Published in final edited form as:

Biochim Biophys Acta. 2015 September ; 1850(9): 1795–1805. doi:10.1016/j.bbagen.2015.05.006.

Iron incorporation into MnSOD A (bacterial Mn-dependent superoxide dismutase) leads to the formation of a peroxidase/catalase implicated in oxidative damage to bacteria

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Abstract

BACKGROUND—Mn/Fe-superoxide dismutase (SOD) is a family of enzymes essential for organisms to be able to cope with oxygen. These enzymes bound to their classical metals catalyze the dismutation of the free radical superoxide anion ($O_2^{\bullet-}$) to H_2O_2 and molecular oxygen. *E. coli* has the manganese-dependent SOD A and the iron-dependent SOD B.

METHODS—Strains of *E. coli* overexpressing SOD A or SOD B were grown in media with different metal compositions. SODs were purified and their metal content and SOD activity were determined. Those proteins were incubated with H_2O_2 and assayed for oxidation of Amplex red or o-phenylenediamine, consumption of H_2O_2 , release of iron and protein radical formation. Cell survival was determined in bacteria with MnSOD A or FeSOD A after being challenged with H_2O_2 .

RESULTS—We show for the first time that the bacterial manganese-dependent SOD A when bound to iron (FeSOD A) has peroxidase activity. The *in vivo* formation of the peroxidase FeSOD A was increased when media had higher levels of iron because of a decreased manganese metal incorporation. In comparison to bacteria with MnSOD A, cells with FeSOD A had a higher loss of viability when exposed to H_2O_2 .

GENERAL SIGNIFICANCE—The biological occurrence of this fundamental antioxidant enzyme in an alternative iron-dependent state represents an important source of free radical formation.

Graphical abstract

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Keywords

superoxide dismutase (SOD); peroxidase; free radicals; metals; iron; manganese

INTRODUCTION

Escherichia coli, a gram-negative and facultative anaerobic bacteria, has two distinct superoxide dismutases (SODs) in the Mn-Fe SOD family [1–3]. SOD A is manganese-dependent [1], and SOD B is iron-dependent [2].

SOD A was diverted from a duplication of the ancient SOD B gene in the ancestral bacterial genome [4]. The acquisition of a manganese-dependent enzyme parallels an increase in the bioavailability of oxygen and manganese in the biosphere [4]. In animals, the SOD2 of eukaryotes is a manganese-dependent superoxide dismutase and is highly homologous with the ancestral SOD A of bacteria [5]. The presence of FeSODs in the stroma of chloroplasts of some plants and SOD2 in the matrix of mitochondria was a major factor suggesting the endosymbiotic theory for the origin of these organelles [6].

The active sites of SOD A and SOD B are superimposable, which indicates that both metals could bind to any Fe-Mn SOD *in vitro* and *in vivo* [7, 8]; however, the superoxide dismutase activity is lost with cross-metallation, i.e., SOD B incorporated with manganese or SOD A incorporated with iron [8]. Since its discovery, the metal specificity for the superoxide dismutase activity of the Mn-Fe SODs has intrigued scientists [2, 7–10]. These enzymes with their correct metallation have a tuned redox potential for the dismutation reaction of $O_2^{\bullet-}$ (superoxide anion) of between +200 and +400 mV [10].

These SODs have different physiological roles: SOD B is constitutively expressed in *E. coli*, while SOD A is the inducible enzyme [11, 12]. In fact, SOD A is part of the SoxRS regulon, which is induced by many oxidative stress-related stimuli such as paraquat and H_2O_2 [12].

The biological acquisition of Mn-Fe SODs is believed to be the most important factor for the existence of aerobic life, since the deletion of the Mn-Fe SODs from the genomes of bacteria through higher animals leads to an unavoidable incompatibility with aerobic life [13–17]. This incompatibility indicates that the dismutation of $O_2^{\bullet-}$ to H_2O_2 and molecular oxygen is the key step in the protection of organisms against oxygen toxicity [13–17].

Human SOD2 is known to be associated with cancer development. Higher levels of this protein are correlated with and detected at advanced stages of many types of cancers [18, 19]. Overexpression of SOD2 in mammalian cells leads to higher production of mitochondrial H_2O_2 [20]. This fact has been explained as a higher efficiency in the $O_2^{\bullet-}$ dismutation by increased levels of MnSOD2 [21]; however, thermodynamics does not support this interpretation [22]. In an attempt to explain these observations, a peroxidase activity of human SOD2 was hypothesized [23]. In support of this proposal, SOD2 reacted with H_2O_2 , oxidizing Amplex red to resorufin, oxidizing hydroxylamine to a nitroxide, and oxidizing itself to a free radical, which ultimately revealed a potential prooxidant activity for SOD2 [23]. Nevertheless, skepticism was expressed regarding those findings [24].

Here we explore the enzymatic mechanism of the peroxidase activity of Mn-Fe SODs using bacterial SOD A and SOD B. We also study the effect of the metal composition of the media in the metallation and final activities of these enzymes.

MATERIAL AND METHODS

Luria broth and other special media

Escherichia coli (Rosetta™ 2 DE3 pLacI competent cells) was routinely grown in Luria-Bertani broth supplied by the Media and Glassware Facility of NIEHS/NIH. The media (pH 7.5, adjusted with NaOH) contains per liter: 10 g bacto-tryptone (Difco™, BD Diagnostics, Sparks, MD), 5 g bacto-yeast extract (Difco™, BD Diagnostics, Sparks, MD) and 10 g NaCl (Sigma Aldrich, St. Louis, MO). The media was sterilized by autoclaving and supplemented with ampicillin (100 µg/mL) under aseptic conditions.

Expression and purification of SODs

SOD A and SOD B genes were recombined from pDONR 221 into a pDEST 527 expression plasmid (destination vector kindly supplied by Dr. Dominic Esposito, SAIC, MD). The vectors were transformed by heat shock into Rosetta 2 DE3 pLacI competent cells. Ampicillin- and chloramphenicol-resistant colonies were grown in LB with the selecting agents. Metallation of SOD A and B overexpressed *in vivo* was studied by supplementing LB with $MnCl_2$ in cultures of the respective bacteria at OD 0.05. After reaching OD 0.6–0.8 (2h, 220 rpm at 37°C), protein expression was induced in cultures by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and cultures were then incubated for 4h at RT (220 rpm).

SOD A and B were purified using HisPur Cobalt Purification kits from Pierce Biotechnology (Thermo Scientific, Rockford, IL) following the supplier's instructions (for more details refer to supplemental materials).

Purified proteins were extensively buffer-exchanged with Chelex-treated 10 mM phosphate buffer, pH 7.4, using ultrafiltration devices (Amicon series, Millipore, Billerica, MA), followed by desalting against the same buffer using Zeba Spin Desalting Columns (Pierce Biotechnology, Thermo Scientific, Rockford, IL).

Unless specified in the text, all samples for the metal analyses, Amplex red oxidation and protein radical formation were prepared in Chelex-treated 100 mM phosphate buffer, pH 7.4, with 25 μ M diethylenetriaminepentaacetic acid (DTPA). 5,5'-dimethyl 1-pyrroline *N*-oxide (DMPO) was from Dojindo (Rockville, MD). Chemicals were of the highest purity grade available and, unless otherwise stated, were supplied by Sigma Aldrich (St. Louis, MO).

Metal analyses

Samples were analyzed for metals using inductively coupled plasma with optical emission spectrometry (ICP-OES) in a Thermo Jarrell-Ash Enviro 36 Inductively Coupled Argon Plasm by the Chemical Analysis Laboratory (CAIS) in the Center for Applied Isotope Studies at the University of Georgia (Athens, GA).

Dismutase and peroxidase activities of SODs

The specific dismutase activity of SOD was measured by the inhibition of cytochrome *c* reduction by a constant flux of superoxide radicals generated by xanthine oxidase in the presence of xanthine [25].

Total cellular homogenates were prepared with native extraction buffer [25 mM Tris buffer, pH 7.4 and 0.1% Triton X-100 with added fresh protease inhibitor cocktail (Roche, Indianapolis, IN)]. The cell pellet with the extraction buffer was sonicated on ice for 1 min, followed by centrifugation at 12,000 g for 10 min at 4°C. The supernatant was collected and the protein concentration was determined using a BCA protein assay kit from Pierce Biotechnology (Thermo Scientific, Rockford, IL).

The peroxidase activity of SOD was assessed by resorufin formation ($\epsilon_{571\text{nm}} = 54.0 \text{ mM}^{-1}\text{cm}^{-1}$) from 100 μ M Amplex red (Molecular Probes®, LifeTechnologies, Grand Island, NY) in the presence of excess H_2O_2 ($\epsilon_{240\text{nm}} = 0.0436 \text{ mM}^{-1}\text{cm}^{-1}$). Due to possible light-dependent artifacts [26, 27], the 96-well plates were prepared in a dark room with minimum light exposure, and the kinetics of resorufin formation was followed by absorbance measurements every 10 minutes, not continuously. Samples without H_2O_2 , samples without SOD and complete samples read for a single final measurement were also prepared to test for the possible contribution of an instrumental light-dependent artifact on the final resorufin yield.

H_2O_2 was determined by catalase-mediated oxygen release. Aliquots of 1.6 mL of samples containing FeSOD A (5 μ M), H_2O_2 (100 μ M) and Amplex red (100 μ M) were transferred to a Clark-electrode-based oxygen monitor (Yellow Springs Instruments, model 53), followed by the addition of beef liver catalase (500 U/mL, Roche Applied Science, Indianapolis, IN). In this method, the concentration of oxygen released is half of the H_2O_2 concentration in the sample.

We determined the consumption of H_2O_2 kinetically using a specific H_2O_2 -selective electrode (Apollo 4000 with an ISO-HPO-100 sensor, World Precision Instruments, Sarasota, FL). Standards were used to calculate the consumption of H_2O_2 ($\epsilon_{240\text{nm}} = 0.0436$

mM⁻¹cm⁻¹) in samples containing FeSOD A (2.14–15 μM), Amplex red (100 μM) and H₂O₂ (100 and 200 μM).

Detection of protein radical formation

Immuno-spin trapping was used for the determination of protein radical formation [28]. In this technique protein radicals react with the spin trap 5,5'-dimethyl 1-pyrroline *N*-oxide (DMPO), resulting in the formation of protein-DMPO adducts that can be specifically detected and quantified using antibodies raised against the nitron of DMPO [29, 30]. ELISAs and Western blots were prepared using mouse monoclonal anti-DMPO. ELISA had anti-mouse IgG conjugated to HRP as a secondary antibody (1:500, Abcam, Cambridge, MA). ELISA was revealed using LumiGlo substrate, and the luminescence was detected on a Genios plate reader (Tecan, Morrisville, NC). For Western blot, the secondary antibody was anti-mouse IgG conjugated to IRDye 800CW (1:15,000, Li-Cor Biosciences, Lincoln, NE), and the images were obtained on an Odyssey scanner (Li-Cor Biosciences, Lincoln, NE).

Bacterial survival

For these assays we used the *E. coli* strain BL21-AITM (Invitrogen, LifeTechnologies, Grand Island, NY). This bacterial strain has the T7 RNAP under the control of the arabinose-inducible araBAD promoter. We chose this bacterial strain because: (a) it shows a tightly regulated and homogenous expression of the protein under control of a *lac* promoter, in our case, the SOD A gene [31]; and (b) the previously used bacterial strain, Rosetta 2 DE3, shows a very low viability after protein expression is induced (at OD 3.0 it has approximately 10⁴ cfus/mL), whereas the BL21-AITM strain shows a robust viability after protein expression (at OD 3.0 it has approximately 3.5 × 10⁸ cfus/mL). The previously described SOD A expression plasmid (pDEST 527) was transformed by heat shock into the BL21-AITM competent cells. Bacteria were routinely grown in Luria-Bertani broth with ampicillin (100 μg/mL). Cultures were prepared with Luria-Bertani broth and 0.02% glucose at OD 0.05/mL. After the cultures reached OD 0.6–0.8 (2h, 220 rpm at 37°C), IPTG (0.1 mM) was added. Protein expression was induced by the addition of 0.0025% arabinose. Cultures were then incubated for 2h at 37°C (220 rpm) before being challenged with 50 mM H₂O₂ for 30 min at 25°C. Aliquots were diluted in sterile saline solution (0.9% NaCl) and plated on regular Luria-Bertani agar plates. Plates were incubated for 18 h at 37°C before colonies were counted.

Statistical analyses

Data are represented as means ± standard deviation. Differences were analyzed using Student's *t*-test and considered significant when the *p*-value < 0.05.

RESULTS

Metal incorporation into SOD A and SOD B

We examined the *in vivo* metallation of SOD A and B using *E. coli* transformed with overexpressing plasmids under control of a *lac* promoter for the expression of each bacterial SOD (Fig. 1). After the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to the

standard Luria-Bertani broth (LB), the bacteria had a robust accumulation of the protein (Fig. 1A, columns 2 and 6); however, the total superoxide dismutase activity in those homogenates showed a high activity in the SOD B-overexpressing strain (1496 ± 93 U/mg prot), but an unexpectedly low SOD activity for the SOD A-overexpressing strain (169 ± 17 U/mg prot) (Fig. 1B, columns 1 and 3). We hypothesized that the overexpressed protein could be accumulating with no manganese incorporated, explaining the lack of activity.

We then added MnCl_2 to the LB before inducing the overexpression of SODs with IPTG as before (Fig. 1). For the SOD A strain, a robust increase in SOD activity (from 169 ± 17 U/mg protein to 790 ± 30 U/mg protein) was found when compared to the activity of bacteria grown in the absence of added MnCl_2 (Fig. 1B, columns 1 and 2). However, for the SOD B overexpressing strain, the total superoxide dismutase activity decreased modestly to 832 ± 86 U/mg protein from 1496 ± 93 U/mg protein (Fig. 1B, columns 3 and 4). Note that SOD A is active as a superoxide dismutase only when incorporated with manganese [7], and SOD B is active only with iron [8, 9].

These SODs were purified (Supplemental Fig. 1) and their specific superoxide dismutase activities were determined. As expected, the supplementation with MnCl_2 increased the final superoxide dismutase activity by more than a factor of 6 for SOD A (1251 ± 118 U/mg SOD A *versus* 7681 ± 872 U/mg SOD A, * p -value < 0.05), but decreased the specific activity by one third for SOD B (8146 ± 653 U/mg SOD B *versus* 5042 ± 423 U/mg SOD B, * p -value < 0.05).

Metal analyses of the purified SOD A (Fig. 1C) showed that the overexpressing bacteria were accumulating SOD A as an apoprotein and also incorporating iron, since: (i) substoichiometric levels of total metal to protein were detected, which indicates the occurrence of unmetallated protein, and (ii) the proportion of manganese to iron increased upon MnCl_2 supplementation. The metal content in the LB is modest for iron ($6.0 \mu\text{M}$), but very low for manganese ($0.3 \mu\text{M}$).

Peroxidase activity of bacterial SODs with different contents of iron and manganese

When we analyzed the proficiency of those proteins at oxidizing Amplex red in the presence of excess H_2O_2 , the SOD A preparation with high FeSOD A showed the highest yield of resorufin formation (Fig. 1D). The kinetics of Amplex red oxidation by SOD B did not follow a classical linear enzymatic catalysis since, after the first 30 min, the oxidation subsided. In the presence of excess H_2O_2 , SOD A had higher protein radical formation that correlated with the abundance of Fe in the purified protein (Fig. 1E). In contrast, SOD B showed a minimal accumulation of protein radicals (Fig. 1E).

FeSOD A has a peroxidase activity

With purified FeSOD A, we were able to detect protein radical formation in the presence of H_2O_2 (Fig. 2A). The formation of protein radicals was dose-dependent on H_2O_2 up to 1:50 (Fig. 2B). The high yields of protein radicals led us to hypothesize that iron bound to SOD A could be released from the native peroxidase by a self-destructive reaction of FeSOD A with H_2O_2 . We performed experiments with our standard buffer (with the chelating agent DTPA)

and detected a dose-dependent iron release from FeSOD A in the presence of excess H_2O_2 up to 1:50 (Fig. 2C).

When we performed the same experiments in phosphate buffer without the chelating agent, we were able to detect the formation of hydroxyl radical ($\text{HO}^\bullet$) by spin trapping with DMPO and ESR detection (Fig. 2D, trace 2); however, the production of this free radical was almost completely suppressed when we used our standard assay buffer with the chelating agent DTPA (Fig. 2D, trace 3). These results indicate that iron can be released from FeSOD A but, in the presence of DTPA, the released metal remains inert when bound to DTPA.

As can be seen in Fig. 2E, the addition of Amplex red, the peroxidase substrate, inhibits the release of iron from FeSOD A exposed to H_2O_2 . In fact, Amplex red completely prevented iron release from FeSOD A up to a 1:10 ratio with H_2O_2 , which further shows that iron release is a consequence of the peroxidase activity of FeSOD A and does not reflect a destruction of the peroxidase by contaminant iron in the buffer of our protein preparations. As shown in Fig. 2F, oxidation of Amplex red in the presence of H_2O_2 cannot be catalyzed by Fe^{3+} or iron bound to the chelating agents ethylenediaminetetraacetic acid (EDTA), DTPA or Desferal (deferrioxamine mesylate); however, Amplex red oxidation in the presence of H_2O_2 can be efficiently catalyzed by FeSOD A (Fig. 2F, column 5). EDTA and DTPA indeed decreased the yield of resorufin formation less than 15% (Fig. 2F, column 6 and 7). On the other hand, as expected for its properties as a well-characterized peroxidase substrate [32], Desferal decreased by half the yield of resorufin formation catalyzed by FeSOD A (Fig. 2F, column 8).

Although instrumental light exposure was minimal in all the experiments shown here (Fig. 2, 5 and 6), it is important to observe that this assay was performed using dark-incubated samples measured for a single final determination of fluorescence. Using this validation experiment, we show that the only known artifactual formation of resorufin (light-dependent chemistry of resorufin) is not responsible for the oxidation of Amplex red in our assays, but reflects the peroxidase activity of intact FeSOD A.

Our data show that FeSOD A in the presence of H_2O_2 not only enzymatically oxidizes Amplex red but also independently forms protein radicals and releases iron dose-dependent on H_2O_2 .

We quantified the consumption of H_2O_2 and the formation of resorufin in samples containing 5 μM FeSOD A, 100 μM H_2O_2 and 100 μM Amplex red (Fig. 3A). It is clear that FeSOD A catalytically consumes H_2O_2 since nearly 90 nmoles of H_2O_2 were consumed by 5 nmoles of the enzyme (monomers) (Fig. 3A, black trace); however, it is also evident that the resorufin formation was not stoichiometric to the H_2O_2 consumed, even though the kinetics had similar traces (Fig. 3A and Supplemental Fig. 2A). Indeed, nearly 95% of the H_2O_2 consumption by FeSOD A led to the production of O_2 , which shows that this protein has a very significant catalase-like activity under the conditions used in our assays (data not shown). We have performed assays with higher concentrations of Amplex red, but the yields of resorufin formation were not significantly changed (Supplemental Fig. 2A).

Using a specific H_2O_2 -sensitive electrode, we determined the rate constant for H_2O_2 consumption by FeSOD A to be $k_2 = 6.18 (\pm 0.25) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (Supplemental Fig. 2B). Even though the formation of resorufin was not stoichiometric to the H_2O_2 consumption, Amplex red efficiently suppressed the protein radical formation by FeSOD A exposed to H_2O_2 , which further demonstrates that this protein has a mixed peroxidase-catalase activity. In addition, by using the alternative peroxidase substrate o-phenylenediamine, it is clear that FeSOD A has a peroxidase activity which is dependent on H_2O_2 (Supplemental Fig. 2C).

Metal composition of the media affects the formation of the peroxidase FeSOD A

We then decided to systematically analyze the effect of the metal composition of the media in the metallation of SOD A. We cultivated bacteria in standard LB and in iron-fortified LB (additional 200 μM as iron-citrate). We added manganese at low concentrations (0.6 and 2.0 μM) to the regular LB and the iron-fortified LB, respectively. As mentioned earlier, the LB media has moderate levels of iron (6–7.5 μM) but low levels of manganese (0.2–0.3 μM).

The SOD A isolated from the iron-fortified LB media has significantly higher levels of iron; however, it is notable that the level of manganese in the media predicts the metallation of SOD A (Fig. 4B). Multiple linear regression analyses showed that the manganese metallation of SOD A can be predicted by the level of manganese in the media when we incorporate a factor in the regression model for low iron (factor “IRON” = 0) or high iron (factor “IRON” = 1) (Fig. 4C). Using this model, but also taking into account the proportion of metal incorporation by SOD A, we could calculate that a fully manganese-metallated SOD A would have 9200 U/mg of protein (per monomer), which is in agreement with previous values reported [8].

We measured the yield of Amplex red oxidation (Fig. 5A) and protein radical formation (Fig. 5B) by those proteins in the presence of excess H_2O_2 and, as expected for independent measurements of the peroxidase activity of FeSOD A, both parameters were highly correlated (Fig. 5C). The absolute concentrations of FeSOD A and MnSOD A in those protein preparations are shown in Fig. 5D and 5E, respectively. Oxidation of Amplex red (Fig. 5F) and protein radical formation (not shown) were highly correlated with the levels of FeSOD A in those preparations, but not with the absolute levels of MnSOD A. By contrast, the specific superoxide dismutase activity (Fig. 5G) was highly correlated with the abundance of MnSOD A in those preparations (Fig. 5H).

Bacteria with FeSOD A has lower survival after being challenged with H_2O_2

We analyzed the consequences of SOD A overexpression to the bacterial in vivo metal incorporation to SOD A, physiology and survival using an arabinose-inducing overexpressing bacterial strain. The arabinose-inducing expression system allowed us to analyze the bacterial physiology of cultures since this strain did not suffer from high losses of viability after the homogenous and tightly regulated protein expression induced by arabinose addition. The bacteria grown in media with or without MnCl_2 (10 μM) equally accumulate SOD A in the presence of arabinose (Fig. 6A). The bacteria grown in regular LB had a very small increase in total SOD activity (increase of 20%, Fig. 6B, column 3), which contrasts with the strong effect observed when the strain was cultured in MnCl_2 -

supplemented LB (increase of 550%, Fig. 6B column 4). Interestingly, the strain overexpressing SOD A in LB showed iron accumulation, whereas the bacteria overexpressing SOD A in MnCl₂-supplemented LB showed manganese accumulation (Fig. 6C). Not surprisingly, the purified SOD A (Fig. 6D) from LB was mostly FeSOD A, whereas that from MnCl₂-supplemented LB was MnSOD A (Fig. 6E). The accumulation of intracellular iron and manganese observed in the overexpressing bacteria grown in LB or MnCl₂-supplemented LB was in agreement with the accumulation of FeSOD A and MnSOD A, respectively. Also as expected, the SOD activities (Fig. 6F) and the Amplex red oxidation (Fig. 6G) clearly correlate with the abundance of MnSOD A and FeSOD A in those preparations. The cultures with higher levels of FeSOD A (LB) showed an order of magnitude lower viability after being challenged with H₂O₂ for 30 min when compared to cultures with higher levels of MnSOD A (manganese-supplemented LB). In fact, this was the only significant difference observed in survival between cells grown in LB and cells grown in manganese-supplemented LB.

DISCUSSION

Metal homeostasis is critical for survival, development and growth [33, 34]. Iron is fundamental for many enzymes involved in respiration and redox reactions as a cofactor or as part of the heme group [34, 35]. Manganese is an optional cofactor for many enzymes, but essential as the cofactor of manganese-dependent SODs [9, 12].

Escherichia coli has two distinct superoxide dismutases (SODs): SOD A is a manganese-dependent superoxide dismutase, and SOD B is an iron-dependent superoxide dismutase [1, 2]. SOD A is known to be a main antioxidant enzyme, since it is inducible by oxidative insults through the SoxRS regulon [12]. Previous reports have shown that iron and manganese can compete *in vitro* and *in vivo* for the active site of SOD A in *E. coli* [8, 36].

Here we show that *E. coli* grown in standard Luria-Bertani broth produced the enzyme mostly bound to iron (Fig. 1C and 6E) because of the limited quantities of manganese in that medium. To a lesser extent, SOD B was also found incorrectly metallated, bound to manganese. In agreement with the literature, the incorrect metallation of the bacterial SODs resulted in lower specific SOD activities [7, 8, 36].

Here we show for the first time that SOD A incorrectly incorporated with iron has a catalytic peroxidase-catalase activity. The reaction of FeSOD A and H₂O₂ leads to oxidation of substrate (Amplex red) and generation of protein radicals that are dose-dependent on H₂O₂. This behavior was not observed for FeSOD B, which indicates that simple iron incorporation into SOD A *per se* is not responsible for the peroxidase activity detected in samples of FeSOD A.

Studies with SOD A show that the site of metal binding is partially gated, which means that protein reorganization would be required for the metal binding; however, the correct protein folding does not depend on the metal coordination [36]. The apoprotein spontaneously binds *in vitro* to different metals added to the protein solution, such as Co, Mn, Fe and Zn [37]; however, complete metallation takes long incubation times and high concentrations of the

metals. The metal is coordinated by three histidines, one aspartate (in *E. coli*, His 26, His 81, His 171 and Asp 167) and one metal-bound solvent molecule firmly fixed by glutamine (Gln 146) and aspartate (Asp 167) [10]. The ligands that coordinate the metal in SOD B are conserved [10, 38].

The lack of SOD activity of the incorrectly metallated bacterial Fe-Mn SODs is due to an untuned reduction potential of the metal for the proper catalysis [39]. Ideally, the midpoint reduction potential should be close to 250 mV, between the reduction potentials for the oxidation of $O_2^{\bullet-}$ ($O_2/O_2^{\bullet-} = -160$ mV) and the reduction of $O_2^{\bullet-}$ ($O_2^{\bullet-}, 2H^+/H_2O_2 = 890$ mV) [10]. Due to a lower reduction potential of SOD A incorrectly metallated with Fe (-240 mV), this enzyme is still able to reduce $O_2^{\bullet-}$ (Scheme 1, reaction 2), but it does not oxidize $O_2^{\bullet-}$ (Scheme 1, reaction 1) [10]. In the manganese-incorporated SOD B, the high oxidation potential (> 870 mV) makes the enzyme very difficult to oxidize [10]. In agreement with our observations, the reaction of the iron-incorporated SOD A with H_2O_2 ($H_2O_2, H^+/H_2O, OH^{\bullet} = 320$ mV) is thermodynamically possible and, as shown here, has a rate constant of $6.18 (\pm 0.25) \times 10^4 M^{-1}s^{-1}$ (Supplemental Fig. 2B). FeSOD A reacts with H_2O_2 in a mixed peroxidase-catalase activity. The fact that FeSOD A also has a catalase activity is not that unusual since a mixed catalase-peroxidase activity is known in other peroxidases, such as HRP-C [40, 41] and KatG from *Mycobacterium tuberculosis* [42].

It is noteworthy that although the active sites are superimposable, the sequence homology of SOD A and B of *E. coli* is only 41.4% [5], which indicates that differences in other amino acids are fundamental for the adequate metallation and activity of each SOD. In fact, a recent report concludes that a conserved glycine located far from the active site (glycine 165 in SOD A of *E. coli* [43]) is important for controlling the metal-specific activity of these enzymes. When threonine is substituted for the conserved glycine, SOD A has higher activity when metallated with iron [43, 44].

Another factor to be considered is that the reaction of H_2O_2 with the active FeSOD B of *E. coli* is known to destroy the enzyme by a localized Fenton-chemistry reaction ($0.36 \pm 0.09 M^{-1}s^{-1}$), which results in the specific oxidation of tryptophan [45]. In our assays we observed higher initial rates of substrate oxidation by FeSOD B and H_2O_2 , but the reaction stopped after 30–40 min (Figure 1D) and did not show any detectable protein radical formation (Figure 1E). We speculate that the protein radicals formed might be inaccessible to the spin-trapping agent used for the determination of protein radical formation, 5,5-dimethyl 1-pyrroline *N*-oxide (DMPO) since the localized Fenton-chemistry would occur in the pocket formed by the interaction of two SOD B monomers [9, 38]. Under the same conditions, Amplex red failed to be oxidized continuously, which implies that FeSOD B is not a peroxidase but, as expected, is irreversibly inactivated by oxidation in the presence of H_2O_2 .

In contrast, the catalytic behavior of the peroxidase activity of FeSOD A is clearly demonstrated in samples in which H_2O_2 consumption is followed in the presence of the substrate Amplex red (Figure 3A). The consumption of H_2O_2 was much higher than the yields of resorufin formation catalyzed by FeSOD A. In fact, resorufin is the product of a 2-electron oxidation of Amplex red, which is formed by the disproportionation of two Amplex

red radicals [46]. Modified horseradish peroxidases, which have lower peroxidase activity, show non-stoichiometric resorufin formation [46], which is the case for FeSOD A. Assays with higher concentrations of Amplex red showed no significant changes in the yields of resorufin formation (Supplemental Fig. 2). Even though resorufin formation in our assays was not stoichiometric to H_2O_2 consumption, the formation of this product was unequivocally correlated with the catalytic consumption of H_2O_2 . This protein has a catalase activity which explains the lack of stoichiometry. As mentioned before, the protein radical formation was also dose-dependent on H_2O_2 and was prevented by the addition of Amplex red, which supports the conclusion that electrons are diverted from the oxidation of Amplex red to induce the formation of protein radicals. Alternatively, the release of iron competes with the peroxidase activity of FeSOD A incubated with H_2O_2 since the enzyme in the presence of the peroxidase substrate Amplex red showed lower iron release. Even more importantly, Amplex red oxidation is insensitive to free iron or iron bound to chelators, and so iron released from FeSOD A does not lead to artifactual Amplex red oxidation.

Iron levels *in vivo* are controlled by its absorption, and many mechanisms exist to absorb, store and reutilize iron in eukaryotic [35] and prokaryotic organisms [12, 47]. Manganese metabolism is less well understood [33]; however, it is clear that its metabolism overlaps with iron since higher levels of iron predispose to lower levels of manganese absorption [48–50].

A clear distinction exists between those two metals regarding their utilization following their absorption, since iron is known to be transported extracellularly and stored intracellularly, bound to transferrins and ferritin, respectively [34, 35], while manganese has no known mechanism for storage either extra- or intracellularly [51, 52]. Many studies explore the toxic properties of both metals [53–55]. A high level of iron is known to poison cells by catalyzing oxidation reactions that lead to the production of reactive free radicals, especially $OH^\bullet$ from H_2O_2 by the Fenton reaction [55–57]. Nevertheless, manganese and iron are known to be fundamental for cellular homeostasis.

In order to study the possible role of iron availability and its role in manganese utilization, we performed experiments with low and high iron and different levels of manganese in the media of our SOD A-overexpressing bacteria. The metallation of SOD A could be predicted by a multiple regression model which includes a factor to account for the levels of iron as high or low. High levels of iron in the medium lead to a reduced metallation of SOD A with manganese. This model further supports the concept that iron and manganese compete *in vivo* for their absorption and utilization. Media with high iron not only favor the metallation of SOD A with iron because of its higher abundance, but also because it reduces the bacterial absorption and assimilation of manganese. In a different *E. coli* strain (BL21-AITM), the observations were reproduced: the overexpression of SOD A in cells in $MnCl_2$ -supplemented medium produced SOD A mostly metallated with manganese. The total homogenate was rich in manganese due to the accumulation of MnSOD A in those cells. In contrast, overexpression of SOD A by the bacteria grown in a regular medium produced mostly FeSOD A, which led to an apparent accumulation of iron in the total homogenate. Since both iron and manganese bind to SOD A, the ratio of manganese-to-iron in the medium dictated whether the major component of the final protein was the peroxidase

FeSOD A or the SOD-active MnSOD A. Finally, compared to bacteria with MnSOD A, cells with FeSOD A had reduced survival after exposure to H₂O₂. Even though we do not have definitive evidence, it is tempting to assume that the lower viability of bacteria with FeSOD A after being exposed to H₂O₂ was due to the prooxidant peroxidase activity of FeSOD A. Nevertheless, the *in vivo* formation of FeSOD A indeed predisposed *E. coli* to lose viability after being exposed to H₂O₂. Recently, knowledge about iron and manganese metabolism during stress in bacteria has been extensively expanded [12, 58, 59] and our results show that an orchestrated activation of these mechanisms must occur for building an adequate response to oxidative challenges. We show that iron incorporation into SOD A not only predisposes cells to oxidative damage by preventing the superoxide dismutase activity of this protein, but also by forming a prooxidant peroxidase. This effect is nutrition-dependent, which shows the importance of environmental factors in the resistance of bacteria to oxidative challenges.

In conclusion, we show that the availability of manganese over iron predicts the metallation of Fe-Mn SODs. The binding of iron to the manganese-dependent SOD A predisposes cells to a biological prooxidant state by synergistically blunting the SOD antioxidant activity and generating a peroxidase enzyme. We show that the biological occurrence of this prooxidant enzyme is nutrition-dependent and is an important source of free radical formation *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors acknowledge Dr. Ann Motten, Ms. Mary Mason and Ms. Jean Corbett for their help in the revision of the manuscript. We also acknowledge Ms. Jean Corbett for her valuable technical assistance. This research was supported by the Intramural Research Program of the NIEHS, National Institute of Environmental Health Sciences/NIH.

ABBREVIATIONS

SOD	superoxide dismutase
DMPO	5,5'-dimethyl 1-pyrroline <i>N</i> -oxide
ESR	electron spin resonance
ICP-OES	inductively coupled plasma with optical emission spectrometry
DTPA	diethylenetriaminepentaacetic acid
EDTA	ethylenediaminetetraacetic acid
Desferal	deferoxamine mesylate

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Highlights

- Iron or manganese incorporate into bacterial manganese-dependent SOD A.
- Only FeSOD A has peroxidase activity, oxidizing substrates in the presence of H_2O_2 .
- Incorporation of iron or manganese into SOD A is nutrition-dependent.
- FeSOD A formation is harmful to bacteria exposed to H_2O_2 .

FIGURE 1. Metallation of Fe/Mn-SODs in *E. coli*

E. coli transformed with overexpressing plasmids for SOD A or SOD B under control of a *lac* promoter were grown in standard Luria-Bertani broth. Overexpression was induced by the addition of IPTG (0.1 mM) to the cultures followed by incubation under gentle agitation for 2h. Where indicated, 100 μ M MnCl₂ was added to the media of the bacteria at the time they were inoculated. Native conditions were used for the whole cell homogenate preparation and protein purification. (A) SDS-PAGE of the different homogenates is depicted. (B) Specific total superoxide dismutase activities of the different homogenates of overexpressing bacteria are shown. For more details refer to Experimental Procedures. (C) SOD A and SOD B were purified from total homogenates and their metal content and specific superoxide dismutase activities were determined. (D) Kinetics of Amplex red oxidation was measured for the purified proteins (Supplemental Fig. 1). The assay was prepared using microplates of 96 wells (200 μ L) with samples containing 0.2 mg/mL of SOD (SOD A is 9.1 μ M and SOD B is 9.3 μ M) and 100 μ M Amplex red prepared in 100

mM phosphate buffer, pH 7.4, with 25 μ M DTPA. The reaction was started by the addition of H_2O_2 1:100 (910 μ M for SOD A and 930 μ M for SOD B). Amplex red oxidation in samples incubated in the dark did not differ from those shown in the figure, in which samples were exposed to instrumental light every 10 min for 60 min. (E) Protein radical formation in the presence of excess H_2O_2 was determined by ELISA. The samples were prepared as described in (D), except that Amplex red was omitted and DMPO (100 mM) was added. Samples were incubated for 1h at 25°C. * p -value < 0.05 *versus* regular medium-grown protein, # p -value < 0.05 *versus* control, and N.S. for not statistically significant (p -value > 0.05).

FIGURE 2. Peroxidase activity of FeSOD A

FeSOD A was prepared as described [8, 60]. The effect of H₂O₂ in the protein radical formation on FeSOD A was determined using (A) Western blot and (B) semi-quantitative ELISA assay for protein radical formation. Samples contained 0.2 mg/mL of FeSOD A (9.1 μM) and 100 mM DMPO, prepared in 100 mM phosphate buffer, pH 7.4, with 25 μM DTPA. After addition of H₂O₂ (9.1–910 μM), samples were incubated for 1h at 25°C. (C) Release of iron into the buffer was measured for samples containing 22.8 μM FeSOD A (0.5 mg/mL) exposed to different concentrations of H₂O₂, prepared in 100 mM phosphate buffer, pH 7.4, with 25 μM DTPA. After incubation for 1h at 25°C, the samples were subjected to

ultrafiltration with 10 kDa-cutoff filters, and the flow through (buffer) was analyzed for iron by ICP-OES. (D) Spin trapping of free radicals formed in samples of 10 μ M FeSOD A, 100 mM DMPO and 500 μ M H₂O₂, prepared in 100 mM phosphate buffer, pH 7.4. Where indicated, 25 μ M DTPA was added before H₂O₂. After incubation for 30 min at 25°C, samples were scanned in a Bruker EMX ESR. (E) Release of iron was measured by ICP-OES in the buffer of samples prepared as described for (C). Where indicated, Amplex red (AR) was used at 100 μ M and DTPA was at 25 μ M. (F) Amplex red (100 μ M) oxidation was measured by the single final-fluorescence measurement of dark-incubated samples. Samples contained 100 μ M H₂O₂, 5 μ M iron (FeCl₃) or 5 μ M FeSOD A, in the absence or presence of 25 μ M chelators [EDTA, DTPA and Desferal (deferrioxamine mesylate)]. # p -value < 0.05, * p -value < 0.05 *versus* control, and N.S. for not statistically significant (p -value > 0.05).

FIGURE 3. FeSOD A catalytically uses H₂O₂ for its peroxidase activity

(A) Consumption of H₂O₂ and formation of resorufin by FeSOD A was followed in samples containing 5 μM of FeSOD A, 100 μM Amplex red and 100 μM H₂O₂ prepared in 100 mM phosphate buffer, pH 7.4, with 25 μM DTPA. The reaction was started by the addition of H₂O₂. At the indicated time points, H₂O₂ was determined by catalase-mediated release of oxygen. Resorufin formation was determined by measuring the absorbance at 571 nm in aliquots taken at the indicated time-points. For more details refer to Experimental Procedures. (B, C) The effect of Amplex red (100 μM) on the protein radical formation on FeSOD A exposed to different concentrations of H₂O₂. Samples were prepared as described in Fig. 2(A) and (B). * *p*-value < 0.05.

FIGURE 4. Metal content in SOD A is dependent on manganese-to-iron ratio in the medium SOD A was expressed in *E. coli* grown in media with different concentrations of MnCl₂. Media used were Luria-Bertani broths with or without added 200 μ M iron citrate. In (A) percentage of metal bound to purified SOD A. In (B) multiple regression analyses of the manganese content of SOD A as a function of the manganese-to-iron ratios in the media.

FIGURE 5. Peroxidase activity of SOD A depends on iron incorporation, while dismutase activity of SOD A depends on manganese incorporation

Purified SOD A expressed in *E. coli* grown in regular Luria-Bertani broth (LB) or LB enriched with 200 μ M iron citrate and different concentrations of MnCl₂ was assayed for peroxidase activity [Amplex red oxidation in (A) was measured by the single final-fluorescence measurement of dark-incubated samples as described in Fig. 1(D), and protein radical formation in (B) was measured as described in Fig. 1(E)], SOD activity (G) and metal content determined by ICP-OES [iron in (D) and manganese in (H)]. Scatter plots and correlation indexes are shown for: (C) Amplex red oxidation *versus* SOD A protein radicals,

(F) iron incorporation *versus* Amplex red oxidation, and (H) manganese incorporation *versus* SOD activity.

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FIGURE 6. After being challenged with H₂O₂, *E. coli* with the peroxidase FeSOD A shows decreased survival

In an arabinose-induced overexpressing strain of *E. coli*, SOD A was overexpressed in bacterial cultures (LB with 0.1 mM IPTG) after the addition of 0.0025% arabinose. Cultures were then incubated under agitation for an additional 2h. Where indicated, 10 μM MnCl₂ was added to the media of the cultures at the time they were inoculated. Cells were then washed twice with water, and cell pellets were stored at -80°C for later analyses and protein purification. Native conditions were used for the whole cell homogenate preparation and protein purification. (A) SDS-PAGE of the different homogenates is depicted. (B) Specific total superoxide dismutase activities of the different homogenates of bacteria were

determined. For more details refer to Experimental Procedures. (C) Concentration of iron and manganese in the total cellular homogenates are shown. (D) SOD A was purified from total homogenates, and (E) their metal content, (F) specific superoxide dismutase activities and (G) yield of Amplex red oxidation [as described in Fig. 5(A)] were determined. In (H) the ability of bacterial cells to grow into colonies after being challenged or not with 50 mM H_2O_2 for 30 min was determined. For more details refer to Experimental Procedures. * p -value < 0.05 *versus* regular medium-grown bacteria, # p -value < 0.05 *versus* control, & p -value < 0.05, and N.S. for not statistically significant (p -value > 0.05).

SCHEME 1. Superoxide dismutation catalyzed by SOD

In the scheme, MnSOD was used to represent the general catalysis by a generic Fe/Mn SOD; MnSOD A can be replaced by FeSOD B.