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Reduction of Hexacoordinate Globins by Cytochrome B5

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Since 2000, several hexacoordinate globins have been discovered. The function of these proteins is still a matter of debate. In general, hexacoordinate globins autoxidize at rates much faster than these of typical oxygen carrier proteins such as hemoglobin and myoglobin. As most putative functions of these proteins involve the ferrous form (Fe^{2+}) or the ferrous oxy form (Fe^{2+} O_2), the biological activity of these proteins may require the presence of a suitable reducing system.

In the case of hemoglobin/myoglobin, the cytochrome b5/cytochrome b5 reductase system converts ferric hemoglobin (methemoglobin) back to ferrous hemoglobin. Based on the structural similarity of globin X, neuroglobin, and cytoglobins to hemoglobin, we hypothesize that cytochrome b5 can effectively reduce these proteins from the ferric form to the ferrous form.

To test this hypothesis, we reacted oxidized globins with cytochrome b5, b5 reductase, and NADH under anaerobic conditions. Specifically, we examined human cytoglobin, and the zebrafish proteins cytoglobin 1 and 2, neuroglobin, and globin X. Globins were oxidized with excess potassium ferricyanide, which was subsequently removed with a filtration column. Oxidized globins were then mixed with b5 and b5 reductase. Following addition of NADH, spectra from 450-700 nm were measured for several minutes to monitor the extent of reduction.

The data gathered indicate that cytochrome b5 is capable of reducing all globins tested. The hexacoordinate globins were reduced at rates comparable to, if not faster than, methemoglobin. This work provides evidence to suggest that the physiologic b5 reductase system may be able to preserve these globins in the reduced state *in vivo*.

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Kinetic Characterization of Sulfenic Acid Reduction in 1-Cys Peroxiredoxins by Ascorbate

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Peroxiredoxins (Prxs) are Cys-based peroxidases with remarkable catalytic efficiency that can be divided into 1-Cys or 2-Cys, depending on the number of Cys residues involved in catalysis. Initially, reduction of Prx was described to be strictly dependent on thiols, but later we showed that ascorbate can also reduce the sulfenic intermediate of 1-Cys Prx (1-Cys Prx-SOH) in various organisms [1]. Here, the kinetic characterization of 1-Cys Prx-SOH reduction by ascorbate is described. Reduction of 1-Cys Prx-SOH by ascorbate was initially analyzed using an enzyme from *A. fumigatus* (AfPrxA) that is 37% similar to PRDX6 (human 1-Cys Prx). H_2O_2 levels were determined by means of a specific electrode (Free Radical Analyzer 4100), using a steady-state bi-substrate approach. AfPrxA decomposed H_2O_2 with good efficiency (k_{cat}/K_M = 7.4 x$10^3M^1.s^1$), through a Bi-Bi Ping-Pong mechanism. To further support these findings, a second, independent approach was also employed: competition between dichlorophenolindophenol (DCPIP) and AfPrxA-SOH for ascorbate. DCPIP is a redox sensor, whose blue color is lost when reduced and its second-order rate constant with ascorbate is 718 $M^1.s^1$, enabling the determination of the rate constant of the reaction between AfPrxA-SOH and ascorbate: 1.5 x $10^3M^1.s^1$. Therefore, by two independent approaches, we showed that ascorbate efficiently reduced AfPrxA-SOH. Next, the reductions of 1-Cys Prx SOH in other organisms (bacteria, yeast and plant) were also investigated and again the constants were in the 10^3M^1.s1 range. We conclude that the reduction of 1-Cys Prx-SOH by ascorbate is probably relevant in the subcellular compartments in which this reductant is present at millimolar levels. We are currently studying the reduction of 1-Cys Prx-SOH by ascorbate in other proteins, which could open new perspectives in cellular redox processes in vivo.

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Site-Specific Radical Formation in DNA Induced by the Potent Oxidizing Agent HOCL, Using ESR, Immuno-Spin Trapping, LC-MS and MS/MS

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Hypochlorous acid (HOCl) is a powerful oxidant generated by the myeloid cells and is likely to contribute to damage mediated by these inflammatory cells. It is a major end product of oxygen metabolism in activated phagocytes and thus its reaction with cellular constituents is of biochemical and clinical interest. The haem enzyme myeloperoxidase, catalyzes the production of HOCl, from hydrogen peroxide and chloride. In addition to its physiological source, HOCl can also be generated by chlorine gas from accidents or a potential terrorist attack. HOCl can cause extensive damage to macromolecules like DNA. In this study we examine the ability of HOCl to damage DNA using spin-trapping, ESR and MS and MS/MS. The radicals generated are trapped by DMPO immediately upon formation. The DMPO adducts formed are initially EPR active but are subsequently oxidized to the stable nitrone, which can then be detected and visualized by IST and further characterized by MS and MS/MS. Here we report the DNA radicals (from cytosine, guanosine and adenine bases) detected from the treatment of DNA with either endogenous or exogenous HOCl in the presence of DMPO.

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Mechanism of the Reaction of Peroxynitrite with Mn-Superoxide Dismutase: Nitration of Critical Tyrosine-34

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Manganese superoxide dismutase (MnSOD) is an antioxidant enzyme that acts as a superoxide detoxifier. Superoxide can react with nitric oxide to lead peroxynitrite and this reaction, that takes place at near diffusion limited rates, can compete with MnSOD for superoxide. One of the most important bio-markers for peroxynitrite formation in vivo is tyrosine nitration, a post-translational modification that can alter both the structure and function of a protein. One of the most widely reported nitrated protein is MnSOD, which, when nitrated at a specific tyrosine (Tyr34), leads to a complete inactivation. The reaction between MnSOD and peroxynitrite occurs via a direct reaction with a second order reaction constant of ~$1.0x10^5 M^{-1}s^{-1}$, according to reports by Quijano et al. Recently, a report by Surmeli et al, reported a constant with a value of several orders of magnitude inferior than the one previously reported and proposed a different mechanism of reaction. Given the controversy, is the aim of this study to settle the value of the second order reaction constant, using both direct and indirect spectroscopic methods. The results obtained showed a value of $1.92x10^4 M^{-1}s^{-1}$, which is in accordance with a metal-catalyzed reaction, involving the oxidation of the metal center: $E\text{-}Mn^{III} + ONOO^- \rightarrow E\text{-}Mn^{IV}{=}O + NO_2^{\bullet}$. In order to clarify the mechanism proposed by the different groups, theoretical methods (QM/MM) were also used, which allowed us the study, at a molecular level, of different approaches to the reaction between MnSOD and peroxynitrite, focusing on the active site of the enzyme. The results obtained by both experimental and theoretical measurements are consistent with a metal-catalyzed reaction that involves the formation of a $Mn^{IV}{=}O$, that explains the site-specific nitration of the nearby Tyr34, located 5Å away from the metal.

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The Advantages of Expressing Dose as Moles Per Cell in Cell Culture Studies: 1,4-Benzoquinone, Oligomycin A, and High Dose Ascorbate as Examples

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The biological consequences upon exposure of cells in culture to a xenobiotic are not only dependent on the cell type, but are also determined by the physical aspects of experiments. We hypothesize that for many xenobiotics (*i.e.* toxicants, pharmaceutical agents, and biochemical tools) specifying dose as moles per cell in addition to extracellular concentration will improve the repeatability of experiments by providing an absolute basis for experimental design and data interpretation, increasing the information content and usefulness of the data. To test this hypothesis we used 1,4-benzoquinone, oligomycin A, and high dose ascorbate as model xenobiotics. A549, MDA-MB231 and MIA PaCa-2 cells were exposed to 1,4-benzoquinone (1,4-BQ), the Effective Dose to achieve 50% survival (ED_{50}) in clonogenic assays was determined as an indicator of proliferative capacity; intracellular [ATP] was measured as a biochemical marker of metabolism. When the dose of 1,4-BQ was specified as moles per cell, ED_{50} was independent of the physical conditions used (*i.e.* number of cells and volume of medium); mechanistic behavior could be directly deduced from the changes in intracellular components *vs*. dose. Clonogenic survival correlated directly with intracellular [ATP]. MIA PaCa-2 cells were exposed to oligomycin A and high dose ascorbate under a range of physical experimental conditions. As with 1,4-BQ, the effects of oligomycin A and high dose ascorbate on intracellular [ATP] were cell density dependent; expression of dose as moles per cell allowed direct, absolute comparison of toxicity between the many different experimental set-ups. This approach is applicable to a wide range of xenobiotics, reduces ambiguity between different experiments, affords better experimental design, and increases the ease of translation of *in vitro* results to *in vivo* settings. We recommend that in addition to traditional expressions of dose, dose as moles per cell be reported. Supported by NIEHS P30ES05605, P42ES013661, NIH R01GM073929, R01CA169046, and P30CA086862.

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Aggregation and Fragmentation of CuZn-SOD by Peroxyl Radicals

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It has been well documented the role that superoxide dismutase (CuZn-SOD) plays in oxidative stress conditions. This enzyme, throughout a redox mechanism, dismute superoxide anion ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) and oxygen (O_2). Additionally, CuZn-SOD plays a role in the developing of Amyotrophic Lateral Sclerosis (ALS), a devastating motor neuron degenerative disease whose etiology and pathogenesis remain poorly understood. Not only specific mutations on CuZn-SOD are associated with familial ALS but also cross-linked dimers of CuZn-SOD are detected in sporadic, as well as, familial ALS. Studies on radical-induced structural changes on CuZn-SOD-1 will contribute in this regard. In the present work we studied the peroxyl radical-induced oxidation of human and bovine SOD (hSOD, bSOD, respectively). Native hSOD and bSOD differ in few amino acids, and have similar 3D structures. hSOD has a single tryptophan residue (Trp) in position 32 and doesn't have a tyrosine (Tyr) residue in its sequence. By contrast, bSOD doesn't have Trp residue and has one Tyr in position 108. Both enzymes were exposed to (2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) 100mM, during 3 hours at 37°C, and changes in size were followed by SDS-PAGE and size exclusion chromatography. hSOD presented some fragmentation and no aggregation, although Trp was oxidized to formylkynurenin (FNK) and kynurenine (Kyn). In contrast, bSOD presented fragmentation, aggregation, oxidation of Tyr and formation of di-Tyr. The results indicate that both proteins are being oxidized by peroxyl radicals in their own aromatic residues but with different oxidation profiles. bSOD suffered mainly aggregation dependent on Tyr oxidation, while no aggregation, but fragmentation dependent on Trp oxidation, was observed for hSOD. These results show that small differences in the sequence of CuZn-SOD impact on its susceptibility to peroxyl radical-mediated aggregation as well as fragmentation, with potential loss of dismutase activity.

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