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Review

Multi-heme proteins: Nature's electronic multi-purpose tool ☆

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ABSTRACT

While iron is often a limiting nutrient to Biology, when the element is found in the form of heme cofactors (iron protoporphyrin IX), living systems have excelled at modifying and tailoring the chemistry of the metal. In the context of proteins and enzymes, heme cofactors are increasingly found in stoichiometries greater than one, where a single protein macromolecule contains more than one heme unit. When paired or coupled together, these protein associated heme groups perform a wide variety of tasks, such as redox communication, long range electron transfer and storage of reducing/oxidizing equivalents. Here, we review recent advances in the field of multi-heme proteins, focusing on emergent properties of these complex redox proteins, and strategies found in Nature where such proteins appear to be modular and essential components of larger biochemical pathways. This article is part of a Special Issue entitled: Metals in Bioenergetics and Biomimetics Systems.

Contents

1. Introduction: types of multi-heme proteins used in nature

While biology is efficient at using metals to achieve electron transfer and catalysis, one of the most prevalent metal cofactors is heme iron [1]. The breadth of heme protein structure and function is an immense subject, even when considering only the diversity of proteins and enzymes that contain a *single* heme cofactor [2]. Here, we will focus on the

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so-called multi-heme proteins that contain multiple units of heme, and in particular heme proteins that can be described as multi-heme cytochromes *c*, where the vinyl groups of iron-protoporphyrin IX are attached to the two cysteine side chains of a $-CX_nCH-$ motif by enzymatic machinery (for bacteria, this has been previously reviewed [3]), where the His residue serves as a proximal heme ligand. Typically, in such motifs, there are two variable residues between the cysteine positions [4], though other *c*-type heme attachment motifs are known, including $-CX_3CH-$ and $-CX_4CH-$ [5], as well as a $-CX_{15}CH-$ motif that requires a dedicated maturation enzyme system [6,7]. However, in the simplest cases, the presence of the $-CXXCH-$ motif itself can be used as sequence-based diagnostic for the *c*-type covalent attachment of heme units in the context of bacterial organisms. In all cases presented here, multiple heme units appear to be essential for their role in supporting electron transfer chemistry within or between proteins and enzymes. Electron transfer is essential for countless biological processes. Efficient electron transfer occurs when the free energy for the electron to be transferred from donor to acceptor is negative meaning that the reduction potential of an intermediary must be fine-tuned to be in between those of the donor and the acceptor. Heme iron reduction potentials can be affected by ligand type, coordination geometry, and solvent accessibility as well as the pH of the environment [8–11]. In electron transfer through proteins, the distance between cofactors plays a critical role in how quickly electrons are passed. To overcome the challenge of long-range electron transport or multi-electron reactions, Nature has evolved chains of redox cofactors such as iron sulfur (Fe–S) clusters or heme groups, thus enabling electrons to be passed across membranes. With respect to these issues, here we will observe that multi-heme proteins display even more elaborate tricks in their capacity for redox chemistry.

Previous reviews of multi-heme proteins or cytochromes have highlighted their evolutionary relationships and the potential for emerging chemistry [12,13], while here we will examine recent, emergent properties that appear to be found in multi-heme proteins and enzymes, considering in turn three different abilities where Nature is expert and Man is novice: redox communication and conformational changes of protein structure (bacterial cytochrome *c* peroxidases), long-range electron transfer through the deployment of many heme cofactors (the multi-heme cytochromes of dissimilatory metal reduction), and storage of multiple reducing and oxidizing equivalents (hydroxylamine oxidoreductase and cytochrome c_{554}). Sadly, many multi-heme proteins and enzymes (e.g. octaheme tetrathionate reductase [14], octaheme nitrite reductase [15,16], thiosulfate dehydrogenase [17]) are beyond the scope of our current review. Instead, here we will highlight the wealth of biochemical and biophysical information that is now available from increasingly divergent multi-heme proteins, while also underscoring the voids in our understanding of chemistry which need to be filled.

2. Redox communication

2.1. A conversation between cofactors: redox communication

Metalloproteins and metalloenzymes are efficient tools in biology using metals to achieve both electron transfer and catalysis. Electron transfer plays a central role in biological energy conversion, photosynthesis or respiration and also in the regulation of gene expression. Redox communication, in the form of oxidation and reduction reactions, is involved in electron transfer between the redox active cofactors within proteins and enzymes. The charge transfer is either initiated by low molecular weight redox mediators, including, but not limited to ubiquinol, oxygen/superoxide or the NAD^+/NADH couple, or by the direct interaction of redox partner proteins. Below we discuss examples of conformational changes associated with inter-protein electron transfer reactions from a partner (protein or mediator) into an enzyme and inter-cofactor electron transfer reaction.

2.2. Bacterial cytochrome c peroxidases

Bacterial diheme cytochrome *c* peroxidases (bCcPs) differ from the canonical monoheme peroxidases such as horseradish peroxidase (HRP) [18] and yeast cytochrome *c* peroxidase (yCcP) [19] in their heme cofactor content and catalytic peroxide reduction mechanisms. Found in the periplasmic space of many gram negative microorganisms, the bCcP family includes not only genuine bCcP enzymes—the main topic of this section—but also diheme orthologs such as MauG, a poor peroxidase that is required for the oxidative installation of the tryptophanyl tryptophane quinone (TTQ) cofactor found in methylamine dehydrogenase (recently reviewed in [20]). The two heme cofactors present in all members of the bCcP family are covalently bound within two separate cytochrome *c*-like domains. In all genuine bCcPs, the high-potential Met-His ligated heme, the H-heme (250–350 mV vs NHE) serves as the electron transfer site, accepting electrons from physiological or artificial electron donors. The other heme, the low-potential *bis*-His ligated L-heme (~−300 mV) serves as the site of peroxide reduction [21]. (In contrast, in the case of MauG the two hemes are known to be very close in potential, displaying redox anti-cooperativity [22].) In the as-isolated state both heme irons are in the ferric oxidation state. The high potential heme, which is not present in monoheme peroxidases, may be responsible for storing a second oxidizing equivalent during the catalytic reaction cycle and is hypothesized to mediate the transfer of electrons from electron donor proteins to the peroxidatic heme [23], potentially abrogating the need for radical-based intermediates in canonical bCcP enzymes.

2.2.1. Redox activation of CcP results in peroxide reduction

The majority of bCcPs are isolated in a catalytically inactive state where both hemes are in the ferric state, exemplified by the diheme peroxidase from *Pseudomonas aeruginosa* (*Pa*) [21,24]. For such bCcP enzymes, the absence of any reducing equivalents provided by an electron donor protein or small molecule redox mediators (such as ascorbate) results in both hemes remaining in the ferric oxidation state where the active site is in a *bis*-His ligated conformation, preventing binding of the substrate peroxide to the active site heme [24] (Fig. 1A, blue). The active form of the enzyme can be achieved by the introduction of one electron through the H-heme electron transfer site. This initiation of activation by a one electron reduction of the H-heme is termed "reductive

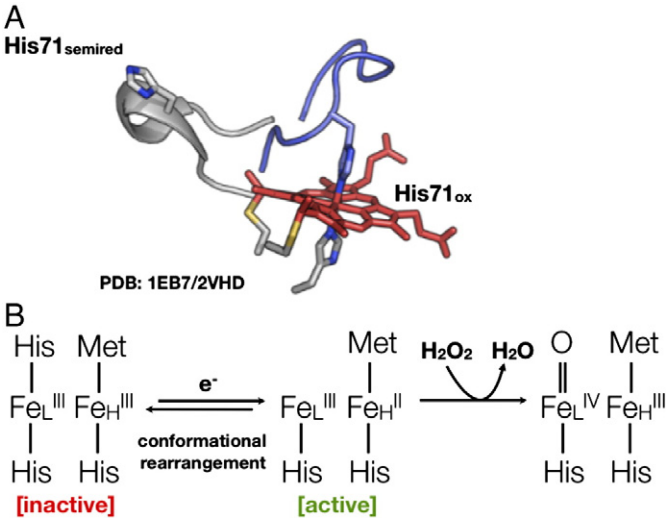

Fig. 1. A. The conformational switch found in the majority of known bCcP enzymes involves the reorganization of the distal face of the peroxidatic heme, as a function of the redox state of a high-potential heme, some 12 Å away. B. The mechanistic impact of reductive activation suggests that by "banking" an electron in the high potential center, the first kinetic intermediate needs to involve the build-up of a radical species.

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