The Virtual Free Radical School

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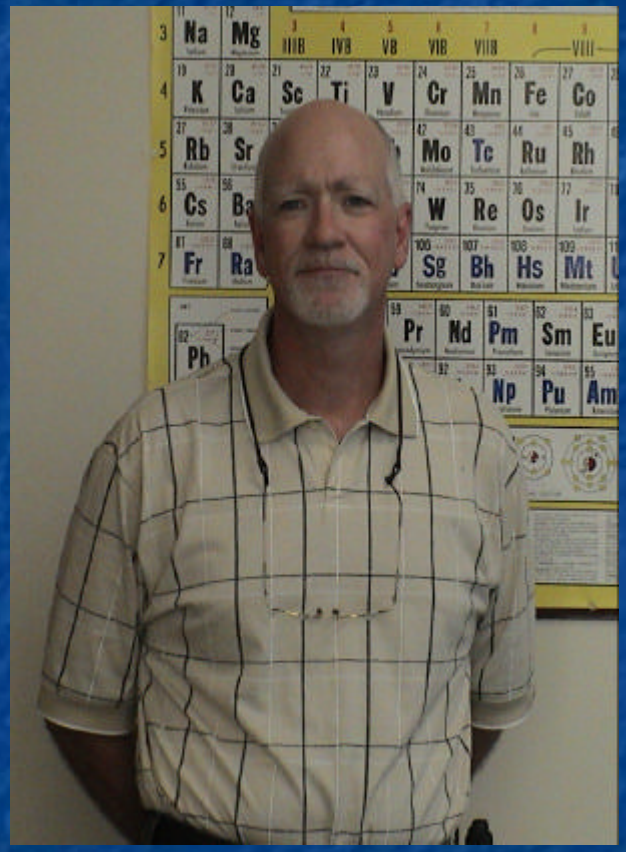

Peroxynitrite: Scavenging for Survival

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Introduction, Background and Essential Rationale

- The rate constant for the reaction of superoxide and nitric oxide (~ 10^{10} M^{-1} s^{-1}) virtually assures that peroxynitrite will be formed in any cell or tissue where both radicals exist simultaneously. (The earliest experiments which demonstrated that SOD enhanced the half-life of "EDRF/NO" remain as the most compelling evidence for peroxynitrite production *in vivo*.)

- While other pathways for *in vivo* nitration may exist, the NO_x species required for such pathways must originate from NO itself (with the exception of limited dietary intake of nitrite and nitrate). Thus, the "opportunity" to form peroxynitrite preceeds all other pathways.

- Because NO is constitutively produced *in vivo* and is uncharged and freely diffusible, production of peroxynitrite is more likely to be governed by local production of superoxide, *i.e.*, a function of NO diffusing into a cellular compartment where superoxide is being generated and retained due to its negative charge.

Introduction, Background and Essential Rationale (Continued)

- Peroxynitrite formation *in vivo* may be "incidental", *e.g.*, NO diffusing into a compartment containing superoxide or "directed", *e.g.*, turning on of a superoxide producing enzyme like NADPH oxidase plus a NOS. In addition, NOS's can become uncoupled and produce both superoxide and NO; at present, it's not clear whether peroxynitrite formation by uncoupled NOS's is "by design" or a merely a consequence of imperfect enzyme control.

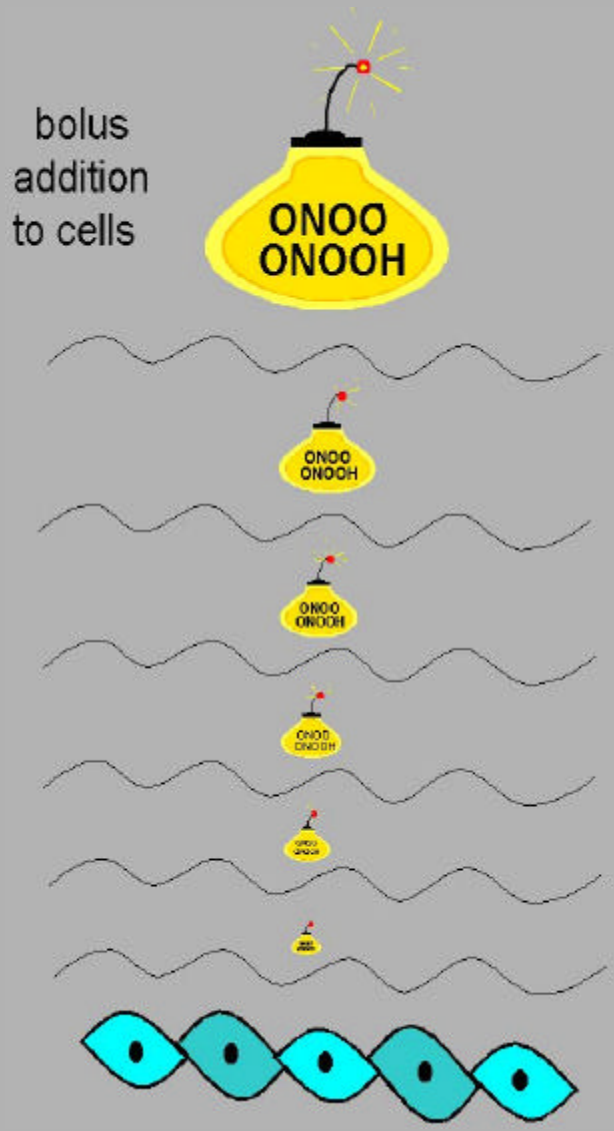
- In the presence of NO, H_2O_2 can drive SOD's in the reverse direction and "co-op" them into becoming peroxynitrite synthases. Thus, in some circumstances, H_2O_2-dependent toxicity may ultimately be mediated by peroxynitrite via a reverse reaction by SOD's.

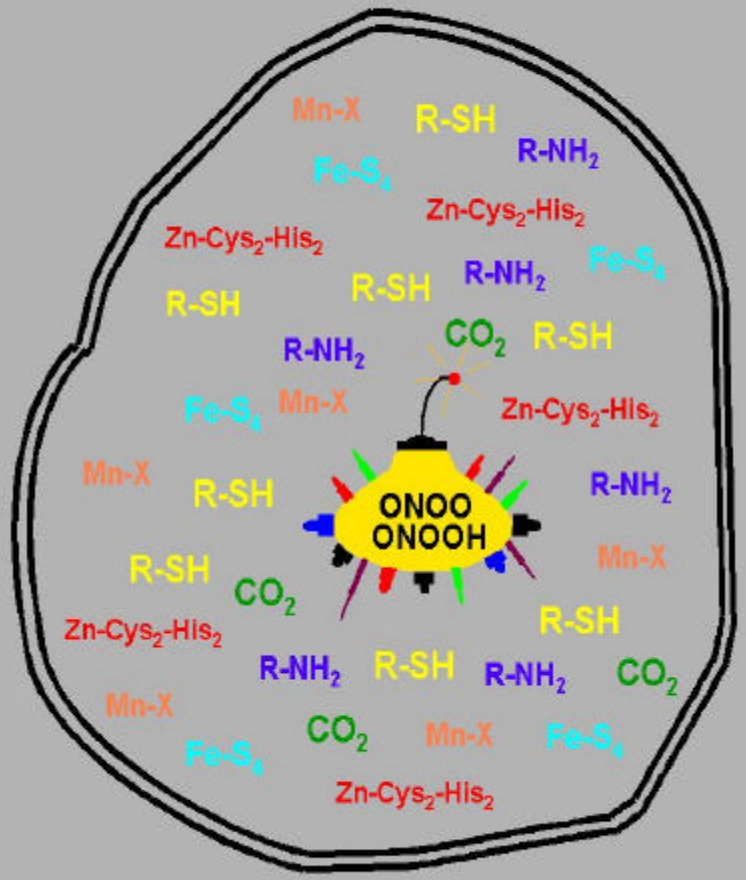
- Although the dose-response varies, and the actual mechanism of toxicity may vary as well, peroxynitrite has been shown to be toxic to many different cell types.

- Because of the diverse, beneficial physiological functions of NO, scavenging of peroxynitrite (as a means of preventing oxidative pathology) is preferable to indiscriminately inhibiting NO production with NOS inhibitors.

Peroxynitrite: fused bomb versus floating mine?

(see next slide for further explanation)





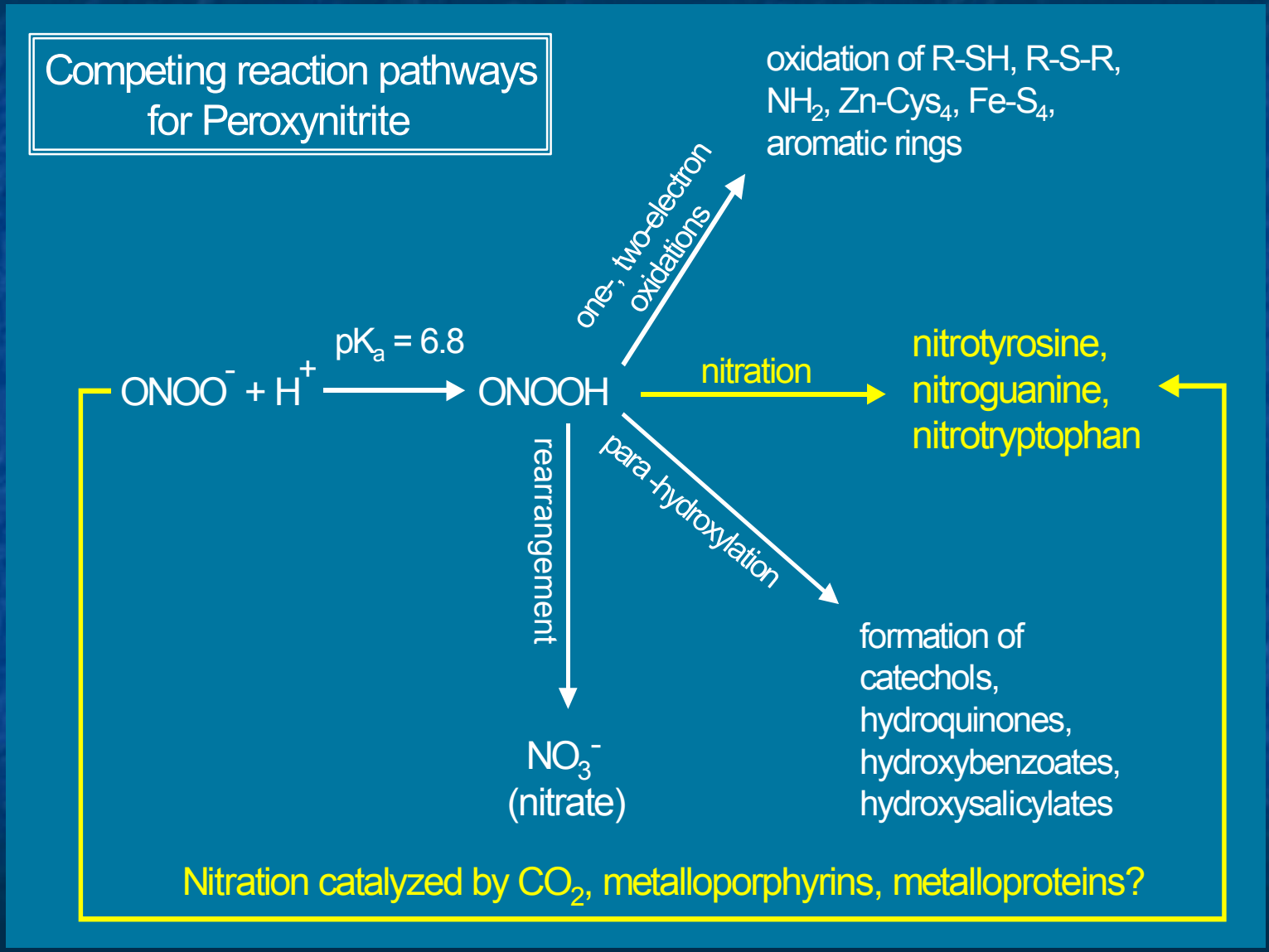
Intracellular formation of $ONOO^-$

Peroxynitrite: fused bomb versus floating mine?

(refer to illustration on previous slide)

- Peroxynitrite has a half-life of ~ 1 s at pH 7.4 and 37 ° C. Thus, it will either "self-destruct" and oxidize proximal target molecules via production of secondary reactive species or harmlessly decompose if no oxidizable targets are present. This "fused bomb" aspect of peroxynitrite means that only a small fraction of a bolus addition to cells will actually reach the cells and interact with intracellular targets.
- Peroxynitrite also acts like a "floating mine" with regard to direct-reacting target molecules such as carbon dioxide, thiols (R-SH, iron-sulfur clusters, zinc fingers, *etc*.), amines, and both free and bound transition metals. In the "crowded" environment of a cell peroxynitrite will tend to be totally consumed via direct reactions with a number of different direct-acting biomolecular targets.

While nitration of phenolics such as tyrosine represents the most "recognizable" modification, peroxynitrite modifies a diverse group of biomolecules, often irreversibly.



Fundamentals of Peroxynitrite Scavenging

- The types and extent of modifications (of biolmolecules) produced by peroxynitrite are highly dependent on the relative concentrations of the target molecules present AND the rate constants for reaction of peroxynitrite with each target molecule (see Slide 6).

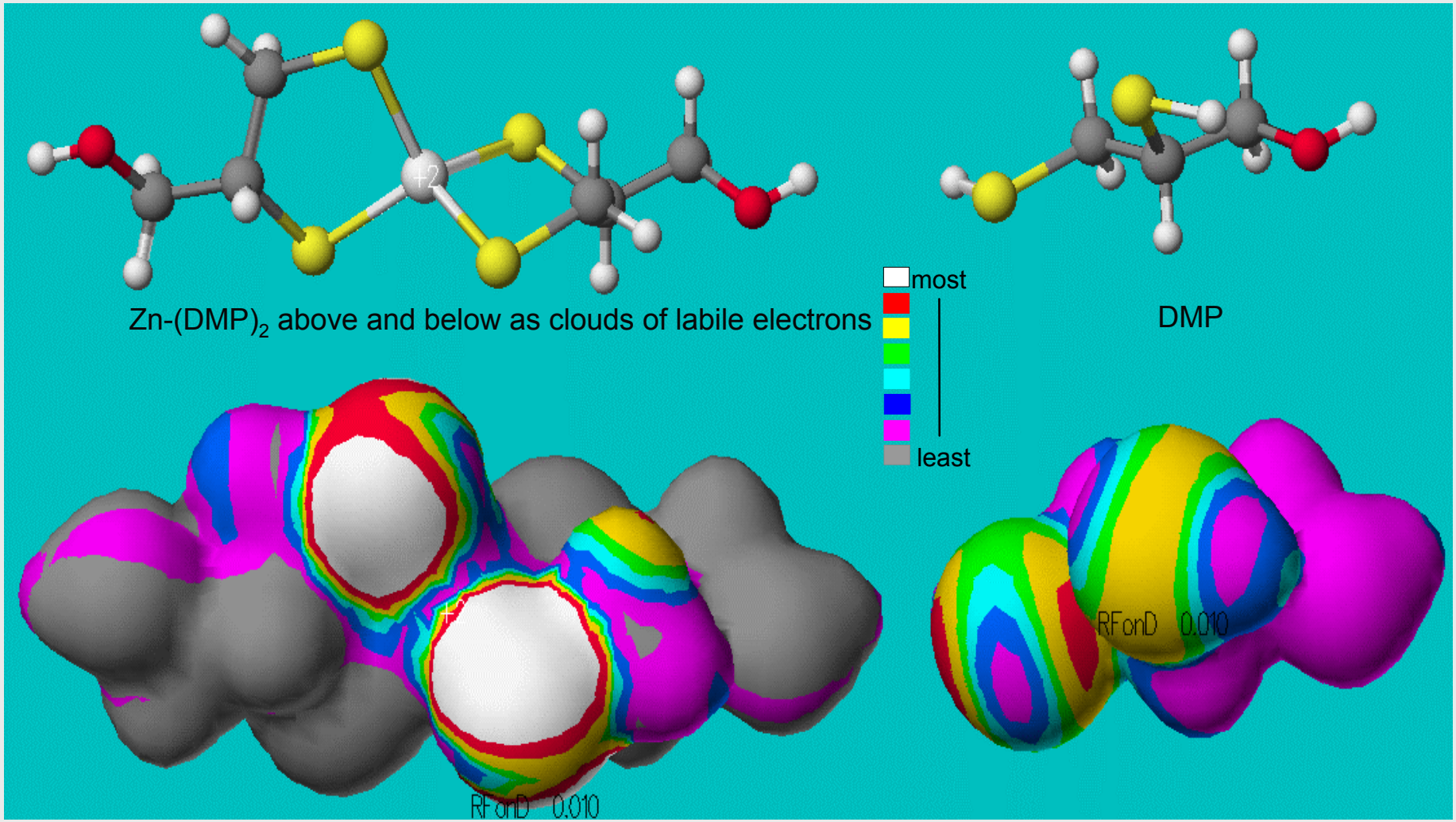
- For example, in a simple buffered system at pH 7.4 and 37 degrees C, containing two direct-reacting targets--targets “A” and “B”--which each react with peroxynitrite at the same rate, and are present at the same concentration, 50% of the added (or endogenously produced) peroxynitrite will react with target “A” and 50% with target “B”. This is based on the simple ratio of [A]/[A+B] x 100%, *i.e.*, 1/(1+1) = 0.5.

- If [A] is increased two-fold relative to [B], then [2]/[2+1] = 0.66 meaning that 66% of added peroxynitrite will react with “A” and 33% with “B”. The same relationship can be used even when the rate constants for reaction with peroxynitrite differ. In that case, the “effective” concentration of “A” would be determined by multiplying [A] and [B] times their respective rate constants before ratioing them.

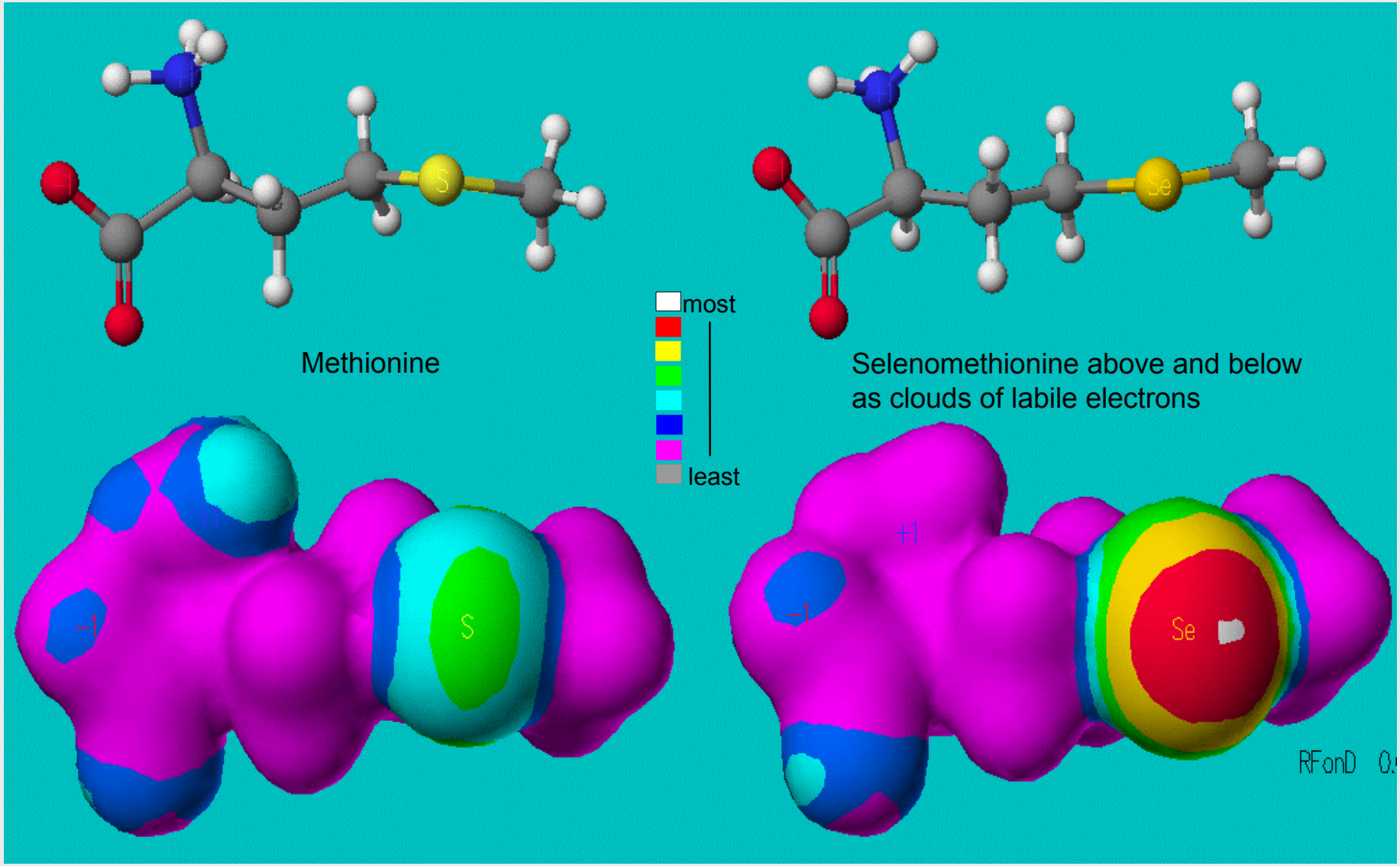
Fundamentals of Peroxynitrite Scavenging (Continued)

- The net effect of this "concentration x rate constant" formula is to permit comparisons between targets which differ dramatically in true concentration. For example, GSH is present at a concentration of 1 mM and has a rate constant of ~ 5 x 10^3 M^{-1} s^{-1} whereas the thiolate-containing tyrosine phosphatase CD45 has a rate constant (with peroxynitrite) of 2.2 x 10^8 M^{-1} s^{-1}-- 44,000-fold faster than the reaction of peroxynitrite and GSH. Thus, if this enzyme is present at a concentration of only 23 nM (nanomolar), it will effectively compete with 1 mM GSH for available peroxynitrite (see Slide 11).
- In biological systems, rarely, if ever, would things be so simple. However, this type of analysis does permit us to make estimates of the rate constants and required concentrations of potential peroxynitrite scavenger compounds.
- The reaction of peroxynitrite with carbon dioxide (CO_2) (rate constant ~ 5 x 10^4 M^{-1} s^{-1}) complicates this simple scheme. The concentration of dissolved CO_2 is ~ 1 mM in mammalian tissues, thus a scavenger present at 10 uM (micromolar) would need to react with a rate constant of 5 x 10^6 M^{-1} s^{-1} to compete with CO_2 for 50% of peroxynitrite added or formed endogenously. However, a scavenger which also reacts with the CO_2 adduct (nitrosoperoxocarbonate or NPC) could overcome this concentration and/or rate constant problem.

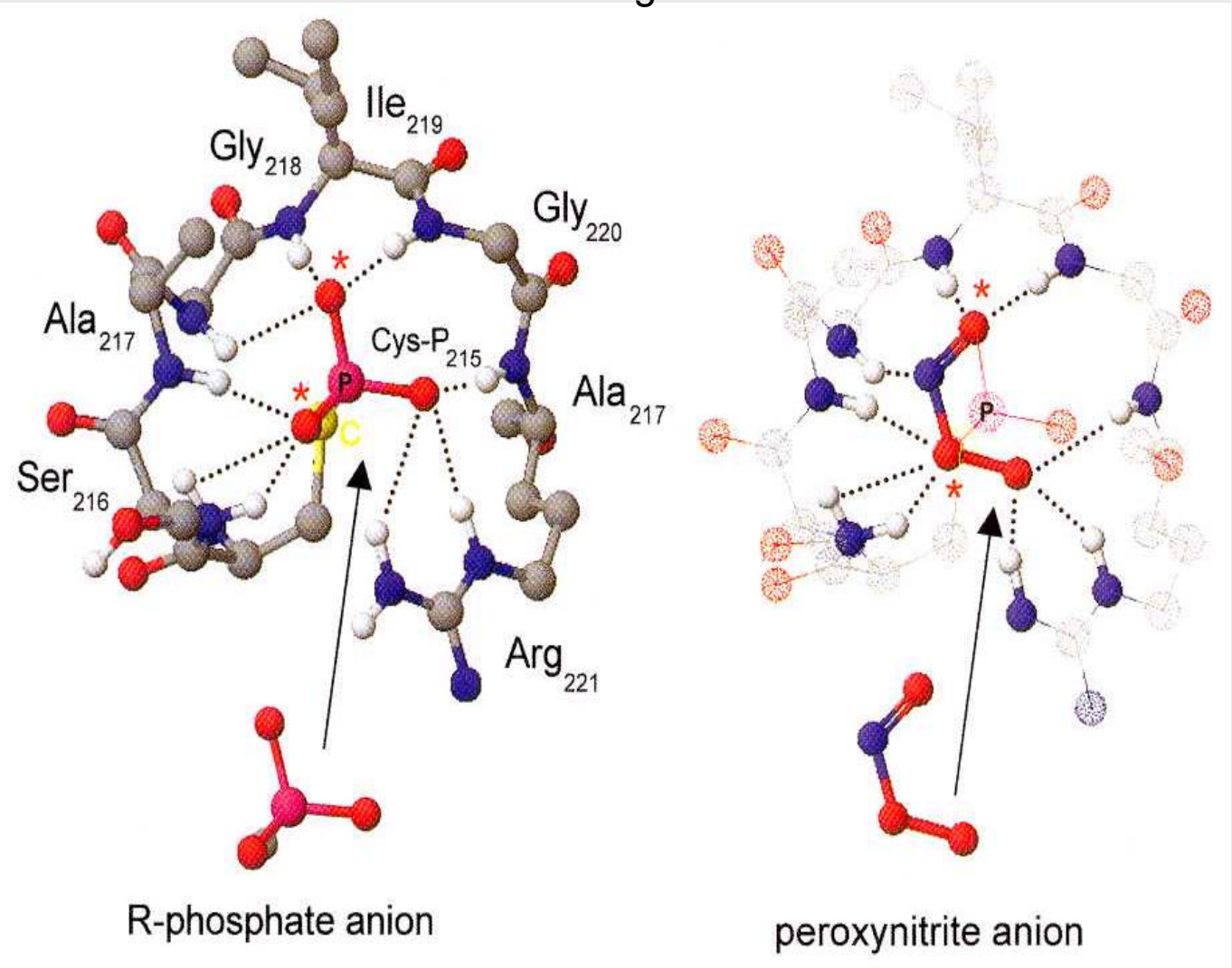
Relative susceptibilities of dimercaptopropanol (DMP, on right) and the 2:1 complex of DMP with zinc (on left) to attack by one electron oxidants. Zn-$(DMP)_2$ reacts ~ 100-fold faster with peroxynitrite than does DMP alone. Zinc stabilizes the thiol groups toward autoxidation while simultaneously "labilizing" them toward reaction with peroxynitrite. This "labilization" can be seen by greatly enhanced susceptibility of the thiolates (in $Zn[DMP]_2$) to attack by one electron oxidants.



Relative susceptibilities of methionine and selenomethionine to attack by one electron oxidants. Such electronic modeling agrees with the relative reactivities of these compounds toward peroxynitrite and thereby allows us to predict which compounds have the potential to act as efficient scavengers.



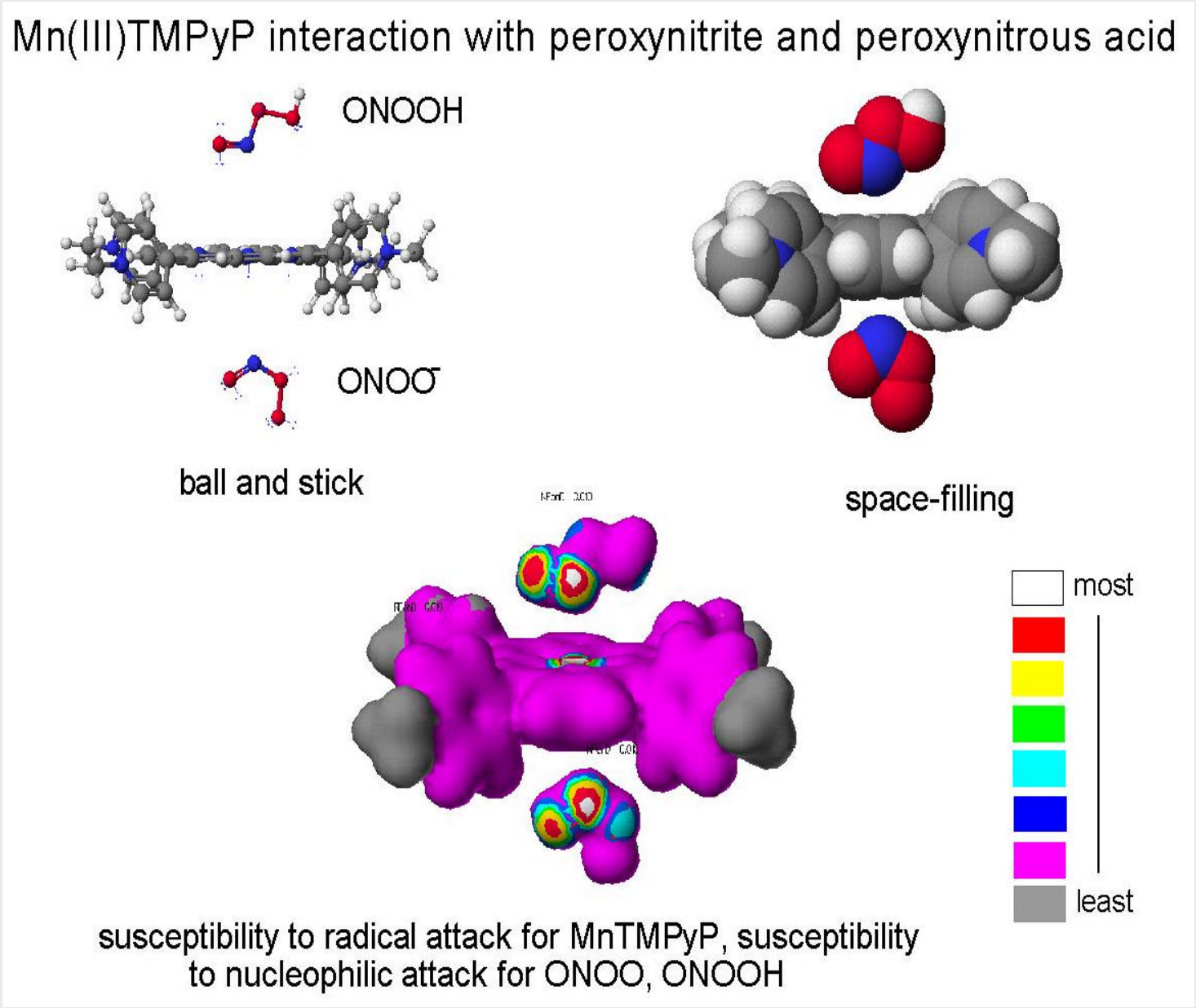
Peroxynitrite reacts very rapidly (rate constant = 2.2 x 10^8 M^{-1} s^{-1}) with the active site thiolate anion of some protein tyrosine phosphatases (active site of PTP 1B shown below). This reactivity is likely due to the fact that the active site thiol (labeled with yellow "C") is ionized at pH 7.4 and that peroxynitrite "resembles" the natural substrate phosphate in terms of molecular radii and charge.



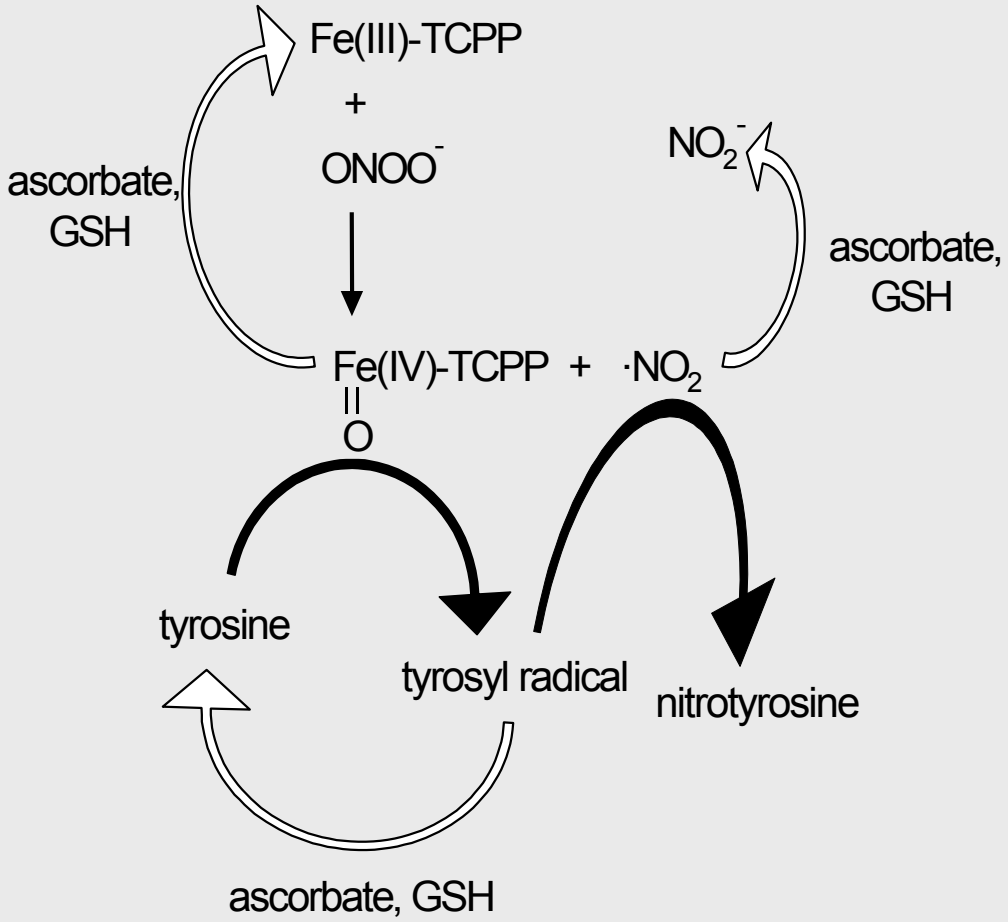
Fundamentals of Peroxynitrite Scavenging (Continued)

- Scavengers can be either sacrificial, such as GSH or other thiols, or catalytic, such as the redox active manganese or iron porphyrins. Thiols can donate two electrons thereby directly reducing peroxynitrite. Mn and Fe porphyrins act to "capture and redirect" the oxidative potential of peroxynitrite, followed by re-reduction of the metalloporphyrin at the expense of cellular reductants like ascorbate and GSH (see Slides 13-15).
- The term scavenger implies complete "absorption" or "quenching" of the oxidative yield of peroxynitrite. This is the case for direct reacting scavengers like GSH (or other thiols) which can donate two electrons to completely reduce peroxynitrite, but is not true for catalytic scavengers like metalloporphyrins; the latter require a two-step pathway involving cellular antioxidants to ultimately quench the reactivity of peroxynitrite.
- Direct-reacting scavengers react, as the name implies, directly with peroxynitrite anion or peroxynitrous acid; such scavengers (*e.g.*, thiols) increase the rate of peroxynitrite decomposition in proportion to their concentration. Indirect-reacting scavengers do not accelerate peroxynitrite decomposition but, instead, scavenge the secondary reactive species produced, *e.g.*, ascorbate and urate inhibit tyrosine nitration by reducing the reactive radical intermediates.

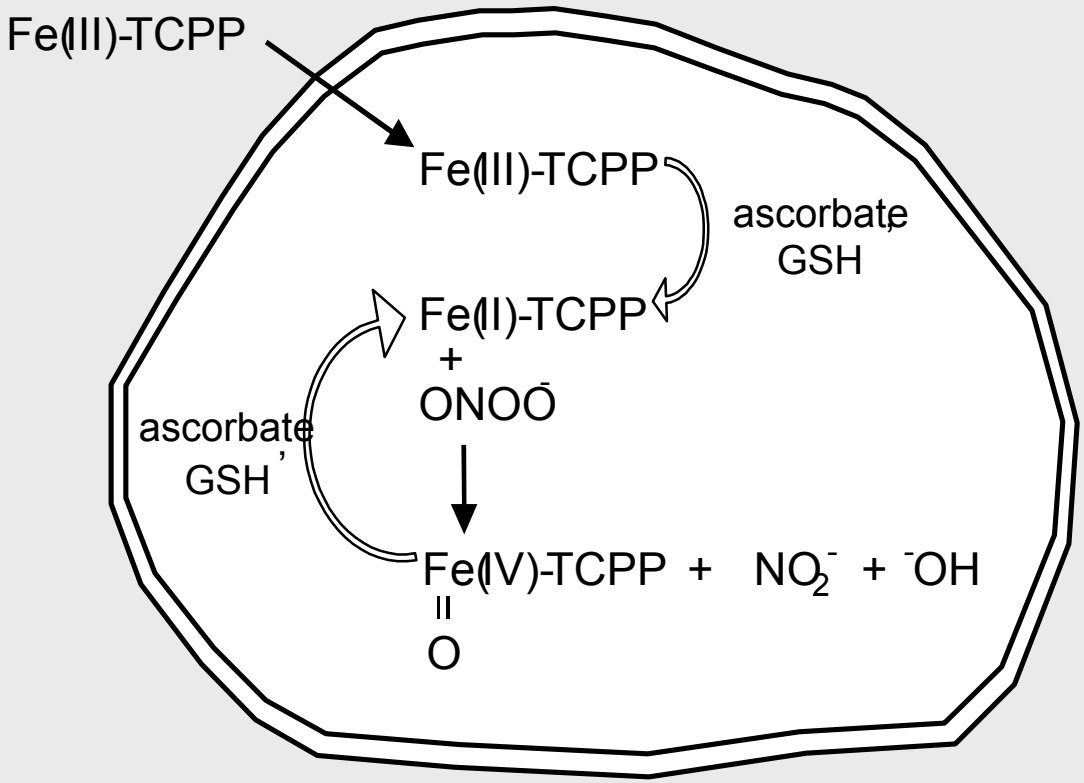
Relative atomic size and reaction susceptibilities of peroxynitrite and a Mn porphyrin



Some Fe and Mn porphyrins react rapidly enough with peroxynitrite to “capture” it and then “redirect” its reactivity toward replenishable cellular antioxidants. Fe and Mn porphyrins act in concert with antixoidants to quench the total oxidative yield of peroxynitrite catalytically. Ascorbate can reduce the +4 porphyrin, reduce nitrogen dioxide, and reduce tyrosyl radical. The fast reaction of Mn and Fe porphyrins with peroxynitrite, combined with the ability of ascorbate to rapidly quench reactive intermediates act synergistically to effectively quench the oxidative yield of peroxynitrite.



While the exact mechanistic details are not known, the Fe(III) and Mn(III) porphyrins may become reduced (and stabilized) intracellularly as their respective +2 forms. Fe(II) and Mn(II) porphyrins could then fully reduce peroxynitrite in one step, followed by re-reduction to the +2 state. This, together with the ability to also react with nitrosoperoxocarbonate, could explain the effectiveness of these compounds as peroxynitrite scavengers *in vivo*.



Intracellular

Scavenger Criteria

- Apart from the quantitative rate constant and target concentration aspects, there are several qualitative aspects which must be considered when designing or evaluating potential peroxynitrite scavenger compounds.

<u>The Ideal Peroxynitrite Scavenger Should:</u>

1. react very rapidly with peroxynitrite anion and/or with nitrosoperoxycarbonate,
2. quench or otherwise "contain" the reactive intermediates produced,
3. be rapidly regenerated under *in vivo* conditions or be non-toxic if irreversibly modified,
4. be stable under *in vivo* conditions, *e.g.*, Mn or Fe remain bound to porphyrin,
5. readily cross cell membranes and distribute evenly in tissues, and
6. not be inherently toxic or undergo toxic metabolism.

- As more is learned about how and where peroxynitrite is formed in vivo, and the specific reactive intermediates that are formed, more specific scavengers can be designed.

Summary

- Rate constants for some thiolate-containing enzymes, metal-thiolate compounds, and Mn and Fe porphyrins suggest that low molecular weight compounds can be designed which can react with peroxynitrite at near diffusion-limited rates and thereby effectively compete with all known reaction pathways *in vivo*.

- From the standpoint of *in vivo* therapuetic agents, catalytic savengers which utilize abundant cellular antioxidants are preferable to sacrificial scavengers which cannot be regenerated. However, such sacrificial scavengers remain useful for probing biochemical mechanisms in well-defined model systems. Compounds such as selenomethionine, GSH and zinc-thiolates are useful scavengers for *in vitro* studies including those involving cultured cells.

- Fe and Mn porphyrins have the advantage of being effective at much lower concentrations due to their high rate constants and ability to be recycled. Porphyrins also appear to react with nitrosoperoxycarbonate, eliminating the need to out-compete CO_2 in order to be effective.

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Selected Reading, Continued

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