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Pro- or anti-oxidant manganese: a suggested mechanism for reconciliation

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Abstract

The neurodegeneration induced by manganese has been attributed to its ability to undergo redox cycling, and catalysis of reactive oxygen species (ROS) formation, as with other transition metals. However, the characterization of manganese as a pro-oxidant is confounded by increasing evidence that the metal may scavenge superoxide anions and protect cells from oxidative damage. The current study was designed to address conflicting reports pertaining to the oxidative capacity of manganese. We found that the metal has distinctive redox dynamics in which the divalent reduced form, unlike iron, possessed no intrinsic oxidative capacity. The apparent ability of Mn^{2+} to promote the formation of ROS within a cortical mitochondrial-synaptosomal fraction was quenched by the depletion of contaminating nanomolar concentrations of trivalent metals. The addition of manganic ions at trace concentrations dose-dependently restored the oxidative capacity attributed to divalent manganese, whereas the presence of the ferric ion retarded the rate of ROS generation. This result was paralleled by the spectrophotometric demonstration that the kinetics of iron oxidation is accelerated by trivalent but not divalent manganese. The markedly different capacities of the lower and higher valence states of manganese to promote free-radical formation in cortical fractions and to modulate the process of iron oxidation may account for earlier contradictory reports of anti- and pro-oxidant properties of manganese.

Keywords: Pro- or anti-oxidant manganese; Reactive oxygen species; Mitochondrial-synaptosomal fraction

1. Introduction

Manganese toxicity, or manganism, can trigger severe psychiatric and extrapyramidal motor dysfunction, which have frequently been attributed to oxidative damage. Manganese-induced brain lesions tend to occur in regions of intense oxygen consumption (Yamada et al., 1986), and are marked by enhanced auto-oxidation and turnover of dopamine, losses of neurons and demyelination (Cotzias et al., 1971; Donaldson et al., 1984; Gerlach et al., 1994; Erikson et al., 1987). The site-specificity of the pathology and the selective targeting of dopamine have led to the comparison of manganese-induced neurodegeneration to that of other transition metals, iron and copper (Triggs and Willmore, 1984; Rauhala and Chiueh, 2000; Sengstock et al., 1993), i.e. the toxicity of manganese has been associated with the general propensity of transition metals to produce cytotoxic levels of free-radicals during redox cycling.

Despite extensive research efforts, the characterization of manganese as a pro-oxidant (Donaldson et al., 1982; Shi and Dalul, 1990; Aschner, 1997) is largely limited to inferences from the histopathological studies and observations of dopaminergic neurotoxicity. The few specific determinations of the oxidative capacity of manganese have yielded conflicting results. Pro-oxidant properties have been attributed to both the divalent and trivalent states of the metal (Donaldson et al., 1984; Soliman et al., 1995; Ali et al., 1995) or to only the latter (Hussain and Ali, 1999; Sziraki et al., 1995). The report that the divalent form does not react with H_2O_2 to produce the hydroxyl radical at a measurable rate (Halliwell and Gutteridge, 1999) challenges the finding that manganese can generate both the hydroxyl and superoxide radicals from H_2O_2 with rises of pH in the physiological range (Yim et al., 1993).

Other studies have ascribed an anti-oxidant role against oxygen-radicals to the divalent state of manganese. Low molecular weight complexes of Mn^{2+} attenuate oxygen toxicity in *Lactobacillus planetarium* and related bacteria deficient in superoxide dismutase (Archibald and Fridovich, 1981). Supplementation with manganous salts

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protects against oxidative damage to glutamine synthetase in *Escherichia coli* that occurs under Mn^{2+}-deprived conditions (Levine, 1989). Divalent manganese inhibits the O_2-dependent inactivation of many enzymes by metal ion-catalyzed reactions (Johnson et al., 1985; Fucci et al., 1983), and facilitates the disproportionation of H_2O_2 in a catalase-like manner (Stadtman et al., 1990). In mammalian tissues, Mn^{2+} inhibits iron-induced lipid peroxidation and dopamine depletion (Sziraki et al., 1995, 1998). The reduction of copper-dependent low-density lipoprotein conjugation (Tsujimoto et al., 1988), and iron-induced phospholipid peroxidation, by Mn^{2+} has also been demonstrated in mammalian cultured cells (Tampo and Yonaha, 1992).

These protective effects contradict reports of a pro-oxidant role for manganese in the divalent state. The recent finding that the trace presence of a trivalent metal ion, most probably manganic, is required for the apparent pro-oxidant activity associated with excess Mn^{2+} (HaMai et al., 2001a) indicates that trivalent ion may have catalytic effects on the oxidation of Mn^{2+} and redox cycling. The catalytic effect of nanomolar concentrations of the trivalent ion on the oxidation of Mn^{2+} and redox cycling allows manganese to assume either a protective or deleterious role during oxidative events.

The current study focused on the interactions between manganese and iron in the promotion of oxidative events. Trace additions of the Mn^{3+} and Fe^{3+} salts alter the oxidant potential of the divalent manganese ion in opposing directions. In the presence of a trivalent chelator, the apparent ability of divalent manganese to promote the formation of reactive oxygen species (ROS) disappeared. These observations were complemented by the finding that trivalent manganese increased the rate of iron oxidation, whereas Mn^{2+} did not alter the ratio of ferrous to ferric. This suggests a mechanism by which manganese has the capacity to be either a pro-oxidant or an anti-oxidant, and highlights the significance of valence and ion speciation in manganese-related oxidative events.

2. Experimental procedures

2.1. Tissue preparation

Brains of 2–3-month-old male B6C351 strain mice were excised on ice, and the cerebral cortex was weighed and homogenized in 10 volumes of 0.32 M sucrose and centrifuged at 1800 × *g* for 10 min. The resulting supernatant fraction was centrifuged at 31,500 × *g* for 10 min to yield a crude pellet (P2), which was comprised largely of mitochondria and synaptosomes. These two organelles are thought to be primary subcellular targets of manganese localization and neurotoxicity (Lai et al., 1997). The pellet was taken up in HEPES buffer to a concentration of 0.1 g eq./ml. The composition of the HEPES buffer was (mM): NaCl, 120; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 0.1; $NaHCO_3$, 5.0; glucose, 6.0; $CaCl_2$, 1.0; and HEPES, 10 at pH 7.4.

2.2. Assay for reactive oxygen species formation

Reactive oxygen species were assayed using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Bass et al., 1983). DCFH is capable of being oxidized to the fluorescent 2′,7′-dichloro-fluorescein by reactive oxygen species. Although the identity of the ultimate species responsible for the oxidation of DCFH is not known (LeBel et al., 1992), the utility of this probe as a sensitive measure of ROS in biological systems, including isolated subcellular cerebral systems, has been documented (LeBel and Bondy, 1991).

All materials were dissolved in a buffer of 20 mM of Tris (hydroxymethyl) aminomethane at a pH of 7.4. Test compounds were pipetted into 96-well cluster plates (Costar, Corning Inc., Corning, NY, USA) with four replicates per sample, and 2.25 μM DCFH-DA was added to each well for a final volume of 200 μl. Given the light sensitive nature of the probe, assays were carried out in a FL600 spectrofluorometer (Biotek Instruments Inc., Winooski, VT, USA). The microplate reader allowed for fast light excitation and precise fluorescence capturing, thus minimizing photooxidation of the probe (Wang and Joseph, 1999). Incubation temperature was maintained at 37 °C. Excitation and emission were set at 485 and 530 nm, respectively. The fluorescence from each well was monitored, digitized, and stored on a computer using KC4 (Version 4.0) (Biotek Instruments Inc., Winooski, VT, USA) software. Parallel blanks with no DCFH-DA were included, and this value was subtracted from readings of samples to correct for autofluorescence of fractions. Additionally, parallel blanks with no P2 loaded with DCFH-DA served as an indicator of photooxidation of DCFH-DA. A DCF standard curve (1–500 nM) was used for conversion of fluorescence to nmol DCF formed/2 h.

2.3. Assay for iron oxidation

The oxidation of ferrous to ferric in an aqueous medium was assayed by using bathophenanthroline-disulfonic acid, which binds to Fe^{2+} to form a red colored complex with maximum absorbance at 530 nm (Ulvik and Romslo, 1979; Yegorov et al., 1993).

All materials were dissolved in a buffer of 20 mM of Tris (hydroxymethyl) aminomethane at a pH of 7.4. Test compounds were pipetted into 96-well cluster plates (Costar, Corning Inc., Corning, NY, USA) with four replicates per sample, and 1.75 mM bathophenthroline was added to each well for a final volume of 200 μl. Assays were conducted using a Opsys MR spectrophotometer (ThermoLabsystems, Chantilly, VA, USA) with the absorbance filter set at 550 nm. The optical density of each well was monitored and digitized using Dynex Revelations software (Version 4.24) (ThermoLabsystems, Chantilly, VA, USA). Blanks contained only bathophenanthroline-disulfonic acid, and values

were subtracted from readings of iron-containing samples to correct for background absorbance. A ferrous iron standard curve (5–500 μM) was used for the conversion of optical density to μmol Fe^{2+} remaining in each sample well.

2.4. Protein determination

Protein content was assayed using the method of Bradford (Bradford, 1976).

2.5. Statistical analysis

Statistical analysis was performed using ANOVA and Tukey's Test. The acceptance level of significance between groups was set at $P < 0.01$ using a two-tailed distribution.

3. Results

3.1. Manganous salts lose pro-oxidant activity in the presence of a trivalent ion chelator

The capacity of redox-active metals to generate ROS has been shown to be enhanced dramatically by the presence of another metal element such as lead or aluminum (Bondy and Guo, 1996; Bondy et al., 1998). In the case of manganese, the pro-oxidant status of the divalent form has been attributed to the trace presence of other trivalent metals (HaMai et al., 2001a).

The extent of the relationship between divalent manganese and trivalent ions was assessed by sequestering traces of the latter, which are constitutively present in trace amounts in cortical fractions, with desferrioxamine (DFO). This trivalent metal chelator was used to discriminate between the effects of divalent and trivalent manganese. To facilitate a comparison of their pro-oxidant effects in mitochondria-synaptosomal fractions, micromolar quantities of 250 μM $MnCl_2$ and 5 μM $MnAc_3$ were used. These concentrations were selected on the basis that they accelerated ROS formation at comparable rates, and that these rates were more than twice that of the control values (HaMai et al., 2001a). Because cortical fractions lack an intact metabolic system, relatively high concentrations of Mn are required to elicit an oxidative stress response comparable to those observed with cultured neural cells. Similar levels of ROS are generated by whole cellular systems with concentrations that are an order of magnitude lower than those used in the subcellular samples (HaMai et al., in preparation).

The chelator was added to samples of P2 incubated with the manganous salt $MnCl_2$ (Fig. 1). Such treatment reduced ROS levels to basal values in treated subcellular fractions. This inhibition occurred even at a molar ratio of 100–1 of Mn^{2+} (500 μM) to DFO (5 μM).

3.2. Manganic and ferric ions oppositely affect manganous-promoted ROS formation

Fe^{3+} and Mn^{3+} salts were added at nanomolar and low micromolar concentrations (0.5–10 μM) to samples of P2 incubated with $MnCl_2$ (250 μM) in order to compare the effects of various trivalent metal salts on the pro-oxidant activity associated with divalent manganese (Fig. 2). Additions of $FeCl_3$ had a slight attenuating effect at 10 μM on Mn^{2+}-promoted ROS formation, whereas increased concentrations of the $MnAc_3$ salt enhanced the pro-oxidant effects

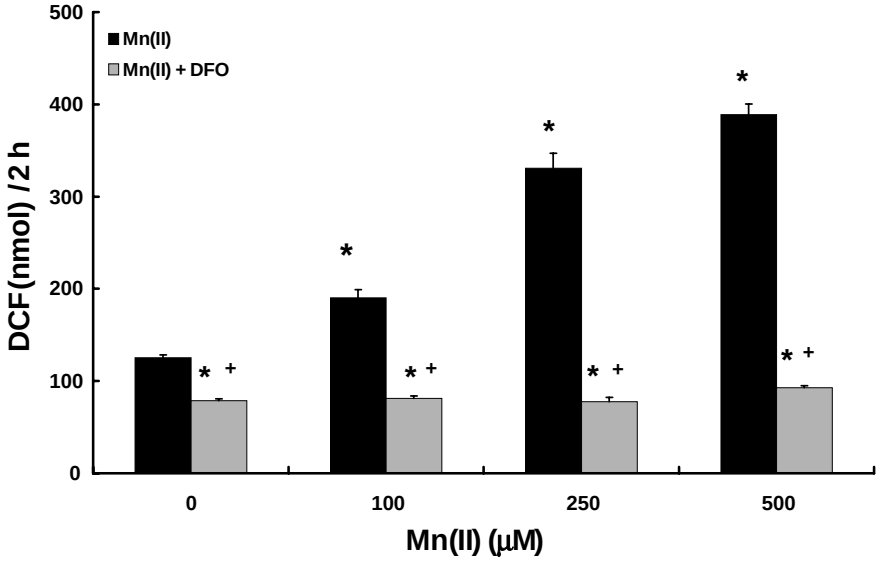

Fig. 1. Effect of (5 μM) desferrioxamine (DFO) on ROS production induced by (0–500 μM) divalent manganese in P2 cortical fractions as determined by DCF formation. Values are mean ± S.E. of three independent experiments performed each with quadriplicates. (✱) Manganous-treated fractions differ ($P < 0.01$) from control; (✚) fractions treated additionally with DFO differ ($P < 0.01$) from fractions treated with $MnCl_2$ alone.

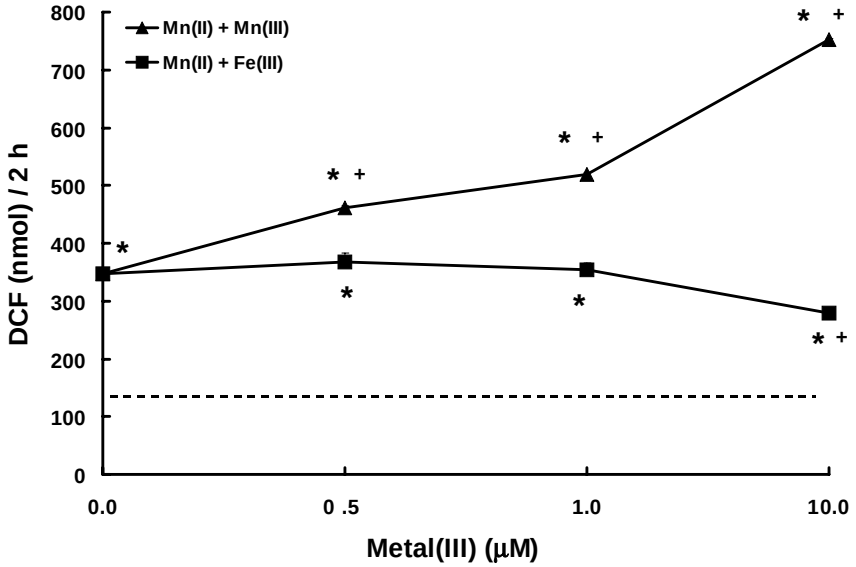

Fig. 2. Effects of trivalent (0.5–10 μM) iron and (0.5–10 μM) manganese on (250 μM) manganous-induced ROS formation. Values are mean ± S.E. of three independent experiments performed each with quadriplicates (error bars are too narrow to visualize). (✱) Manganous-treated fractions differ ($P < 0.01$) from control; (✚) fractions treated additionally with either $FeCl_3$ or $MnAc_3$ differ ($P < 0.01$) from the corresponding fractions treated with $MnCl_2$ alone.

associated with the divalent manganese in a dose-dependent manner.

3.3. Addition of manganic ion restores pro-oxidant activity of the manganous ion

Nanomolar concentrations of $MnAc_3$ (0.5 μM) were added to different P2 samples containing $MnCl_2$ (250 μM) and DFO (0.5 μM) to investigate the enhancing effect of Mn^{3+} salts on the pro-oxidant activity associated with divalent manganese. The rate of manganous-based ROS formation was measured in 15 min increments over a 2 h incubation period (Fig. 3). The sequestration of trace amounts of trivalent metals by DFO was sufficient to impede ROS formation, maintaining it at basal levels throughout the entire incubation with manganous salts and the chelator. The trace addition of Mn^{3+} salts to the samples treated similarly as above accelerated the kinetics of manganous-based generation of ROS, and restored a significant level of the manganous pro-oxidant activity that was observed in

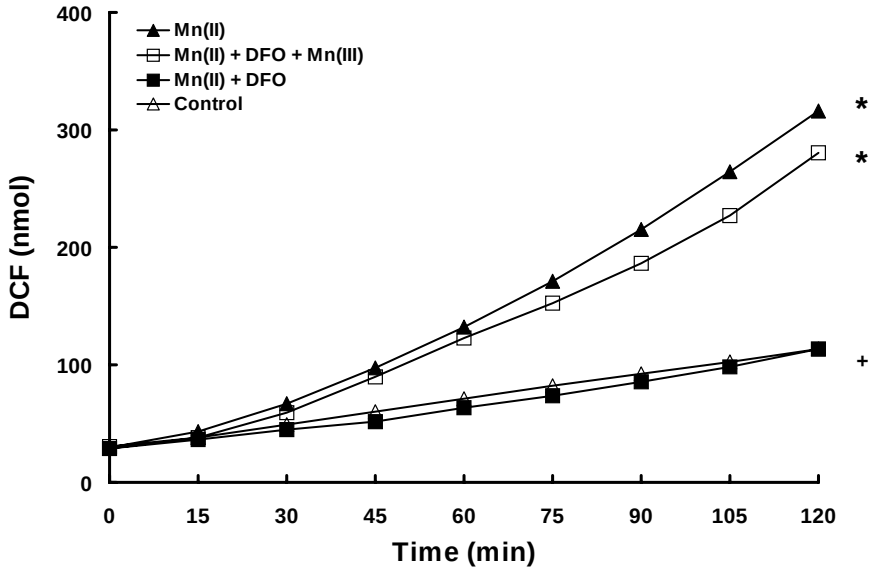

Fig. 3. Manganous-induced ROS formation following additional treatments with (0.5 μM) DFO and/or (0.5 μM) trivalent manganese. Values are mean ± S.E. of three independent experiments performed each with quadriplicates (error bars are too narrow to visualize). (✱) Manganous-treated fractions differ ($P < 0.01$) from control; (✚) fractions exposed additionally to either DFO or DFO and $MnAc_3$ co-treatment differ ($P < 0.01$) from the corresponding fractions treated with $MnCl_2$ alone.

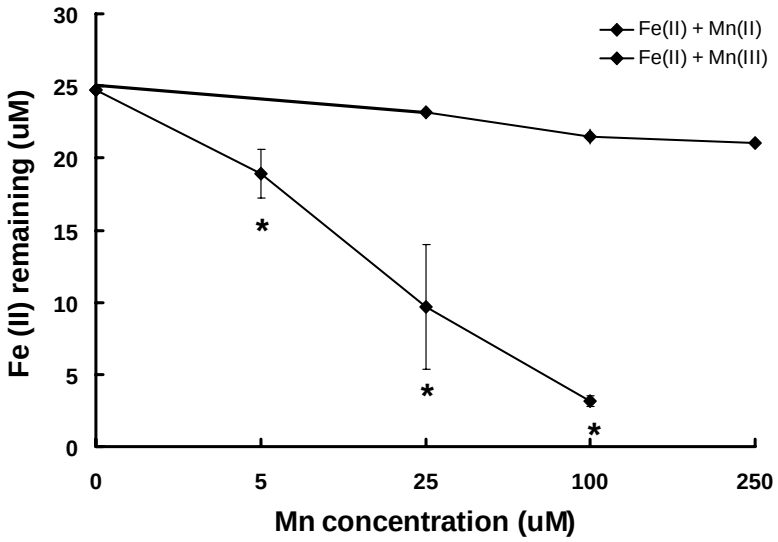

Fig. 4. (20 μM) Iron oxidation in the presence of (25–250 μM) divalent and (5–100 μM) trivalent manganese as determined by formation of bathophenanthroline–iron colored complex. Values are mean ± S.E. of three independent experiments performed each with quadruplicates. (✱) Samples exposed additionally to either $MnCl_2$ or $MnAc_3$ differ ($P < 0.01$) from corresponding samples treated with $FeSO_4$ alone.

samples incubated without DFO. In contrast, the addition of ferric salts (0.5 μM) did not raise the rate of ROS production above that of control (data not shown).

3.4. Manganic ion accelerates ferrous oxidation

Since the capacity of manganese to promote ROS formation was dependent upon the valence state, the possibility that the redox status of iron, and its oxidation rate may be altered by its interaction with manganese salts was investigated (Fig. 4). The addition of $MnAc_3$ (5–100 μM) increased the rate of the oxidation of the ferrous salt $FeSO_4$ (20 μM) in a concentration-dependent manner, whereas the presence of $MnCl_2$ (25–250 μM) did not alter the kinetics of this reaction.

4. Discussion

In spite of claims that manganese is a pro-oxidant, experimental efforts to investigate the role of the metal in oxidative stress have largely ignored the relationship between valence and the capacity to promote ROS formation. This oversight is in part due to the erroneous assumption that the initial valence of the manganese salt and its redox tendencies are irrelevant to the subsequent biological response. Our findings illustrate the significance of valence and ion speciation in metal-mediated oxidative events. Interactions between metal ions of different valences may exert influence on manganese-related ROS generation, and determine whether ROS generation will be reduced or enhanced. Demonstration of the capacity of manganese, specifically in the divalent state, to act in both a pro- and anti-oxidant manner, and the significance of trace concentrations of Mn^{3+} in oxidative events, reconciles earlier conflicting reports concerning the oxidant capacity of manganese.

Although divalent manganese showed an apparent ability to promote the formation of ROS within a cortical mitochonria-synaptosomal fraction, its pro-oxidant activity was blocked in the presence of a chelator of trivalent metals. The possibility that the inhibition of ROS generation was due to Mn^{2+} being sequestered by desferrioxamine, a trivalent metal chelator, was negated by the 1000-fold difference in molar concentrations between Mn^{2+} and DFO. Since the stability constants of complexes between divalent ions such as Cu^{2+} and Zn^{2+} are at least eleven orders of magnitude lower than those of trivalent ones (Halliwell and Gutteridge, 1999), the trace presence of a trivalent metal as a promoting factor in Mn^{2+}-related ROS generation could be inferred.

In view of the high affinity of DFO for Fe^{3+}, the trivalent ion is most probably the metal specie sequestered by the chelator, and involved in manganous-related ROS generation. Contrary to expectations, nanomolar increases of Fe^{3+} slightly reduced the oxidative potential of Mn^{2+} while similarly low concentrations of Mn^{3+} markedly promoted the ROS-generating capacity of Mn^{2+}. Although DFO complexes most stably with Fe^{3+}, the ability of DFO to sequester other trivalent metals including Mn^{3+} has been used in studies of chelation therapy and metal coordination chemistry (Jorge et al., 1999; Blanusa et al., 1986; Beyer and Fridovich, 1986). The opposing effects of manganic and ferric ions on manganous-related pro-oxidant activity suggest that the trace presence of Mn^{3+} rather than Fe^{3+} was a critical factor in enabling the oxidizing effects attributed to divalent manganese.

Even in manganous-treated fractions that were co-incubated with DFO, low micromolar increases of Mn^{3+} accelerated ROS formation and restored a significant level of Mn^{2+}-related pro-oxidant activity. However, the addition of the same low concentrations of Fe^{3+} to samples incubated with $MnCl_2$ and DFO maintained the ROS generation at basal levels. These results further supported the proposition that the apparent pro-oxidant activity of Mn^{2+} was dependent on the trace presence of Mn^{3+} rather than Fe^{3+}.

Increases of manganic ions at nanomolar and low micromolar ranges concentration-dependently restored the oxidant capacity attributed to divalent manganese, whereas the presence of the ferric ion modestly retarded the rate of ROS generation. This was paralleled by the observation that Mn^{3+} but not divalent manganese accelerates iron oxidation. Low micromolar concentrations of manganic salts were sufficient to trigger an immediate oxidation of ferrous ion, whereas divalent manganese at concentrations of 100-fold higher did not promote the conversion of ferrous to ferric.

Collectively, these findings indicate the significance of valence in manganese-related oxidative events. The homogenous oxidation rate of manganous ion is calculated to be slower than that of ferrous ion by approximately six orders of magnitude (Morgan, 2000). The symmetry of its half-filled 3d shell accounts, in large part, for the reluctance of Mn^{2+} to lose one of its five d-electrons, and for its poor reducing ability (HaMai et al., 2001a,b).

In spite of its electrochemical stability, the reactivity of divalent manganese can be enhanced by its contact with surfaces of metals colloids, or precipitates with metal ligands (Morgan, 2000), such as the oxy-hydroxides that are formed by Mn^{3+} in aqueous solutions. A localized increase in Mn^{2+} concentration at the surfaces of such metal-containing insoluble bodies may induce a shift in the electron density at the interface between two different metal ionic species. Thus, the oxidation of divalent Mn can be catalyzed by Mn^{3+}, which may exist as a trace contaminant, to generate reaction products that are together substantially more oxidizing than the original reactants.

The four unpaired electrons of Mn^{3+} in the usual high-spin configuration render the ion unstable. As a result, the ion is inclined either to lose its one electron in the antibonding e_g set of orbitals or to gain an electron to maximize electron exchange energy (Larson and Pecoraro, 1992). Since increased ROS generation attributed to divalent manganese was observed with nanomolar increases of Mn^{3+} in mitochondria-synaptosomal fractions, the more reactive trivalent ion may be the critical rate-limiting factor in the kinetics of Mn^{2+}-related pro-oxidant activity.

The inhibition of manganous-promoted ROS generation by trace additions of Fe^{3+} suggests that the trivalent ion alters the redox status of Mn^{2+}. Similarly, the promotion of iron oxidation from ferrous to ferric does not occur in the presence of Fe^{3+} and Mn^{2+} together. The finding that oxidizing conditions are attenuated by the presence of both Fe^{3+} and Mn^{2+} demonstrates that the presence of both ions confer a stability to the respective redox couples of manganese ($Mn^{2+} \leftrightarrow Mn^{3+}$) and iron ($Fe^{2+} \leftrightarrow Fe^{3+}$). This stability is derived from the comparably low redox potential of Mn^{2+} and Fe^{3+}, and the symmetry of their electrochemical configurations. In direct interactions with one another, shifts in electron density that occur during redox cycling may be evenly distributed in the symmetry of their electron configuration. Alternatively, their similar electrochemical profile may enable the ferric ion to compete with the manganous ions for ligands or colloidal surfaces that enhance the reducing ability of Mn^{2+} and enable its oxidation to Mn^{3+}. The anti-oxidant activity often ascribed to Mn may be derived from the stabilizing effect of an electron exchange between the two comparatively stable metal species, or the buffering effect provided by the electrochemically similar Fe^{3+} ion competing for oxidizing ligands.

In contrast to other transition metals, Mn^{2+} demonstrates a resistance to becoming oxidized to higher valence states while instability characterizes trivalent manganese. The prototypical transition metals iron and copper are characterized by their lower valence state having greater redox potential, higher reactivity, and greater readiness to catalyze free-radical generating reactions. The marked similarities of manganese to closed shell ions may lead to its acquisition of redox bioenergetics uncharacteristic of transition metals. This highlights the importance of considering the electrochemical profiles of different manganese ionic species in evaluations of their biological activity.

Although divalent manganese has no intrinsic oxidant properties, the trace presence of Mn^{3+} is sufficient to promote Fenton-type redox cycling in its interaction with divalent manganese or iron. In contrast, the interaction between Fe^{3+} and Mn^{2+} is a stabilizing one that may attenuate the process of ROS generation and iron oxidation. These findings suggest a mechanism by which reports of inhibition of iron-based ROS production by manganese and protection against oxidative stress may be reconciled by descriptions of the apparent ROS-generating capacity of manganese and the potential to inflict oxidative damage.

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