MITOCHONDRIAL CYTOTOXICITY OF CARBON MONOXIDE

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Introduction

The intracellular effects of carbon monoxide are, to say the least, obscure. It seems fairly clear that with a structure closely approximating oxygen, a likely place for action would be the intracellular molecules with a high affinity for oxygen. These molecules are the hemoproteins, and it is in their possible interaction that carbon monoxide's intracellular toxicity may lie. What follows is an overview of these hemoproteins.

Cytochrome Oxidase

General Biochemical Structure - Functional Organization. By way of an introduction, one can look on the cell as a system of membranes organized for regulation primarily by the specificity of what passes through those membranes. The initial barrier to compounds entering the cell is the cytoplasmic membrane whose receptors for hormones, such as insulin or glucagon, regulate the metabolism of the cytoplasm. The mitochondrial membrane offers further regulation by relying on specific shuttles to ferry ATP, citrate, and glycerol phosphate out of the fatty acid-carnitine complex, NADH pigments, etc., into the inner matrix of the mitochondria. Inside the inner membrane of the mitochondria, further reactions of the Krebs cycle catalyze oxidation-reduction changes that generate the $NADH + H^+$ that can start the successive reduction of the electron transport chain components that culminates in the generation of ATP from ADP and inorganic phosphate. The ultimate determiner of the rates of cellular catabolism lies in the regulation of oxidative phosphorylation through respiratory controls (the coupling of ATP synthesis with electron transport). A key enzyme in the electron transport chain is the cytochrome oxidase, which catalyzes the reduction of oxygen to water.

Oxygen Utilization. After an estimated billion years of anaerobic living, the ability to utilize the high potential electron acceptor oxygen allowed the development of metazoan life. Without oxygen, glucose catabolism generates 2 ATP/mole utilizing a 220 mv potential drop (-400 mv to -180 mv), but requires the generation of a relatively strong acid. By use of oxygen as the terminal acceptor in electron transport, the efficiency of glucose catabolism was elevated to 38 ATP/mole, with the generation of carbon dioxide, not only a strong acid, but also a buffer.

The Perils of Oxygen Utilization. Use of oxygen, however, generated a number of problems for the cell, which took a long time to be solved in the evolution of life. First, electrons come down the electron transport system one at a time, yet it takes four electrons to reduce an oxygen molecule. Second, the intermediate reduction steps of oxygen are very toxic. Adding a single electron generates the superoxide radical - a very reactive species - so potent that all aerobic life necessitates a detoxification system involving the enzyme superoxide dismutase. Superoxide dismutase catalyzes the formation of H_2O_2 and O_2 from two moles of O_2^-. The elegant molecular studies of this enzyme by Fridovich and his co-workers[1] indicate that just two classes of protein, one microbiological and one found in metazoa (exclusive of their mitochondria), have been retained in all aerobes.

Addition of a second electron generates the peroxide molecule. Peroxide is destroyed by multiple enzymes, catalases, and peroxidases which have appeared during evolution. It is interesting to note that the polymorphonuclear leukocyte may use the superoxide radical or the peroxide in the process by which it kills bacteria in their phagocytic vacuoles. Adding the third electron produces an

unstable state for which no enzyme has been detected, and addition of the fourth electron results in reduction to the highly stable H_2O.

The Structures of Cytochrome Oxidase. The requirements to reduce oxygen without generating unstable and very toxic intermediates were met by a complex molecule, cytochrome oxidase. The molecule has a number of properties which make it unique and difficult to study. It contains unique hemes, heme a and heme a_1, which are formed from protoporphyrin as other hemes, but which contain aldehyde and lipid isoprenoid side chains. Each molecule contains two hemes with the same structure, but with different electronic environments, so each reacts differently.[2] The molecule also contains two copper atoms/mole, again unique for intracellular proteins. The molecule is membrane-bound and closely associated with a phospholipid found only in the inner mitochondrial membrane. That lipid is cardiolipin.

Work in our laboratory has established that synthesis of cardiolipin is essential for the function, but not the synthesis, of cytochrome oxidase in bacteria. (Fan and White, unpublished results.)

Biosynthesis of Cytochrome Oxidase. As might be expected, the synthesis of this complex molecule is almost as mystifying as its structure. In yeasts, which are both eukaryotic and genetically versatile, this huge molecule consists of seven to nine non-identical subunits, four or five of which are coded for in the nuclear DNA (translation blocked by cycloheximide), and at least three of which are coded for in the mitochondrial DNA (translation inhibited by chloromycetin).[3] Unfortunately, the detergent treatment necessary to separate the subunits also removes the prosthetic groups. In the intact membrane, two heme a's can be distinguished spectrophotometrically. Reduced cytochrome a shows absorption maxima at 439 and 600 nm; cytochrome a_3, when reduced, shows absorption maxima at 443 and 603 nm. During the synthesis of the cytochrome oxidase molecule, the ratio of a_3 : a goes from near 0.15, when cytochrome oxidase is first detectable, to 1.0 when the mitochondria are fully functional, indicating that the electronic environment necessary for the a_3 function develops more slowly than the a type environment.[4] Studies in our laboratory have documented a remarkable effect of carbon monoxide on the synthesis of cytochrome oxidase. Carbon monoxide induces a four-fold increase in cytochrome oxidase concentration on *Haemophilus aegyptius* as compared with air.[5]

Function of Cytochrome Oxidase. To date, studies of the molecule with four prosthetic groups (heme a, heme a_3, Cu_1, Cu_2) are indirect. Unfortunately, data are readily generated, but the interpretation is difficult, as the voluminous literature can attest. In general, the molecule can be isolated and its reaction with the electron donor cytochrome c and its acceptor oxygen studied. In an elegant series of studies, Dr. Lucile Smith established the importance of how the assay is performed if the rate of cytochrome c or oxygen diffusion is not to be rate-limiting.[6,7]

The molecule has been studied using three major tools: spectrophotometrically for the a - a_3 chromogens, with electron spin resonance for certain copper complexes, and lately, by means of redox titrations in attempts to unravel the complexities of the molecule.[8-11]

Allosteric Molecules. Cytochrome oxidase is possibly the premier allosteric molecule, where reaction with one mole of substrate drastically changes the binding of further substrate molecules. This is detectable in plots of rate versus substrate concentration, where simple enzymes follow the Michaelis-Menten rectangular hyperbola. Allosteric enzymes produce a sigmoid curve in the rate versus substrate concentration curve. For example, to increase a simple enzyme's rate from 10% to 90% of V_{max} requires an 81-fold increase in substrate concentration, whereas the allosteric enzyme can increase to 90% of V_{max} with a nine-fold increase in substrate.

Not only are there heme a - heme a_3, Cu_1 - heme, Cu - Cu interactions, but also the ATP level apparently affects the binding to hemes and Cu. Currently, the most popular model of oxygen reduction bridges the oxygen molecule between the heme a_3 and a Cu, where it is reduced in two steps.[4, 12, 13]

Reaction of Carbon Monoxide with Cytochrome Oxidase. The addition of carbon monoxide to the exceedingly complex system further mystifies the system. The increasing ATP enhances carbon monoxide binding.[14] The binding of carbon monoxide clearly depends on the redox level of the

prosthetic groups; reduced Cu^{++} and heme Fe^{+++} more avidly bind carbon monoxide. The redox state in turn depends on the oxygen concentration. Grossly, carbon monoxide and oxygen act as competitive inhibitors. The fact that the carbon monoxide apparently interacts with intermediate complexes in the oxygen reduction makes the understanding of carbon monoxide effects exceedingly difficult.[8, 13]

Another inhibitor which acts like cyanide or some volatile isocyanates has been found in fresh cigarette smoke. This inhibitor also affects the carbon monoxide binding by changes in the steric configuration of the reactive structure.

Volatile Cytochrome Oxidase Inhibitor in Tobacco Smoke. Dr. Lucile Smith of Dartmouth Medical School detected the presence of a very powerful inhibitor in tobacco smoke. We were able to develop an assay using the cytochrome oxidase from the bacterium *Micrococcus denitrificans*, which is astonishingly similar to the mammalian enzyme,[6, 7] and with this to show that the inhibitor was related specifically to cytochrome oxidase in a clear dose-response relationship. The inhibitor was present in the gas phase of tobacco smoke with a half-life in water of 12 hours. Its activity paralleled that of the cyanide or cyanate levels of the gas phase of smoke using different smoking parameters or different tobaccos. Activated charcoal or specific filters can remove this inhibitor. Attempts to fractionate smoke and identify the factors are currently under study at the University of Kentucky in the laboratory of Dr. T. T. Lillich. This smoke inhibitor is clearly distinct from carbon monoxide, but it has marked effects on cytochrome oxidase.

Localization of the Cellular Effects of Carbon Monoxide. Clearly, the major immediate effect of carbon monoxide results from its high affinity for hemoglobin, as discussed by Dr. Rink in this Symposium. The affinity of hemoglobin for carbon monoxide is about 250 times greater than for oxygen, and the results of acute carbon monoxide intoxication are those of a reduced effective blood supply. Even though relative affinity of carbon monoxide for cytochrome oxidase depends on the state of the molecule, as we have discussed, in mitochondrial or bacterial preparations a molar ratio of oxygen to carbon monoxide of 1 to 10 is sufficient for a 50% inhibition of the activity of the enzyme. However, carbon monoxide very likely has long-term effects on the cellular metabolism. The many studies reviewed in Kjeldsen's monograph[15] or in the Surgeon General's report on "The Health Consequences of Smoking"[16] link the presence of elevated carbon monoxide to the increase in vascular atheroma.

The P_{450} System An intracellular site for the activity of carbon monoxide that could affect steroid metabolism, and thereby possibly affect atheroma formation, might be the P_{450} system. The P_{450} system has an affinity for carbon monoxide between that of cytochrome oxidase and hemoglobin. The P_{450} system is actually a complex of proteins located in the endoplasmic reticulum that couples the electrochemical energy of reduced pyridine nucleotides to oxygen reduction and, in the process, accomplishes the hydroxylation of lipid molecules using a sort of miniature electron transport system. The terminal hemoprotein is called P_{450}, named for its absorptive maximum when reduced. This hemoprotein reacts with carbon monoxide. This enzyme complex has a number of unusual properties. It is inducible, and the induction can be relatively non-specific.[17] In addition, the P_{450} systems form a family of relatively non-specific enzymes.[18]

A multitude of drugs, pesticides, carcinogens, hormones, and other natural products are hydroxylated or o-methylated through the activity of a small number of relatively non-specific oxidases.[18] These are particularly important in the synthesis of steroids in their inactivation and in progressive increase in solubility. Elegant studies of these enzyme complexes in the adrenal gland show a shuttling between the endoplasmic reticulum, where the formation and condensation of squalene, the formation of lanosterol, the 17α and 2′ hydroxylation are localized; and the mitochondria, where the cholesterol is transformed to pregnandone and 11β hydroxylation, occurs.[19] Carbon monoxide has been demonstrated to inhibit P_{450} enzymes involved in N-oxide formation[20] or biosynthesis of cholesterol.[21] Consequently, it may be appropriate to speculate that interruption in the delicate balance between these P_{450} systems might somehow be related to increased atherosclerosis, which appears to be the major intracellular effect of carbon monoxide.

Conclusion

Carbon monoxide can seriously disrupt the function of organ systems by its effects on hemoproteins, primarily hemoglobin. At the cellular level, effects of carbon monoxide appear to exert less of the effect on the cytochrome oxidase, the terminal acceptor in the electron transport system, than on the other major hemoproteins of the cell, the P_{450} mixed function oxidase system. The P_{450} mixed function oxidase system is involved in synthesis and inactivation of critical steroid hormones. Since this intracellular system is inhibited by carbon monoxide, the mechanism of the carbon monoxide-induced increase in atherosclerosis may have its basis in this system. The cytochrome oxidase is, however, affected by other components of the gas phase of tobacco smoke.

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