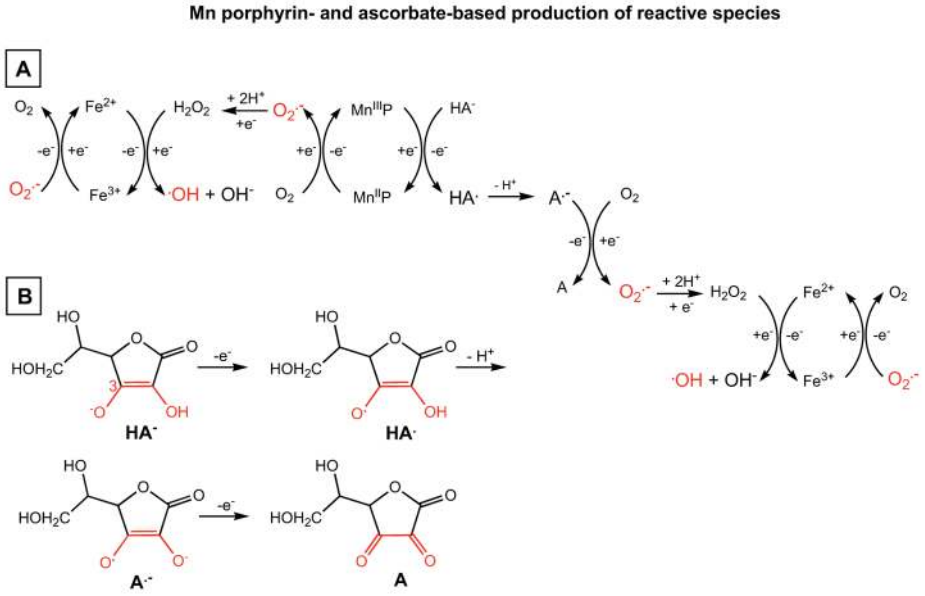

Fig. 3.

The pro-oxidative action of Mn porphyrins in the presence of cellular reductant, ascorbate. The protonation and oxidation of ascorbate is shown in Fig. (**3B**). The pK_a for HA_2 <==> $HA^- + H^+$ is 4.17 and for HA^- <==> $A^{2-} + H^+$ is >11.5 [31]. A^{2-} stands for doubly deprotonated ascorbic acid, and A stands for two-electronically oxidized dehydroascorbate. *In vivo*, ascorbate, rather than superoxide would be the preferred reductant for Fe^{3+}. In the absence of metal catalyst, doubly deprotonated, A^{2-} can autooxidize with O_2 to produce $O_2^{\cdot -}$ ($k \sim 10^2\ M^{-1}\ s^{-1}$) and orders of magnitude faster than HA^- [84]. The possibility that Fe site but not Mn center of metalloporphyrins reacts with peroxide producing $^{\cdot}OH$ radical has been indicated [85].

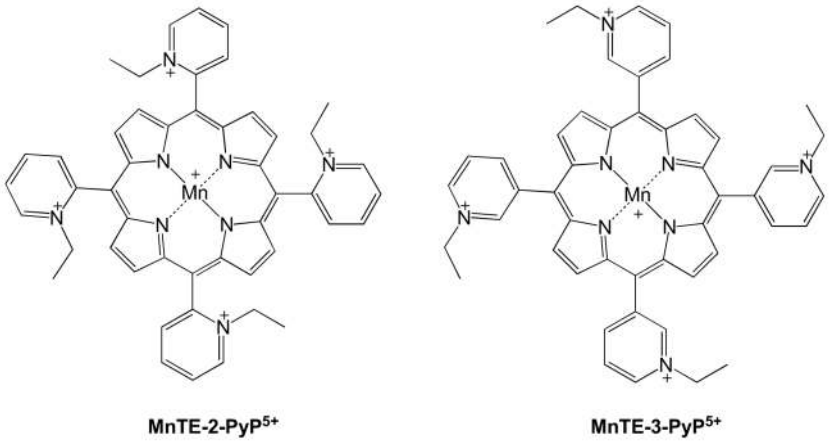

Fig. 4.
Structures of MnTE-2-PyP^{5+} and MnTE-3-PyP^{5+}.

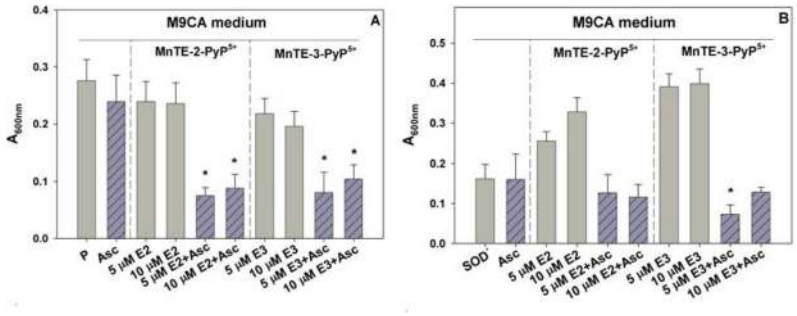

Fig. 5.

Growth of wild type parental *E. coli* AB1157 (**Panel A**) and SOD-deficient, JI132 (**Panel B**) *E. coli* with 5 and 10 μM MnP −/+ ascorbate (1 mM) in nutrient-rich M9CA medium at 6 h. (*) represents statistical significance when compared to untreated controls. P=parental or SOD^{-}=SOD-deficient *E. coli*, Asc=ascorbate, E2/E3= MnTE-2-PyP^{5+}/MnTE-3-PyP^{5+}.

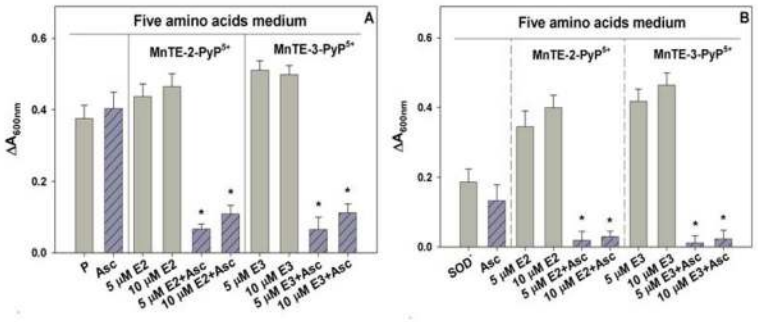

Fig. 6.

Growth of wild type parental *E. coli* AB1157 (**Panel A**) and SOD-deficient JI132 *E. coli* (**Panel B**) with 5 and 10 μM MnP −/+ ascorbate (1 mM) in minimal, five amino acids medium at 14 h. (*) represents statistical significance when compared to untreated controls. P=parental or SOD^{-}=SOD-deficient *E. coli*, Asc=ascorbate, E2/E3= MnTE-2-PyP^{5+}/ MnTE-3-PyP^{5+}.

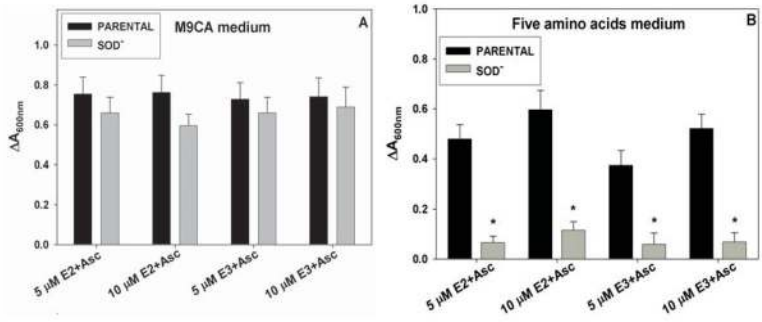

Fig. 7.

Growth of wild type AB1157 (parental) and SOD-deficient *E. coli* JI132 with 5 and 10 μM MnP/ascorbate (1 mM) in M9CA medium at 18 h (**Panel A**), and in minimal 5 amino acids medium at 24 h (**Panel B**), respectively. (*) represents statistical significance, Asc=ascorbate, E2/E3= MnTE-2-PyP^{5+}/MnTE-3-PyP^{5+}.

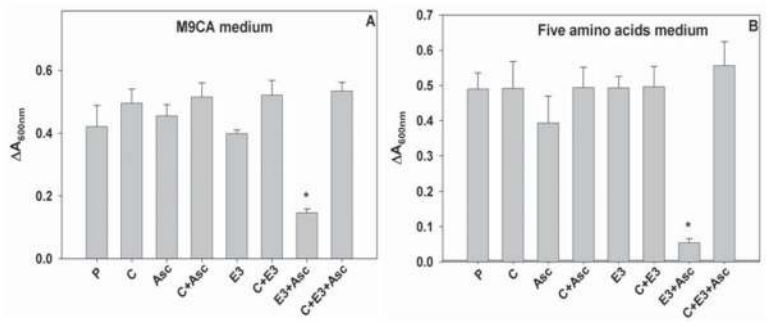

Fig. 8.

Effect of catalase on MnTE-3-PyP^{5+}/ascorbate toxicity on the growth of *E. coli*. The parental AB1157 *E. coli* (P) grew with or without 1 μM MnP and 1 mM ascorbate. Catalase (C) was added to the growth medium at 1,000 units/mL five minutes before the addition of MnP and ascorbate. **Panel A** shows growth at 9 hours in M9CA nutrient rich medium, and **Panel B** growth at 18 hours in five amino acids medium. P=parental, C=catalase, Asc=ascorbate, E2/E3= MnTE-2-PyP^{5+}/MnTE-3-PyP^{5+}.

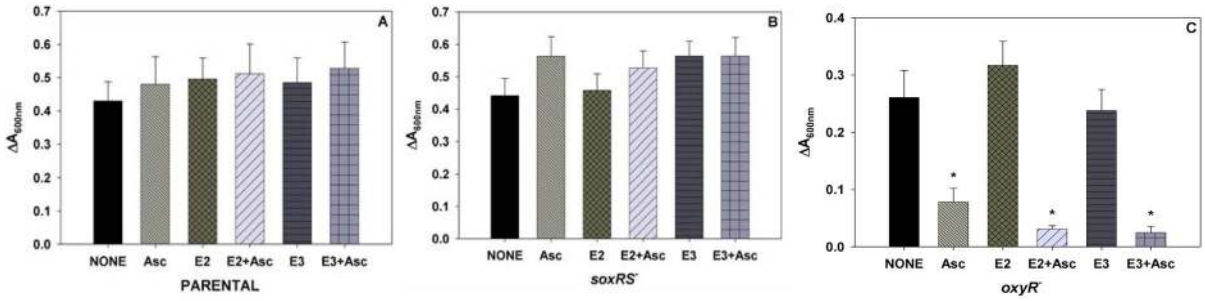

Fig. 9.

The growth of *E. coli* in the presence of 1 mM ascorbate, 1 μM MnTE-2-PyP^{5+}, 1 μM MnTE-3-PyP^{5+} and the combination of Mn porphyrins with ascorbate in M9CA nutrient-rich medium at 15 hours. Three strains were tested, the parental GC4468 (**Panel A**), a mutant unable to induce the s*oxRS* regulon (*soxRS*$^{-}$) (**Panel B**) and a mutant unable to induce the *oxyR* regulon (*oxyR*$^{-}$) (**panel C**). The growth was followed at 600 nm. Asc=ascorbate, E2/E3= MnTE-2-PyP^{5+}/MnTE-3-PyP^{5+}.

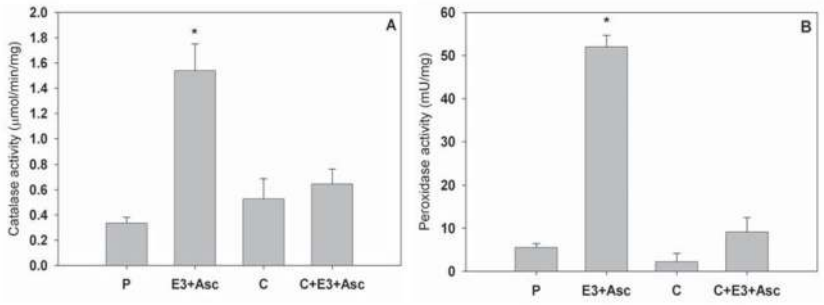

Fig. 10.

The effect of MnP/ascorbate on the upregulation of catalases (**A**) and peroxidases (**B**). The parental (GC4468) cells were incubated for 2 hours in the presence of 1 μM MnTE-3-PyP^{5+} and 1 mM ascorbate. *E. coli* responds to the increased peroxide levels as a consequence of MnTE-3-PyP^{5+}/ascorbate-based peroxide formation by upregulating catalases and peroxidases (E3+Asc bar *vs* P bar). No adaptive response of parental GC4468 *E. coli* strain was observed when 1,000 units/mL of catalase was added into M9CA medium 5 min after the addition of MnP/ascorbate to remove peroxides. (*) represents statistical significance. P=parental, C=catalase, Asc=ascorbate, E3= MnTE-3-PyP^{5+}.

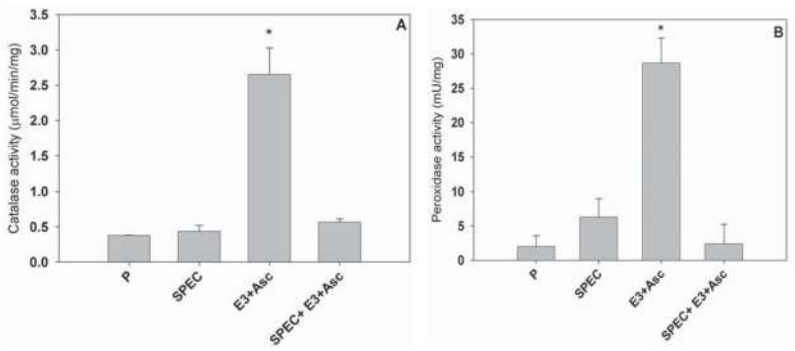

Fig. 11.

The adaptive response of parental (P) GC4468 *E. coli* to oxidative insult is prevented when the synthesis of proteins was suppressed by the addition of spectinomycin. *E. coli* was incubated in the presence of 1 mM ascorbate + 1 μM MnTE-3-PyP^{5+} in M9CA medium for 2 hours. Catalase (**Panel A**) and peroxidase activities were determined (**Panel B**). (*) represents statistical significance. P=parental, C=catalase, Asc=ascorbate, E3= MnTE-3-PyP^{5+}, SPEC=spectinomycin.

Table 1

The Metal-Centered Reduction Potential for $Mn^{III}P/Mn^{II}P$ Redox Couple, $E_{1/2}$, log k_{cat} for the MnP-Catalyzed $O_2^{\cdot -}$ Dismutation and the Lipophilicity of MnTE-2-PyP^{5+} and MnTE-3-PyP^{5+} Expressed as the Partition of the Compound between n-Octanol and Water, P_{OW}

Mn Porphyrins	$E_{1/2}$[a]/mV *vs* NHE	log k_{cat}[b]	log P_{OW}
MnTE-2-PyP^{5+}	+228 [72,95]	7.76 [95]	−6.89 [96]
MnTE-3-PyP^{5+}	+54 [21,95]	6.65 [21]	−5.98 [96]
SOD enzymes	~ +300 [97]	8.84–9.30 [2,97,98]	

[a] $E_{1/2}$ was determined in 0.05 M phosphate buffer (pH 7.8, 0.1 M NaCl),

[b] k_{cat} was determined by cytochrome *c* assay in 0.05 M potassium phosphate buffer (pH 7.8, at 25±1 °C).