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The impact of electrostatics in redox modulation of oxidative stress by Mn porphyrins: Protection of SOD-deficient *E. coli* via alternative mechanism where Mn porphyrin acts as a Mn-carrier

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Abstract

Understanding the factors that determine the ability of Mn porphyrins to scavenge reactive species is essential for tuning their *in vivo* efficacy. We present herein the revised structure-activity relationships accounting for the critical importance of electrostatics in the Mn porphyrin-based redox modulation systems and show that the design of effective SOD mimics (*per se*) based on anionic porphyrins is greatly hindered by inappropriate electrostatics. A new strategy for the β -octabromination of the prototypical anionic Mn porphyrins Mn(III) *meso*-tetrakis(*p*-carboxylatophenyl)porphyrin ([Mn^{III}TCPP]³⁻ or MnTBAP³⁻) and Mn(III) *meso*-tetrakis(*p*-sulfonatophenyl)porphyrin ([Mn^{III}TSPP]³⁻), to yield the corresponding anionic analogues [Mn^{III}Br₈TCPP]³⁻ and [Mn^{III}Br₈TSPP]³⁻, respectively, is described along with characterization data, stability studies, and their ability to substitute for SOD in SOD-deficient *E. coli*. Despite the Mn^{III}/Mn^{II} reduction potential of [Mn^{III}Br₈TCPP]³⁻ and [Mn^{III}Br₈TSPP]³⁻ being close to the SOD-enzyme optimum and nearly identical to that of the cationic Mn(III) *meso*-tetrakis(*N*-methylpyridinium-2-yl)porphyrin (Mn^{III}TM-2-PyP⁵⁺), the SOD activity of both anionic brominated porphyrins ([Mn^{III}Br₈TCPP]³⁻, $E_{1/2} = +213$ mV vs NHE, $\log k_{\text{cat}} = 5.07$; [Mn^{III}Br₈TSPP]³⁻, $E_{1/2} = +209$ mV, $\log k_{\text{cat}} = 5.56$) is considerably lower than that of Mn^{III}TM-2-PyP⁵⁺ ($E_{1/2} = +220$ mV, $\log k_{\text{cat}} = 7.79$). This illustrates the impact of electrostatic guidance of O₂^{•-} toward the metal center of the mimic. With low k_{cat} , the [Mn^{III}TCPP]³⁻, [Mn^{III}TSPP]³⁻, and [Mn^{III}Br₈TCPP]³⁻ did not rescue SOD-deficient *E. coli*. The striking ability of [Mn^{III}Br₈TSPP]³⁻ to substitute for the SOD enzymes in the *E. coli* model does not correlate with its $\log k_{\text{cat}}$. In fact, the protectiveness of [Mn^{III}Br₈TSPP]³⁻ is comparable to or better than that of the potent SOD mimic Mn^{III}TM-2-PyP⁵⁺, even though the dismutation rate constant of anionic complex is 170-fold smaller. Analyses of the medium and *E. coli* cell extract revealed that the major species in the [Mn^{III}Br₈TSPP]³⁻ system is not the Mn complex, but the free-base porphyrin [H₂Br₈TSPP]⁴⁻ instead. Control experiments

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with extracellular MnCl_2 showed lack of *E. coli* protection, indicating that “free” Mn^{2+} cannot enter the cell at significant extent. We proposed herein the alternative mechanism where a labile Mn porphyrin $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSP}]^{3-}$ is not a SOD mimic per se but carries Mn into the *E. coli* cell.

Keywords

Mn transport; Electrostatics; *Escherichia coli*; SOD mimics; Redox modulators; Mn porphyrins

Introduction

Mn porphyrins (MnP) have been originally developed as SOD mimics based on a structure-activity relationship (SAR) correlating the metal-centered reduction potential ($E_{1/2}$) and the rate constant for the catalysis of $\text{O}_2^{\bullet-}$ dismutation, $k_{\text{cat}}(\text{O}_2^{\bullet-})$ [1]. The initial SAR included a number of Mn(III) and Fe(III) porphyrins and showed that in general an increase in $E_{1/2}$, resulted in higher k_{cat} values [1]. The introduction of electron-withdrawing groups on the porphyrin core is often accompanied by increase in $E_{1/2}$. A convenient strategy in developing Mn porphyrin-based SOD mimetics has been to modify *ortho* cationic *N*-alkylpyridylporphyrins [2–5], as these *ortho*-charges impose electron-withdrawing effect upon the metal center and concomitantly afford electrostatic guidance of $\text{O}_2^{\bullet-}$ to the Mn site [5]. Incidentally, these *ortho*-*N*-alkylpyridylporphyrins are also among the most (if not the most) potent scavengers of peroxynitrite so far [6,7]. These basic concepts on the *ortho*-*N*-alkylpyridinium-2-ylporphyrin framework have been extensively explored to prepare a myriad of metal-porphyrinoid complexes as redox modulators [2–4,8–10].

Major cellular pathways are redox-controlled. Mn porphyrins have several biologically accessible redox states and when properly chosen they are able to reduce the levels of reactive species, which results not only in a smaller damage of biological molecules, but also in the modulation of transcription factors, protein and gene expression [11–14]. Direct interaction between Mn porphyrins and transcription factors has also been described [14]. The most potent Mn porphyrins have been tested *in vivo* and showed remarkable efficacy in radioprotection [15], tumor suppression [13], diabetes [14,16,17], sickle cell disease [18], cardiac [19], kidney [20], and liver ischemia [21], and disorders of central nervous system [22,23], among others. However, a good understanding of the factors, such as stericity, lipophilicity, electrostatics, and charge distribution, underlying the activity of Mn porphyrin remains imperative to tailor and increase their bioavailability, *i.e.*, *in vivo* efficacy. Our efforts on increasing cellular accumulation led us to the design of a 5-fold more lipophilic, but equally effective antioxidant, MnTnHex-2-PyP⁵⁺ [3] (Fig. 1). This compound was up to 120-fold more effective than its ethyl analogue, MnTnHex-2-PyP⁵⁺, in several different models of oxidative stress injury, such as radiation [15], kidney ischemia/reperfusion [20], and SOD-deficient *E. coli* [24].

Utilizing pentacationic compounds of similar stericity, we have recently studied the effect of charge distribution on the thermodynamic and electrostatic facilitation of $\text{O}_2^{\bullet-}$ dismutation [25]; such a work resulted in a more refined structure-activity relationship between $\log k_{\text{cat}}$ and $E_{1/2}$ as the designed series of compounds presented minimal steric variations. We have reported also on the effect of electrostatics on the catalytic potency of cationic Mn porphyrins [5,25].

Up to now anionic porphyrins were not extensively studied as SOD mimics, with the exception of $[\text{Mn}^{\text{III}}\text{TCP}]^{3-}$ ($[\text{Mn}^{\text{III}}\text{TBAP}]^{3-}$) [1,46], which is now known *not* to be an SOD mimic when pure [27]. For a compound to be a potent SOD mimic the $E_{1/2}$ must be in between the potential for the reduction and oxidation of $\text{O}_2^{\bullet-}$, *ca.* +300 mV vs NHE, as is the case with the SOD enzymes themselves [28–30] (Figure 2). We have achieved this potential range with cationic

Mn(III) *ortho*-*N*-alkylpyridylporphyrins. However, most of the anionic porphyrins thus far studied are either out of range ($[\text{Mn}^{\text{III}}\text{TCPP}]^{3-}$; $[\text{MnTBAP}]^{3-}$) or near the end of the range for thermodynamically achievable reaction with superoxide ($[\text{Mn}^{\text{III}}\text{TSPP}]^{3-}$) [1,26,27]. With no electrostatic facilitation and very negative $E_{1/2}$ values for $\text{Mn}^{\text{III}}/\text{Mn}^{\text{II}}$ reduction, their efficacy in dismuting $\text{O}_2^{\bullet-}$ or reducing ONOO^- has been poor or none [1,6,27]. The rate constants for $\text{O}_2^{\bullet-}$ dismutation were $\leq 10^4 \text{ M}^{-1}\text{s}^{-1}$ as compared with k_{cat} of $\geq 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for the cationic derivatives.

The increase in $\text{Mn}^{\text{III}}/\text{Mn}^{\text{II}}$ reduction potential as a response to the presence of electron-withdrawing groups on the porphyrin ring has been extensively studied [31–33]. Octabromination of the β -pyrrole positions, for example, has provided a convenient derivatization route to tune the steric and electronic properties of Mn porphyrin to yield complexes with metal-centered reduction potential increased by 360–420 mV [5,33–36]. Such remarkable modulation of the redox properties upon halogenation prompted us to investigate the two classical anionic porphyrin complexes (namely $[\text{Mn}^{\text{III}}\text{TCPP}]^{3-}$ and $[\text{Mn}^{\text{III}}\text{TSPP}]^{3-}$) in an attempt to improve their unfavorable $\text{Mn}^{\text{III}}/\text{Mn}^{\text{II}}$ reduction potential and poor (to none) SOD activity [1,27]. The β -octabromination of $[\text{Mn}^{\text{III}}\text{TCPP}]^{3-}$ ($E_{1/2} = -194 \text{ mV vs NHE}$ [27]) and $[\text{Mn}^{\text{III}}\text{TSPP}]^{3-}$ ($E_{1/2} = -160 \text{ mV vs NHE}$ [1]) is, thus, expected to yield complexes whose $\text{Mn}^{\text{III}}/\text{Mn}^{\text{II}}$ reduction potentials are of *ca.* +200 mV vs NHE. Incidentally, such a value is comparable to that of the pentacationic $\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+}$ ($E_{1/2} = +220$) complex [25].

As mechanistic studies are essential for the design of Mn porphyrin-based therapeutics, we undertook and report herein a detailed study on the anionic porphyrin series, which resulted in more comprehensive structure-activity relationships for this class of compounds. The synthesis and characterization of the anionic $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ and $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ porphyrins are also described here along with a comparison of their properties with their parent compounds, $[\text{Mn}^{\text{III}}\text{TSPP}]^{3-}$ and $[\text{Mn}^{\text{III}}\text{TCPP}]^{3-}$ (Fig. 1). We extended, thus, our earlier study on the monocationic/pentacationic Mn porphyrins [5], to include anionic and cationic porphyrins and gain, thus, further insights into the impact of electrostatics on the SOD activity of this class of compounds.

We have been routinely using SOD-deficient *E. coli* aerobic growth to assess the potency of the compounds in search for possible drug candidates [1,25]. This model has proven applicable unambiguously and was tested here on all the anionic porphyrins studied. We were greatly surprised that $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ showed a remarkable potency (comparable if not higher than the efficacy of our lead compounds, MnTM-2-PyP^{5+} or MnTE-2-PyP^{5+}) in protecting SOD-deficient *E. coli*. The mechanism of action of this anionic Mn porphyrin was found to be different from that of MnTM-2-PyP^{5+} and is discussed herein.

Experimental procedures

General

Xanthine (X) and equine ferricytochrome *c* (lot number 7752 [2,37]) were from Sigma. Xanthine oxidase (XO) was prepared by R. Wiley [38] and was a gift from Dr. K.V. Rajagopalan (Duke University). Acetone (Quimex) and CHCl_3 (Quimex) were freshly distilled prior to use. *N,N*-dimethylformamide (DMF; Aldrich) was dried over KOH, followed by distillation over P_2O_5 and stored over 4 Å molecular sieves. Sodium salt of *meso*-tetrakis(4-sulfonatophenyl)porphyrin ($[\text{H}_2\text{TSPP}]^{4-}$) was purchased from MidCentury Chemicals. Other chemicals and solvents were of reagent grade or higher from Fisher Scientific, Mallinkrodt, or Sigma-Aldrich, and were used as received.

Mn porphyrins

$\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+}$ (chloride salt) [39], $[\text{Mn}^{\text{III}}\text{TCPP}]^{3-}$ (acid form) [27], $\text{H}_2\text{Br}_8\text{TCMPP}$ [34], and $\text{Mn}^{\text{III}}\text{Br}_8\text{TCMPP}^+$ (chloride salt) [34,40] were prepared and characterized as previously described.

$[\text{Mn}^{\text{III}}\text{TSPP}]^{3-}$ (sodium salt) purchased from Porphyrin Products (now Frontier Scientific) gave satisfactory elemental, spectroscopic, and spectrometric analyses and was used as received. Anal. Calcd for $\text{H}_3[\text{Mn}^{\text{III}}\text{TSPP}] \cdot 9\text{H}_2\text{O}$, $\text{C}_{44}\text{H}_{45}\text{N}_4\text{O}_{21}\text{S}_4\text{Mn}$: C, 45.99; H, 3.95; N, 4.88. Found: C, 46.29; H, 3.80; N, 5.14. UV-vis (0.05 M phosphate buffer, pH 7.8): 219.5 nm ($\epsilon/\text{M}^{-1}\text{cm}^{-1}$, 75,061), 287.0 (25,375), 379.5 (59,712), 399.5 (59,673), 420 (sh; 39,080), 466.5 (102,069), 512.5 (7,684), 563.0 (13,164), 596.5 (9,229), 689.5 (1,761), 776 (sh; 1,869), 801.5 (1,456). ESI-MS (1:1 $\text{H}_2\text{O}:\text{MeCN}$): clusters centered at m/z 245.8 ($\{[\text{Mn}^{\text{III}}\text{TSPP}]^{3-} + \text{e}^{-}\}^{4-}/4$), 328.0 ($[\text{Mn}^{\text{III}}\text{TSPP}]^{3-}/3$), 492.4 ($\{[\text{Mn}^{\text{III}}\text{TSPP}]^{3-} + \text{H}^{+}\}^{2-}/2$); peak distribution within the cluster is consistent with the isotope pattern simulation.

$[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ was prepared *via* bromination of $[\text{Mn}^{\text{III}}\text{TSPP}]^{3-}$ as follows: to a refluxing solution of $[\text{H}_2\text{TSPP}]^{4-}$ (sodium salt) (65 mg, 0.063 mmol) in a 1:1 $\text{CHCl}_3:\text{MeOH}$ (10 mL) were added 10 equivalents of $\text{Mn}(\text{OAc})_2 \cdot 4\text{H}_2\text{O}$ (155 mg, 0.63 mmol) dissolved in 5 mL of 1:1 $\text{CHCl}_3:\text{MeOH}$. The reaction was monitored by UV-vis and SiO_2 -TLC plate eluted with a 1:2 $\text{CHCl}_3:\text{MeOH}$ mixture (disappearance of the fluorescent spot associated with the free-base porphyrin). After 24 h of reflux, the heating was discontinued, and the mixture was taken to dryness in a rotary evaporator. The resulting solid was dissolved in a minimal volume of an 1:2 $\text{CHCl}_3:\text{MeOH}$ mixture and chromatographed on SiO_2 eluted with the same solvent mixture. The brown band was collected and evaporated to dryness to yield $[\text{Mn}^{\text{III}}\text{TSPP}]^{3-}$. The UV-vis data of this solid is consistent with that reported above for the $[\text{Mn}^{\text{III}}\text{TSPP}]^{3-}$ complex. The solid was dissolved in DMF (3 mL) and was carried forward to the bromination step without further purification. This Mn complex was brominated using an adapted procedure [41]. A Br_2 (0.25 mL, 4.9 mmol) solution in DMF (1.5 mL) was added dropwise over 30 min to the well-stirred solution of $[\text{Mn}^{\text{III}}\text{TSPP}]^{3-}$ in DMF at room temperature (25–30 °C). The color of the solution changed immediately from greenish brown to dark green and some demetallation occurred. The reaction was monitored by UV-vis and after 24 hours the reaction was quenched by the addition of CH_2Cl_2 (30 mL), which precipitated the brominated compounds. The solids were filtrated and washed with CH_2Cl_2 until the washings were colorless. (Excess Br_2 in the filtrate can be quenched by sodium metabisulfite prior to disposal). The solid (a mixture of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ and $[\text{H}_2\text{Br}_8\text{TSPP}]^{4-}$) was dissolved in a minimal amount of MeOH and chromatographed in a Sephadex LH-20 column eluted with MeOH. The first fractions that analyzed (UV-vis) for $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ were combined and concentrated to a minimal volume. This solution was percolated slowly through a cation-exchange resin, Dowex 50X2-200 (H^+ form), with MeOH. The single fraction collected was taken to dryness in a rotary evaporator to yield $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ (acid form). The solid was kept over P_2O_5 and CaCl_2 in a desiccator under vacuum at room temperature. Yield: 66 mg (64 % based on starting $[\text{H}_2\text{TSPP}]^{4-}$). Anal. Calcd for $\text{H}_3[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}] \cdot 7\text{H}_2\text{O} \cdot 2\text{MeOH}$, $\text{C}_{46}\text{H}_{41}\text{Br}_8\text{N}_4\text{O}_{21}\text{S}_4\text{Mn}$: C, 30.55; H, 2.29; N, 3.10. Found: C, 30.53; H, 2.33; N, 3.34. UV-vis (0.05 M phosphate buffer, pH 7.8): 224.5 nm ($\epsilon/\text{M}^{-1}\text{cm}^{-1}$, 56,551), 316.5 (25,068), 397.0 (46,461), 432.0 (45,944), 491.0 (101,822), 591.5 (11,624), 633.5 (9,162). ESI-MS (1:1 $\text{H}_2\text{O}:\text{MeCN}$): clusters centered at m/z 538.7 ($[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}/3$); peak distribution within the cluster is consistent with the isotope pattern simulation. The UV-vis data for this compound are in agreement with those previously published using a different synthetic route [42,43].

The Sephadex fractions that analyzed for $[\text{H}_2\text{Br}_8\text{TSPP}]^{3-}$ (UV-vis data given in [44,45]) may be metallated with Mn to improve yields on the complex. Briefly, $[\text{H}_2\text{Br}_8\text{TSPP}]^{3-}$ and $\text{Mn}(\text{OAc})_2 \cdot 4\text{H}_2\text{O}$ (10 equiv) were refluxed in an 1:1 $\text{CHCl}_3:\text{MeOH}$ mixture for 2 h and monitored by UV-vis. The mixture was purified by three chromatographic steps: first on SiO_2 (1:2

CHCl₃:MeOH), then on Sephadex LH-20 (MeOH), and finally on a cation-exchange resin (MeOH), Dowex 50X2-200 (H⁺ form). The resulting compound had the same spectral features as those described above for [Mn^{III}Br₈TSP]³⁻ (acid form).

[Mn^{III}Br₈TCPP]³⁻ was prepared *via* the acid-hydrolysis of the corresponding Mn porphyrin tetramethyl ester followed by Mn re-metallation. A solution of MnBr₈TCMPP⁺ (chloride form, 70 mg, 0.045 mmol) in a mixture of H₂SO₄(conc) (10 mL) and H₂O (0.5 mL) was left under stirring at room temperature for 3 weeks, after which part of the material precipitated. The reaction vessel was placed in an ice bath and H₂O (20 mL) was added dropwise to the cooled greenish orange mixture to precipitate the remaining colored material. The solid, which corresponded to the free-base [H₂Br₈TCPP]⁴⁻, was recovered by filtration and washed with water until the washings had neutral pH, then washed with CHCl₃ and dried under vacuum. The crude [H₂Br₈TCPP]⁴⁻ was dissolved in ~15 mL of a 2:1 acetone:H₂O mixture and refluxed with 10 equivalents of Mn(OAc)₂·4H₂O (111 mg, 0.45 mmol). The reaction was monitored by UV-vis spectroscopy, which revealed that a considerable amount of [H₂Br₈TCPP]⁴⁻ was metallated immediately upon the addition of the Mn salt. Reflux was continued for 1 hour, after which part of the porphyrin precipitated. The reaction mixture was concentrated in a rotary evaporator and H₂O (20 mL) was added to precipitate the remaining compound. The precipitate was filtered off and washed with H₂O (5 × 4 mL). The resulting solid was further washed with a 2:1 acetone:H₂O mixture (5 × 4 mL) to remove the unreacted free-base that co-precipitated with the Mn complex. The crude Mn porphyrin was purified by a precipitation/re-precipitation procedure as follows: the solid was dissolved in about 10 mL of a 1:1 mixture of acetone and 0.1 M phosphate buffer (pH 7.6), and then the solvent was removed in a rotary evaporator; the resulting solid material was dissolved in water (5 mL) and re-precipitated with ~2 mL of glacial acetic acid; the suspension was filtered and the solid was washed, first with H₂O (until neutral pH), and then with a 2:1 acetone:H₂O mixture (until the washings were colorless; ~20 mL total). This precipitation/reprecipitation purification procedure was repeated three times. The filtrate was suction dried for one day and the resulting [Mn^{III}Br₈TCPP]³⁻ complex was then stored over P₂O₅ and CaCl₂ in a desiccator under vacuum at room temperature. Yield: 29 mg (45 % based on starting MnBr₈TCMPP⁺). The recovered unreacted free-base [H₂Br₈TCPP]⁴⁻ can be re-metallated with Mn to improve the yield on the complex. Anal. Calcd for H₃[Mn^{III}Br₈TCPP]·3HOAc, C₅₄H₃₁Br₈N₄O₁₄Mn: C, 39.21; H, 1.89; N, 3.39. Found: C, 39.19; H, 2.04; N, 3.80. UV-vis (0.05 M phosphate buffer, pH 7.8): 237.5 nm (ε/M⁻¹ cm⁻¹, 69,827), 309.5 (30,951), 400.5 (58,233), 441.0 (62,223), 492.5 (124,741), 593.0 (14,994), 635.0 (11,981). ESI-MS (40:40:1 H₂O:MeCN:HCO₂H): cluster centered at *m/z* 1474 ([Mn^{III}Br₈TCPP]³⁻ + 4H⁺); peak distribution within the cluster is consistent with an isotope pattern simulation.

Superoxide dismutase activity

The catalytic rate constants for the O₂^{•-} dismutation were determined by cytochrome *c* assay as previously described [25,46]. Briefly, rate constants for the reaction of the Mn porphyrins with O₂^{•-} were based upon the competition with 10 μM cyt *c* (*k*_{cyt *c*} = 2.6 × 10⁵ M⁻¹s⁻¹ [47]). The O₂^{•-} was produced at 1.2 μM/min by the X/XO (40 μM xanthine, ~2 nM XO) generator, and ferricytochrome *c* was used as the indicating scavenger of O₂^{•-}. The reduction of cyt *c* was followed at 550 nm. Assays were conducted at 25 ± 1 °C in 0.05 M phosphate buffer, pH 7.8, 0.1 mM EDTA in the presence or absence of 15 μg/mL catalase. The possible inhibition of X/XO system (O₂^{•-} production) by Mn porphyrin was examined following urate formation at 295 nm in the absence of cyt *c*, all other conditions being the same. We have shown previously for three compounds, namely MnTE-2-PyP⁵⁺, Mn-biliverdin-dimethylester, and MnCl₂, that identical results are obtained by pulse-radiolysis and cyt *c* assay [46]; additionally, the catalytic rate constant reported for MnTM-4-PyP⁵⁺ by stopped-flow [48] is in agreement with that

determined by the cyt. *c* assay [1]. Kinetic salt effect measurements were carried out as described in [25].

***E. coli* growth experiments**

Escherichia coli strains used in this study were AB1157, wild type (*F-thr-1*; *leuB6*; *proA2*; *his-4*; *thi-1*; *argE2*; *lacY1*; *galK2*; *rpsL*; *supE44*; *ara-14*; *xyl-15*; *mtl-1*; *tsx-33*), and JI132, SOD-deficient, *sodA*[−]*sodB*[−] (same as AB1157 plus (*sodA::mudPR13*)25 (*sodB-kan*)1-Δ2). Both strains were obtained from J. A. Imlay [49]. The experiments were carried out as described in detail in [25]. Briefly, cultures were grown aerobically in a 5 amino acid restricted medium (L-leucine, L-threonine, L-proline, L-arginine, L-histidine) in flasks on a water bath shaker at 37°C and 200 rpm. The effect of Mn porphyrins on the growth of these strains was followed turbidimetrically at 700 nm (to minimize the interference of compounds studied) and compared to the growth curves of both strains in the absence of Mn porphyrin (controls). Deionized water was used throughout the study.

Results and Discussion

Synthesis, characterization, and stability of anionic β-octabromoporphyrin Mn complexes

The preparation of [Mn^{III}Br₈TSPP]^{3−} has been usually accomplished *via* a multi step procedure starting from the commercially available *meso*-tetraphenylporphyrin (H₂TPP). According to the literature reaction scheme, H₂TPP is β-octabrominated (directly or *via* a Cu porphyrin intermediate), followed by phenyl sulfonation to yield [H₂Br₈TSPP]^{4−} ([42,43,45], overall yields not given), which is then Mn-metallated to afford [Mn^{III}Br₈TSPP]^{3−} ([42,43], experimental details and yields not reported). We report here an alternative, shorter procedure using as starting materials the commercially available [H₂TSPP]^{4−} or [Mn^{III}TSPP]^{3−} compounds. If starting from the free-base [H₂TSPP]^{4−}, metallation with Mn affords quantitatively [Mn^{III}TSPP]^{3−}, which is then brominated directly with Br₂ to yield [Mn^{III}Br₈TSPP]^{3−}. The byproduct in this reaction is the brominated free-base [H₂Br₈TSPP]^{4−}, which was characterized spectroscopically [44,45] and can be easily metallated with Mn to increase the yield of the corresponding Mn complex. The direct bromination of Mn porphyrin complexes, although synthetically very convenient, remains relatively unexplored; there are only two reports on this strategy so far and in both occasions the bromination was carried out on neutral porphyrin complexes [34,40]. The synthesis of [Mn^{III}Br₈TSPP]^{3−} described here represents, thus, the first example of a direct bromination of a charged (water-soluble) Mn porphyrin by using a synthetic protocol adapted from that of Richards *et al.* [41], in which the bromination of a cationic Cu porphyrin was described [41]. Of note, the synthesis of the Mn counterpart of the cationic Cu octabromoporphyrin complex required Cu-demetalation, isolation, and purification of the free-base ligand prior to Mn-metallation [36]. The isolated [Mn^{III}Br₈TSPP]^{3−} sample showed consistent elemental analysis and mass spectrometric data. The UV-vis data for this sample were in agreement with those previously reported [42,43], in which the compound was prepared *via* Mn-metallation of the corresponding free-base. Alternatively, acid demetallation of this Mn complex provided further evidence for the β-octabromination as the resulting free-base had identical spectral features to those of an authentic, independently prepared [H₂Br₈TSPP]^{4−} [44,45]. The electrochemical characterization of [Mn^{III}Br₈TSPP]^{3−} is described in the next section.

The derivatization of porphyrins bearing the carboxylato groups, such as [H₂TCPP]^{4−} ([H₂TBAP]^{4−}), is more conveniently achieved *via* the corresponding methyl ester analogues; hydrolysis of the ester moieties restores the carboxylato functionality. This methyl ester-mediated strategy has been recently explored to prepare pure samples of [Mn^{III}TCPP]^{3−} ([Mn^{III}TBAP]^{3−}) [27]. Convenient routes to the preparation of the β-octabrominated methyl ester analogue of [Mn^{III}TCPP]^{3−}, *i.e.*, Mn^{III}Br₈TCMPP⁺, has been described [34,40].

Although the hydrolysis of this methyl ester porphyrin by 2 M NaOH(aq) was claimed to yield the corresponding anionic complex $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ [40], multiple attempts to reproduce this base-hydrolysis were unsuccessful, yielding a complex mixture of decomposition products [50]. Of note, the instability of octabrominated Mn porphyrins in highly basic solutions has been verified (see below). Different routes to the preparation of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ were explored and an acid-hydrolysis procedure emerged as the most convenient alternative. Given the severely acidic conditions (conc. H_2SO_4) required for hydrolysis of the methyl ester moieties of $\text{Mn}^{\text{III}}\text{Br}_8\text{TCMPP}^+$, demetallation of the porphyrin was unavoidable; upon work-up the free-base $[\text{H}_2\text{Br}_8\text{TCPP}]^{4-}$ was obtained, which upon Mn metallation gave the desired Mn complex. The isolated sample showed elemental analysis and mass spectrometric data consistent with the $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ formulation.

The UV-vis data for $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ are very similar to those of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$, which is in agreement with their related electronic and structural properties. Additionally, an indirect characterization evidence for this complex is that its acid demetallation restores the initial free-base, which has spectral data very similar to those of $[\text{H}_2\text{Br}_8\text{TSPP}]^{4-}$ [42,43]. Interestingly, it was noted that the UV-vis spectra of all TCPP derivatives in 0.05 M phosphate buffer (or H_2O) share very similar features to those of the parent methyl ester compound in MeOH (Table 1), as we observed previously with the $[\text{Mn}^{\text{III}}\text{TCPP}]^{3-}$ and MnTCMPP^+ compounds [27]. Such information may prove valuable in assisting the characterization of other carboxylato-containing porphyrins (*e.g.*, the partially brominated ones), as these water-soluble derivatives are consistently more difficult to handle/purify than their corresponding methyl ester analogues. The electrochemical characterization of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ is described in the next section.

The overall chemical stability of the metal complexes are an important factor to be considered, especially when these are to be used in biological systems; some studies on the β -octabromo Mn porphyrins described here were undertaken to address the most usual instability problems of these samples. Türk and Berber [42] have reported that $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ decomposes under basic conditions, which was confirmed in the present work. Based on our several unsuccessful attempts to prepare $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ *via* base-hydrolysis and given its resemblance to $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$, we were prompted to check on the stability of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ to base. We verified that, similarly to $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$, $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ is not stable in basic conditions either. A small test monitored by UV-vis spectroscopy revealed that in 2 M NaOH (described previously [40] as the conditions for base-hydrolysis of $\text{Mn}^{\text{III}}\text{Br}_8\text{TCMPP}^+$), $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ ($\sim 2.3 \mu\text{M}$) is instantaneously reduced to $[\text{Mn}^{\text{II}}\text{Br}_8\text{TCPP}]^{4-}$ and some loss of metal is also observed; within 5 min the species present is mostly the demetallated analogue $[\text{H}_2\text{Br}_8\text{TCPP}]^{4-}$, which undergoes fast decomposition to a complex mixture of products ($t_{1/2} \sim 1 \text{ h}$, at room temperature).

In phosphate buffer (pH 7.8), these β -octabrominated Mn^{III} complexes are stable toward decomposition/demetallation. The UV-vis spectra of mM solutions of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ and $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ at pH 7.8 remained essentially unaltered within one year, which is consistent with the high stability of Mn^{III} porphyrins toward demetallation. Because of the high $\text{Mn}^{\text{III}}/\text{Mn}^{\text{II}}$ reduction potential of these complexes (see next section), and the fact that Mn^{II} porphyrins are notoriously unstable toward hydrolysis [36,51], it was of interest to gather some insights on the Mn^{II} analogues of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ and $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$. The reduction of these Mn^{III} complexes was monitored by UV-vis spectroscopy and carried out using sodium ascorbate (of biological relevance) as reducing agent. To a μM solution of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ in 0.05 M phosphate buffer under aerobic conditions, an excess of ascorbate was added and the spectrum was recorded immediately. A dramatic spectral change was observed, consistent with the fast formation of $[\text{Mn}^{\text{II}}\text{Br}_8\text{TSPP}]^{4-}$. This reduced species, however, was surprisingly extremely unstable and underwent demetallation spontaneously (at

pH 7.8!) to yield the corresponding free-base within 5 min; the spectral changes are illustrated in Fig. 3. An identical reductive demetallation process was observed for $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ (Table 1). Such a process was found of remarkable importance in the experiments with *E. coli* (see below).

This quantitative reduction and demetallation of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ and $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ allowed an estimation of the molar absorptivity of the Soret band of the corresponding Mn^{II} and free-base species. Since the total concentration of the species generated *in situ* is related to the initial total concentration of the Mn^{III} complex prior to the addition of ascorbate (Fig. 3), the ratio between the absorbance of the Soret band of $[\text{Mn}^{\text{II}}\text{Br}_8\text{TSPP}]^{4-}$ (or $[\text{H}_2\text{Br}_8\text{TSPP}]^{4-}$) and that of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ equals the ratio between their corresponding epsilons; given that the ϵ of the isolated and purified Mn^{III} species was determined experimentally, it follows that the molar absorptivity of $[\text{Mn}^{\text{II}}\text{Br}_8\text{TSPP}]^{4-}$ and $[\text{H}_2\text{Br}_8\text{TSPP}]^{4-}$ may be roughly estimated. Of note, our estimated ϵ value for $[\text{H}_2\text{Br}_8\text{TSPP}]^{4-}$ ($\log \epsilon = 5.16$) in phosphate buffer (pH 7.8) is very close to that reported for an authentic sample of $[\text{H}_2\text{Br}_8\text{TSPP}]^{4-}$ ($\log \epsilon = 5.30$ [44,45]) in H_2O (pH 7.0). An identical approach was applied to the $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ derivatives and all the values are summarized in Table 1.

SOD activity and $\text{Mn}^{\text{III}}/\text{Mn}^{\text{II}}$ reduction potential of the anionic Mn porphyrins

In order to evaluate the impact of electrostatics on the redox-modulating abilities of Mn porphyrins, we studied a group of pentacationic ($\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+}$) and trianionic Mn^{III} porphyrin complexes ($[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ and $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$) possessing virtually the same $\text{Mn}^{\text{III}}/\text{Mn}^{\text{II}}$ reduction potential (Table 2). The reduction potential of Cu,Zn-SOD [28–30] and that of the non-brominated analogues of the anionic porphyrins ($[\text{Mn}^{\text{III}}\text{TSPP}]^{3-}$ and $[\text{Mn}^{\text{III}}\text{TCPP}]^{3-}$) [1,27] were included here for comparison. The $\text{Mn}^{\text{III}}/\text{Mn}^{\text{II}}$ reduction potential of the $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ and $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ complexes are 369 and 407 mV, respectively, more positive than that of the non-brominated analogues, which corresponds to a shift of 46 and 51 mV/Br, respectively, upon bromination and is in agreement with closely related systems [5,34–36].

The reduction potential within this series falls into two classes: those of *ca.* +200 mV ($\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+}$, $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$, $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$) and those of *ca.* –200 mV *vs* NHE ($[\text{Mn}^{\text{III}}\text{TSPP}]^{3-}$, $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$). Conversely, the SOD activity ($\log k_{\text{cat}}$ and IC_{50}) of the compounds within this series varies greatly in the following order: $\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+} \gg [\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-} \sim [\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-} \gg [\text{Mn}^{\text{III}}\text{TSPP}]^{3-} > [\text{Mn}^{\text{III}}\text{TCPP}]^{3-}$ (Table 1).

Although the $\text{Mn}^{\text{III}}/\text{Mn}^{\text{II}}$ reduction potential of the anionic β -brominated complexes are close to the optimum and nearly identical to that of the cationic $\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+}$ compound, the SOD activity of both $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ and $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ is approximately 300-fold lower than that of $\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+}$ (Table 2). This is consistent with an unfavorable electrostatic facilitation in the anionic porphyrins, whose negative charges behave as a barrier that hampers the approach of the incoming superoxide anion. The influence of electrostatics on these systems was further confirmed by measurement of kinetic salt effects for the $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ complex, which showed an increase in the rate of superoxide dismutation upon increase of the ionic strength (in the presence of 0.1 M NaCl, $\log k_{\text{cat}} = 5.70$).

Revised structure-activity relationships: Implications to the design of Mn-porphyrin-based redox mediators

The dismutation of superoxide, catalyzed by Mn porphyrins, occurs *via* a redox mechanism, where the Mn complex cycles between the +3 and +2 states, as represented by equation (1) and equation (2) [26]. The Mn^{III} porphyrin is initially reduced (in the rate-determining step)

by $O_2^{\bullet-}$ to yield O_2 , and the resulting Mn^{II} complex is then rapidly re-oxidized to the initial Mn^{III} species by $O_2^{\bullet-}$ to yield H_2O_2 . It follows, therefore,

that the catalytic rate must depend upon the Mn^{III}/Mn^{II} reduction potential, which has been confirmed experimentally [1–5,25–27,36]. Other parameters that affects the dismutation rate constants by modulating the metal reduction potential and/or the access of $O_2^{\bullet-}$ to the active site, include the spatial charge distribution [5,40], the stericity/lipophilicity around the metal center [2,4] and electrostatics [5]. Among these general features, which may certainly be of interest to the design of related metal-based mimics, such as Fe porphyrins, Fe/Mn corroles, expanded metalloporphyrins, salens, and cyclic polyamines, the impact of electrostatics remains the least explored [5] and not fully appreciated. This is not the case, however, for the SOD enzymatic systems [52,53], where the critical role of electrostatics has been thoroughly investigated. These studies have demonstrated that the fast, diffusion controlled reactivity of the SODs is greatly dependent on the electrostatic facilitation that cationic amino acid residues close to the catalytic site exert on channeling the superoxide anion to the enzyme active site. In the biomimetic systems, such facilitation is achieved by designing cationic porphyrin ligands in which the positive charges are distributed unsymmetrically either below or above the plane (an extension of the so-called “*ortho*-effect” [39]) to channel the negatively-charged superoxide toward the axial positions of the Mn porphyrin more efficiently [25]. There has been so far only one example demonstrating the impact of electrostatic effects on the design of SOD mimics [5], in which we showed that the 100-fold difference in SOD activity between a pentacationic and a monocationic Mn porphyrin of same $E_{1/2}$ is a result of a diminished electrostatic guidance in the mono-cationic analogue. In the present study, the data for $[Mn^{III}Br_8TCPP]^{3-}$, $[Mn^{III}Br_8TSP]^{3-}$, and $Mn^{III}TM-2-PyP^{5+}$ provide further evidence for such an effect.

These results imply that the structure-activity relationships attempting to describe the SOD activity of Mn porphyrins must account not only for the dependence of the rate constant on the Mn^{III}/Mn^{II} reduction potential [1], but also on the electrostatic channeling of superoxide, which is governed by the charges on the macrocycle periphery. Thus, a reevaluation of the available SOD activity and $E_{1/2}$ data for the most common series of Mn porphyrins, namely the $Mn^{III}P^{5+}/Mn^{II}P^{4+}$, $Mn^{III}P^+/Mn^{II}P$, and $[Mn^{III}P]^{3-}/[Mn^{II}P]^{4-}$ complexes, was carried out and is illustrated in Fig. 4. The relative position of the curves for each of these series indicates that, at any given Mn^{III}/Mn^{II} reduction potential, the SOD activity increases as the overall charge of the compound becomes more positive (or less negative), which is a direct consequence of the impact of electrostatics effects on the dismutation. In each of the series the $\log k_{cat}$ vs $E_{1/2}$ relationship show a bell-shaped behavior. The curvature observed in these plots is consistent with our recent work [25] on a series of minimum stericity demand and constant overall charge and suggests the approach of a change in the rate-limiting step from the Mn^{III} reduction reaction (eq. 1) to the Mn^{II} oxidation reaction (eq. 2). This is reminiscent of the behavior described by the Marcus theory [4], *i.e.* at higher reduction potentials the stabilization of Mn^{II} porphyrins is increased and the Mn^{II}/Mn^{III} oxidation (eq. 2) may become rate-limiting. Notably, the range of $E_{1/2}$ covered within the anionic Mn porphyrin series (from –200 to +200 mV) has been large enough to reach a well-defined maximum in the SOD activity at *ca.* +100 mV. It is worth noting that this optimum SOD activity for the $[Mn^{III}P]^{3-}/[Mn^{II}P]^{4-}$ series is relatively low (being easily achieved by even the most simple and un-optimized compounds of the $Mn^{III}P^{5+}/Mn^{II}P^{4+}$ series) and corresponds roughly to that of common Mn^{2+} salts, such as $MnCl_2$. These results show that the design of effective SOD mimics (*per se*) based on *anionic* porphyrins is

greatly hindered by inappropriate electrostatics and, furthermore, that the complexation of Mn by anionic porphyrins is essentially detrimental to the SOD activity of free Mn^{2+} ion.

Cell studies: Protection of SOD-deficient *E. coli*

In order to examine whether a compound that shows SOD activity *in vitro* is able to substitute for the SOD enzymes in biological systems, we test its ability to restore the aerobic growth of the SOD-deficient *E. coli* in a minimal medium. The anionic porphyrin complexes and the potent cationic SOD-mimic $\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+}$ were added to final concentrations of 1, 10, and 20 μM , and their effects were assessed by following the densities of the cell suspensions at definite time intervals. Given the easy demetallation of the β -octabromo derivatives upon reduction (Fig. 3), MnCl_2 (1 and 10 μM) was tested as a source of “free” Mn^{2+} . The growth of the SOD-proficient, wild type *E. coli* was followed in parallel under identical conditions to that of the SOD-deficient strain and used as control.

For simplicity, the relative densities of the cell suspensions at a fixed time point (24th hour) are presented on Fig. 5. In the absence of an SOD mimic, the SOD-deficient mutant did not grow. MnTM-2-PyP^{5+} at 10 and 20 μM restored its growth almost to the level of the wild type strain, which is consistent with our previous reports [25,39].

As anticipated from the physico-chemical properties of these compounds (Table 2), $[\text{Mn}^{\text{III}}\text{TCPP}]^{3-}$ (which is not an SOD mimic in aqueous systems [27]) and $[\text{Mn}^{\text{III}}\text{TSPP}]^{3-}$ (which has considerably low $\log k_{\text{cat}}$ value) do not protect SOD-deficient *E. coli* grown aerobically in 5AA restricted medium. The modest protection of SOD-deficient *E. coli* observed with $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ is in agreement with the low SOD activity ($\log k_{\text{cat}} = 5.07$) measured for this compound (Table 2). Conversely, $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ ($\log k_{\text{cat}} = 5.56$), which has thermodynamic and kinetic properties (including k_{cat}) similar to those of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$, showed good to excellent protection efficiency at 1 and 10 μM ; at 1 μM $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ is significantly better than MnTM-2-PyP^{5+} , but at 20 μM the efficiency of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ dropped (compared to the 10 μM dose), which suggests that it might become toxic at high concentrations. Of note, no toxicity (at 30 μM) of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TCPP}]^{3-}$ and $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ was observed on wild type *E. coli* experiments.

The striking ability of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ to substitute for the SOD enzymes in the *E. coli* model does not correlate with its $\log k_{\text{cat}}$. In fact, the protectiveness of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ is comparable to or better than that of the potent SOD mimic $\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+}$, even though the dismutation rate constant of anionic complex is 170-fold smaller (Fig. 5). Such an unexpected discrepancy between the physico-chemical and the *E. coli* data for $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ prompted us to investigate this system in greater detail.

$[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ as an efficient Mn-carrier: uptake into *E. coli* and reductive demetallation

As a first working hypothesis, we supposed that the ability of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ to sustain the aerobic growth of the SOD-deficient mutant could be due to a better uptake of this porphyrin, which would, thus, compensate for its modest $\log k_{\text{cat}}$. The *E. coli* pellet recovered at the end of the experiment with $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ was highly colored (as opposed to that recovered from the $\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+}$ experiments) indicating qualitatively that the uptake of the anionic porphyrin in to *E. coli* is greater than that of the cationic one. Remarkably, the UV-vis spectra of the medium and the *E. coli* cell extract from the $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ experiment (Fig. 6) indicate that the major species in the system is no longer $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$, but the free-base porphyrin $[\text{H}_2\text{Br}_8\text{TSPP}]^{4-}$ instead. This is in direct contrast with the $\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+}$ systems [24,39] and imply that a reductive demetallation of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ is taking place *in situ*. Thus, the Soret band of $\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+}$ and $[\text{Mn}^{\text{III}}\text{TSPP}]^{3-}$ appear at 452 and 466 nm, respectively, indicating these Mn porphyrins are not demetallated within the cell (data not

shown). It is worthwhile noting that a control experiment in 5AA medium in the absence of *E. coli*, showed no appreciable demetallation of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$, which shows that the demetallation is an *E. coli*-mediated process. In fact, $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ was found stable toward demetallation for at least one year in phosphate buffer and for at least for 2 days in the presence of 100-fold excess EDTA. Its reduced form $[\text{Mn}^{\text{II}}\text{Br}_8\text{TSPP}]^{4-}$, however, loses metal almost immediately in phosphate buffer (no EDTA needed) at pH 7.8 (Fig. 3).

That the protective effect of the $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ sample does not arise from “free” Mn^{2+} (if released directly in the medium) was demonstrated by control experiments with MnCl_2 (Fig. 5). Whereas $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ rescues the growth of SOD-deficient *E. coli* by 61 and 78 % (relative to the wild type strain) at 1 and 10 μM , respectively, *E. coli* growth does not even reach 15% if MnCl_2 is substituted for $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ at identical doses.

The high protectiveness and substantial uptake of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ by SOD-deficient *E. coli* associated with its modest $\log k_{\text{cat}}$ value and *in situ* reductive demetallation strongly suggest that this complex operates not as a redox modulator itself, but as a Mn-carrier. Once internalized by *E. coli*, the speciation of Mn^{2+} remains unknown. Although “free” Mn^{2+} ion is able to dismutate superoxide, it is most likely that this metal cation is bound to some cellular ligand/component, which may, in turn, exert the SOD mimicking activity required to protect the SOD-deficient *E. coli* from O_2 toxicity. Significant SOD-like activity of non-porphyrin Mn complexes containing simple carboxylic acids or ionophores as ancillary ligands or oxo/hydroxo Mn clusters has been reported [27,54–56]. The true nature of the intracellular ligands of Mn in this system remains, however, unknown.

Figure 7 illustrates some of the possible mechanisms by which $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ may mediate the Mn delivery to *E. coli*. As the key point in the process is the reduction of Mn^{III} to Mn^{II} , and this requires some cellular reductant (this reductive demetallation of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ is *E. coli*-dependent), one may envision that the reduction may take place in the cytoplasm (Process A), in the cellular membrane (Process B), or in the medium (Process C).

In Process A, $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ is uptaken and delivered to the cytoplasm, where reduction and concomitant demetallation take place; exporting of the ligand $[\text{H}_2\text{Br}_8\text{TSPP}]^{4-}$ to the extracellular medium needs to be considered to explain the high concentration of free-base porphyrin found in the medium. In Process B, the reduction of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ occurs in the membrane and the resulting Mn^{II} complex is translocated into the cytoplasm, where it is demetallated; again exporting of the free-base $[\text{H}_2\text{Br}_8\text{TSPP}]^{4-}$ to the extracellular medium is evoked. In Process C, *E. coli* would excrete into the medium compounds of reducing ability that could bring about the reduction and demetallation of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ extracellularly. Whereas these three routes are not mutually exclusive and may indeed be happening simultaneously at some extent, Process C is the least likely to explain the high protection of SOD-deficient *E. coli*, given that in the best case scenario (total reductive demetallation of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$) the levels of Mn^{2+} would be similar to those of the control experiments with MnCl_2 , which is not protective.

It is worth noting that the use of Mn porphyrins as Mn-carriers is, however, neither straightforward nor easily achievable by employing familiar complexes. The ability of the Mn porphyrin to release Mn *in situ* requires a delicate balance of the kinetics and thermodynamics of the metallation/demetallation reactions, which is only achievable by a controlled tuning of the porphyrin-ring electronics and deformation [51]. Mn^{III} porphyrins (similarly to Fe^{III} heme) are thermodynamically and kinetically stable toward demetallation, but this reaction becomes more favored with the more kinetically and thermodynamically unstable 2+ oxidation state [51]. Such a reduction is achievable by introducing electron-withdrawing groups (*e.g.*, halogens) onto the macrocycle, which increases the $\text{Metal}^{\text{III}}/\text{Metal}^{\text{II}}$ reduction potential to

values compatible with common cellular reductants [5,34–36,57]. The metallation/demetallation reactions in these systems are also very much dependent on the deformability of the porphyrin ligand [51]. The presence of bulky substituents, such as Br, in the β -pyrrole positions brings about a large deviation from planarity, which increases dramatically the metallation/demetallation rate constants, as the macrocycle ruffling resembles the metallation/demetallation transition state (*i.e.*, an entatic condition) [51]. The critical importance of steric tuning in these systems has also been conveniently explored by Nature in the metallation of protoporphyrin IX *in vivo* by the ferrochelatase enzymes to yield the heme group [58]. Of note, similarly to the common non- β -substituted porphyrins, demetallation of heme is neither electronically (low reduction potential) nor sterically (high planarity) favored and metal release requires usually porphyrin destruction by catabolic pathways. Likewise, the planar complexes $[\text{Mn}^{\text{III}}\text{TCPP}]^{3-}$ and $[\text{Mn}^{\text{III}}\text{TSPP}]^{3-}$ share unfavorable reduction potential and low deformability; they are neither good SOD mimics nor efficient Mn carriers. Conversely, $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ and $\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+}$ are readily reduced by common cellular reductants, such as ascorbate, but only $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ is an efficient Mn donor. The deformation of the $[\text{H}_2\text{Br}_8\text{TSPP}]^{4-}$ ligand, due to the β -octabromination, results in ready metallation and demetallation processes, on which the fast forward/backward equilibria favor, markedly, the demetallated species ($[\text{H}_2\text{Br}_8\text{TSPP}]^{4-}$ and “free” Mn^{2+}) under micromolar or higher dilution conditions. The high planarity and low deformability of $\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+}$ [59] stabilizes the Mn porphyrin, favoring redox cycling as opposed to Mn loss; no spectroscopic evidence of $\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+}$ demetallation under relevant biological conditions has been described so far. We believe therefore, that, although $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ and $\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+}$ are both exceptionally good at providing protection to SOD-deficient *E. coli*, they may operate by remarkably different mechanisms: $\text{Mn}^{\text{III}}\text{TM-2-PyP}^{5+}$ as an SOD mimic in its own right, and $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ as a Mn carrier.

Concluding remarks

The concepts guiding the development of the Mn porphyrin complexes as superoxide/peroxynitrite scavengers and redox modulators may be applied to the design of related metal-based therapeutic systems, such as Fe porphyrins, Fe/Mn corroles, and expanded metalloporphyrins.

The revised structure-activity relationships show the critical importance of electrostatics in these Mn porphyrin-based redox modulation systems. The impact of electrostatic guidance of $\text{O}_2^{\bullet -}$ toward the metal active site is a common feature shared between the Mn porphyrin and the enzymatic systems. The design of effective SOD mimics based on *anionic* porphyrins is greatly hindered by inappropriate electrostatics. Furthermore, once the “free” Mn^{2+} is complexed to anionic porphyrin, the SOD activity of the resulting Mn porphyrin is lower than that of “free” Mn^{2+} itself.

Although *anionic* porphyrins are of limited SOD activity, if the Mn porphyrin stability (*i.e.*, non-planar porphyrins of high $\text{Mn}^{\text{III}}/\text{Mn}^{\text{II}}$ reduction potential), type of porphyrin substituents, and the cellular uptake and distribution are properly tuned, *in vivo* redox modulation may be achieved *via* an alternative mechanism, in which the Mn porphyrin acts not as an SOD mimic *per se*, but as a suitable Mn-carrier.

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Abbreviations

[Mn^{III}Br₈TCPP]³⁻, Mn(III) β-octabromo-*meso*-tetrakis(4-carboxylatophenyl)porphyrin (also [Mn^{III}Br₈TBAP]³⁻)
 [Mn^{III}Br₈TSPP]³⁻, Mn(III) β-octabromo-*meso*-tetrakis(4-sulfonatophenyl)porphyrin
 [Mn^{III}TC₂SPP]³⁻, Mn(III) *meso*-tetrakis(2,6-dichloro-3-sulfonatophenyl)porphyrin
 [Mn^{III}TCPP]³⁻, Mn(III) *meso*-tetrakis(4-carboxyphenyl)porphyrin (also [Mn^{III}TBAP]³⁻)
 [Mn^{III}TF₂SPP]³⁻, Mn(III) *meso*-tetrakis(2,6-difluoro-3-sulfonatophenyl)porphyrin
 [Mn^{III}TSPP]³⁻, Mn(III) *meso*-tetrakis(4-sulfonatophenyl)porphyrin
 5AA medium, five amino acid medium
 cyt, cytochrome
 E_{1/2}, half wave potential
meso, means 5, 10, 15, 20 positions of the porphyrin ring
 Mn^{III}Br₈TCMPP⁺, Mn(III) β-octabromo-*meso*-tetrakis(4-carbomethoxyphenyl)porphyrin
 Mn^{III}TE-2-PyP⁵⁺, Mn(III) *meso*-tetrakis(*N*-ethylpyridinium-2-yl)porphyrin (AEOL10113)
 Mn^{III}TM-2-PyP⁵⁺, Mn(III) *meso*-tetrakis(*N*-methylpyridinium-2-yl)porphyrin (AEOL10112)
 Mn^{III}TnHex-2-PyP⁵⁺, Mn(III) *meso*-tetrakis(*N*-hexylpyridinium-2-yl)porphyrin
 MnP, Mn porphyrin
 ONOOH, peroxyntirite includes both ONOO⁻ and its protonated analogue
 SOD, superoxide dismutase
 SOD-def., SOD-deficient, *sodA*⁻*sodB*⁻ *E. coli* strain (JI132)
 WT, wild type, SOD-proficient *Escherichia coli* strain (AB1157)
 X, xanthine
 XO, xanthine oxidase
 β, means 2,3,7,8,12,13,17, 18 pyrrole positions of the porphyrin ring

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Figure 1.
 Structural diagram of the Mn porphyrins relevant to this work.

Figure 2.

Design of SOD mimics based on thermodynamic grounds. Reduction potential of selected Mn porphyrins ($\text{Mn}^{\text{III}}/\text{Mn}^{\text{II}}$ couple), the SOD enzymes, and relevant species for superoxide dismutation half reactions. Good Mn porphyrin-based SOD mimics have $\text{Mn}^{\text{III}}/\text{Mn}^{\text{II}}$ reduction potential around the midway potential for the reduction and oxidation of $\text{O}_2^{\bullet-}$, and close to those of SOD enzymes.

Figure 3.

Instability of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ upon reduction. *In situ* reductive demetallation of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ by sodium ascorbate in 0.05 M phosphate buffer (pH 7.8) as followed by UV-vis spectroscopy: ●, $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ (no ascorbate added); ○, $[\text{Mn}^{\text{II}}\text{Br}_8\text{TSPP}]^{4-}$ (immediately upon ascorbate addition); ▼, $[\text{H}_2\text{Br}_8\text{TSPP}]^{4-}$ (~4 min after ascorbate addition; spectrum coincides with that of an authentic $[\text{H}_2\text{Br}_8\text{TSPP}]^{4-}$ sample).

Figure 4.

Revised structure-activity relationships between $\text{Mn}^{\text{III}}/\text{Mn}^{\text{II}}$ reduction potential and SOD dismutation ability of Mn porphyrins accounting for electrostatic facilitation. Data for Mn porphyrin systems are from this or previous [1–5,25–27,36,39] works: (●) $[\text{Mn}^{\text{III}}\text{P}]^{3-}/[\text{Mn}^{\text{II}}\text{P}]^{4-}$ systems; (○) $\text{Mn}^{\text{III}}\text{P}^+/\text{Mn}^{\text{II}}\text{P}$ systems; (▲) $\text{Mn}^{\text{III}}\text{P}^{5+}/\text{Mn}^{\text{II}}\text{P}^{4+}$ systems. Data for MnCl_2 [26], MnSalen^+ [26], M40403 [48], and SOD [28–30] are given for comparison. The relative position of the curves for each of these series indicates that, at any given $\text{Mn}^{\text{III}}/\text{Mn}^{\text{II}}$ reduction potential, the SOD activity increases as the overall charge of the compound becomes more positive (or less negative), which is a direct consequence of the impact of electrostatics effects on the dismutation. In each of the series the $\log k_{\text{cat}}$ vs $E_{1/2}$ relationship show a bell-shaped behavior.

Figure 5.

Effects of Mn porphyrins or MnCl_2 (at 1, 10, or 20 μM) on the aerobic growth of SOD-deficient *E. coli* JI132 strain in 5AA medium. Aerobic growth of the wild type *E. coli* AB1157 was used as a control. Experiments were carried out in flasks in quadruplicate and data of the 24th hour of growth is presented. At 1mM, $[\text{Mn}^{\text{III}} \text{Br}_8 \text{TSPP}]^{3-}$ is the most potent among the porphyrins studied, including the standard potent drug MnTM-2-PyP^{5+} .

Figure 6.

Instability of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ upon exposure to *E. coli*. UV-vis spectra of the medium (▼) and the *E. coli* cell extract (○) from experiment of the aerobic growth of SOD-deficient *E. coli* JI132 strain in 5AA medium in the presence of the $20\ \mu\text{M}$ $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$. $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ spectrum (●) is given for comparison. The UV-vis spectra of the medium and *E. coli* cell extract from the $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ experiment indicate that the major species in the system is no longer $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$, but the free-base porphyrin $[\text{H}_2\text{Br}_8\text{TSPP}]^{4-}$ instead.

Figure 7.

Alternative mechanism explains the remarkable efficacy of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ in protecting SOD-deficient *E. coli* through carrying Mn into the cell. Two possible processes A and B are in agreement with our data. In Process A, $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ is uptaken and delivered to the cytoplasm, where reduction and concomitant demetallation take place; exporting of ligand $[\text{H}_2\text{Br}_8\text{TSPP}]^{4-}$ to the extracellular medium needs to be considered to explain the high concentration of free-base porphyrin found in the medium. In Process B, the reduction of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ occurs in the membrane and the resulting Mn^{II} complex is translocated into the cytoplasm, where it is demetallated; again exporting of the free-base $[\text{H}_2\text{Br}_8\text{TSPP}]^{4-}$ to the extracellular medium is evoked. In Process C, *E. coli* would excrete into the medium compounds of reducing ability that could bring about the reduction and demetallation of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$ extracellularly. Whereas these three routes are not mutually exclusive and may indeed be happening simultaneously at some extent, Process C is the least likely to explain the high protection of SOD-deficient *E. coli*, given that in the best case scenario (total reductive demetallation of $[\text{Mn}^{\text{III}}\text{Br}_8\text{TSPP}]^{3-}$) the levels of Mn^{2+} would be similar to those of the control experiments with MnCl_2 , which is not protective.

Table 1

Selected UV-vis data for some sulfonato, carboxylato and methyl ester porphyrins and Mn porphyrins

Compound	Solvent	Soret, $\lambda_{\text{max}}/\text{nm}$	Visible bands, $\lambda_{\text{max}}/\text{nm}$	Ref.
[Mn ^{III} TSPP] ³⁻	Buffer ^a	467 (5.01) ^b	513, 563, 597	This work
[Mn ^{III} TCPP] ³⁻	Buffer ^a	468 (5.04) ^b	515, 566, 599	[43]
Mn ^{III} TCMPP ⁺	MeOH	466 (5.11) ^b	515, 563, 596	[43]
[Mn ^{III} Br ₈ TSPP] ³⁻	Buffer ^a	491 (5.01) ^b	592, 634	This work
[Mn ^{III} Br ₈ TCPP] ³⁻	Buffer ^a	493 (5.10) ^b	593, 635	This work
Mn ^{III} Br ₈ TCMPP ⁺	MeOH	495	592, 634	This work
[Mn ^{II} Br ₈ TSPP] ^{4- c}	Buffer ^a	469 (5.16) ^{b,e}	576, 624, 723	This work
[Mn ^{II} Br ₈ TCPP] ^{4- c}	Buffer ^a	470 (5.28) ^{b,e}	581, 625, 728	This work
Mn ^{II} Br ₈ TCMPP ^d	MeOH	474	584, 629, 734	This work
[H ₂ Br ₈ TSPP] ⁴⁻	H ₂ O ^f	478 (5.30) ^b	657, 760	[63,64]
	Buffer ^a	471 (5.16) ^{b,e}	654, 749	This work
[H ₂ Br ₈ TCPP] ⁴⁻	Buffer ^a	477 (5.24) ^{b,e}	660, 760	This work
H ₂ Br ₈ TCMPP	MeOH	476	668, 768	This work

^a 0.05 M phosphate buffer (pH 7.8).^b log $\epsilon/\text{cm}^{-1}\text{M}^{-1}$.^c Prepared *in situ* via reduction of the Mn^{III} complex with ascorbate.^d Prepared *in situ* via reduction of Mn^{III}Br₈TCMPP⁺ with zinc amalgam.^e Estimated value (see text).^f pH 7.0.

Table 2

Metal-centered reduction potential ($E_{1/2}$) and $O_2^{\bullet-}$ scavenging properties of Mn porphyrins and Cu,Zn-SOD in 0.05 M phosphate buffer (pH 7.8).

Compound	$E_{1/2}$ /mV vs NHE ^a	Log k_{cat} ^b	IC ₅₀ /nM ^c	Ref.
[Mn ^{III} TCPP] ³⁻	-194	< 3.5	> 822,190	[44]
[Mn ^{III} TSPP] ³⁻	-160	3.95	291,725	this work, [1]
[Mn ^{III} Br ₈ TCPP] ³⁻	+213	5.07 ^d	22,130	this work
[Mn ^{III} Br ₈ TSPP] ³⁻	+209	5.56	7,160	this work
Mn ^{III} TM-2-PyP ⁵⁺	+220	7.79	43	[1]
Cu,Zn-SOD	ca. +300	ca. 9	ca. 1.3	[44–46]

^a $E_{1/2}$ (± 3 mV) vs NHE determined in 0.1 M NaCl, 0.05 M phosphate buffer (pH 7.8).

^b k_{cat} /M⁻¹ s⁻¹ ($\pm 20\%$) determined by cyt *c* assay.

^c IC₅₀ is the concentration of compound that causes a 50% inhibition of cyt *c* reduction by $O_2^{\bullet-}$.

^d In the presence of catalase. At concentrations of [Mn^{III}Br₈TCPP]³⁻ greater than 10 μ M, there is a significant effect of H₂O₂, which results in an apparent log k_{cat} of ~5.13 in the absence of catalase.