On and beyond O_2 binding: hemoglobin and myoglobin revisited

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Abstract

The past decade of our laboratory's investigations into some aspects of heme proteins at first glance may seem marginal to the primary biological function of these proteins in O_2 binding and transport. But in fact, understanding long-range electron transfer mechanisms and the participation of hemoglobin and myoglobin in the generation of oxidative stress may provide relevant new biochemical insights.

We first consider hemoglobin, myoglobin and other heme proteins as potent reducing agents of Fe(III) and Cu(II) with concomitant generation of the methemeprotein under aerobic as well as anaerobic conditions. The mechanisms for electron transfer are short-range, outer-sphere, over the heme edge, and long-range from the heme to specific binding sites for Fe(III) and Cu(II) on the protein surface. Myoglobin was subjected to site directed mutagenesis to modify the surface Cu(II) binding sites, and the effects on the kinetics of protein oxidation were observed. Amino acid residues around the heme pocket of myoglobin were also mutated to change the redox character of the myoglobin and its kinetics, as well as to alter the binding of H_2O_2 and its activity as a peroxidase. Finally we will examine oxidative stress in vitro and in vivo as a function of the important role of reduced metals in the Fenton reaction for the generation of hydroxyl free radicals (•OH). Particular attention will be given to the importance of site-specific oxidation as a function of metal binding and the participation of heme proteins in this process.

Hemoglobin is the reducing agent responsible for the generation of Fe(II) in aerobic red cells[1]. The reduction of Fe(III)- and Cu(II)-chelates by hemoglobin is mediated by site-specific binding of the Cu(II) to β-Cys-93 and of Fe(III) to as yet undefined sites on both alpha- and beta- chains[2–8]. Ferricyanide is reduced by outer-sphere transfer. The reduction is enhanced by anaerobiosis and blocked by CO. Myoglobin has no specific site for Fe(III), and several sites for Cu(II)[7,8]. NMR identified several surface histidines on myoglobin which bind Cu(II) and also characterised the binding sites on leghemoglobin[9]. Cytochrome b_5 also site-specifically reduces Cu(II) and Fe(III)[10]. Isolation and characterisation of the reducing behaviour of alpha- and beta-subunits confirmed the activities of tetrameric hemoglobin[11]. The β-Cys-93 of hemoglobin reacts with Cu(II) to form a thyl free radical which in the presence of a variety of thiol compounds forms a disulphide which blocks the recycling of the redox metal[12].

Electron transfer mechanisms operate in accord with the Marcus equation. There is little agreement among investigators with respect to defining the path of electron transfer in myoglobin. In some systems the rate appears to be a function of the distance between the two redox centers assuming a uniform dielectric through which the electron passes. In other systems it would appear that the electron moves along the covalent bonds of the protein seeking the path of least resistance and 'jumping' through space where bonds are absent. Kinetic data from our mutants defines the appropriate pathways involved with each of the simultaneously operating multiple metal-binding sites[13].

The electrical potential between the two redox sites is an important parameter of the Marcus equation. It has been recognised that naturally occurring mutant myoglobins exhibit atypical oxygen binding properties and redox half-cell values[14]. We have measured redox kinetics in a series of myoglobins with mutated amino acid residues surrounding the heme. Not only are the rate constants for electron transfer altered, but significant 'gating' of potential can be shown by cyclic voltametry. Similar strategies were applied to modify the binding of H_2O_2 and alter the hydrogen bonding characteristics of myoglobin to mimic the structure and function of the similar protein, cytochrome *c* peroxidase. More than a doubling of peroxidase activity has been achieved.

A wide variety of physical and chemical conditions induce oxidative stress and the ensuing pathologies[15]. Oxidative stress initiated by the Fenton chemistry of Cu(II)-chelates and ascorbate in human red cells mimicked that observed in many hemoglobinopathies[16]. Methemoglobin was produced along with characteristic changes in membrane structure. The role of redox metals in the production of hydroxyl free radicals, •OH, in paraquat killing of *E. coli*, arrhythmias of ischemia reperfused rat hearts, and other systems has been characterised[17–20]. Redox metals bound to specific sites in the cells react with H_2O_2 and release •OH to oxidise the site or to form a reactive hypervalent metal as the active oxidant. Mechanisms for binding and displacing metals from labile sites to protect cells and tissues have been developed[21,22]. The insensitivity of many analytical techniques for measuring free radicals is a function of competition among metal binding sites, buffers, and chemical detectors and can be avoided[23–27].

Hemoglobin reduces redox metals as well as reacts with O_2 to form both superoxide and H_2O_2. We have characterised both •OH and amino acid side chains oxidised to carbonyl groups with hemoglobin in the presence of O_2 and Cu(II). Chinese hamster ovary cells exposed to low levels of oxidative stress exhibit novel behaviour. They are structurally and metabolically intact, but are unable to undergo cell division. DNA is not oxidised. The site of oxidation appears to be localised on the membrane of the cell and involves redox metals. The mechanism of this signalling pathway for the regulation of the cell cycle is under study.

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About hemoglobins, G6PD and parasites in red cells

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Introduction

Kaspar Winterhalter is a towering figure in more ways than one. For those who are fortunate to know him in person, he is a congenial analyst of any topic germane to human endeavour and to the relationships between human beings and human societies. For the scientific community, he is a scientist who has made substantial contributions to the study of hemoglobins and to the understanding of the nature and function of the extracellular matrix. My own interactions with him are full of pleasant memories, and they are related to the fact that both of us have been caught throughout our working life on the '*Grenzgebiet*' between biochemistry, molecular genetics and hematology. I feel greatly privileged to have been invited by our mutual colleague Ernesto Di Iorio to pay tribute to Kaspar on the occasion of his 60th birthday. I thought on this occasion it may be appropriate to review some examples of how hematology and molecular biology have been mutually beneficial in understanding human disease.

Hemoglobin

Kaspar Winterhalter carried out some pioneering work on the subunit structure of hemoglobin (Hb), starting some 30 years ago[22], and he has contributed impressively to the study of the pathophysiology of hemolytic anemias associated with abnormal hemoglobins. Therefore I shall mention some recent developments which I find pertinent.

Unstable hemoglobin disease. This condition, which hematologists refer to also by the phrase of Congenital Heinz Body Anemia (CHBA), is a classical example of