ACCOUNTS of chemical research

Article

pubs.acs.org/accounts

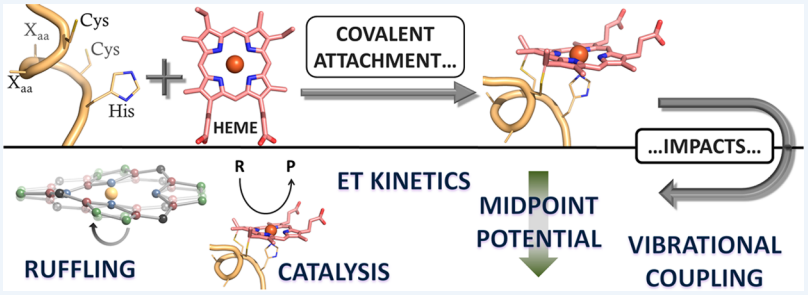
Biological Significance and Applications of Heme *c* Proteins and Peptides

Jesse G. Kleingardner[†,‡] and Kara L. Bren*[,‡]

[†]Department of Chemistry, Ithaca College, Ithaca, New York 14850, United States

[‡]Department of Chemistry, University of Rochester, Rochester, New York 14618, United States

Supporting Information



CONSPECTUS: Hemes are ubiquitous in biology and carry out a wide range of functions. The heme group is largely invariant across proteins with different functions, although there are a few variations seen in nature. The most common variant is heme *c*, which is formed by a post-translational modification in which heme is covalently linked to two Cys residues on the polypeptide via thioether bonds. In this Account, the influence of this covalent attachment on heme *c* properties and function is discussed, and examples of how covalent attachment has been used in selected applications are presented.

Proteins that bind heme *c* are among the most well-characterized proteins in biochemistry. Most of these proteins are cytochromes *c* (cyts *c*) that serve as electron carriers in photosynthesis and respiration. Despite the intense study of cyts *c*, the functional significance of heme covalent attachment has remained elusive. One observation is that heme *c* reaches a lower reduction potential in nature than its noncovalently linked counterpart, heme *b*, when comparing proteins with the same axial ligands. Furthermore, covalent attachment is known to enhance protein stability and allow the heme to be relatively solvent exposed. However, an inorganic chemistry perspective on the effects of covalent attachment has been lacking. Spectroscopic measurements and computations on cyts *c* and model systems reveal a number of effects of covalent attachment on heme electronic structure and reactivity. One is that the predominant nonplanar ruffling distortion seen in heme *c* lowers heme reduction potential. Another is that covalent attachment influences the interaction of the heme iron with the proximal His ligand. Heme ruffling also has been shown to influence electronic coupling to redox partners and, therefore, electron transfer rates by altering the distribution of the orbital hole on the porphyrin in oxidized cyt *c*. Another consequence of heme covalent attachment is the strong vibrational coupling seen between the iron and the protein surface as revealed by nuclear resonance vibrational spectroscopy studies. Finally, heme covalent attachment is proposed to be an important feature supporting multiple roles of cyt *c* in programmed cell death (apoptosis).

Heme covalent attachment is not only vital for the biological functions of cyt *c* but also provides a useful handle in a number of applications. For one, the engineering of heme *c* onto an exposed portion of a protein of interest has been shown to provide a visible affinity purification tag. In addition, peptides with covalently attached heme, known as microperoxidases, have been studied as model compounds and oxidation catalysts and, more recently, in applications for energy conversion and storage. The wealth of insight gained about heme *c* through fundamental studies of cyts *c* forms a basis for future efforts toward engineering natural and artificial cytochromes for a variety of applications.

1. INTRODUCTION

Iron protoporphyrin IX (heme, Figure 1) is an essential biological cofactor that performs a diverse array of functions, including electron transfer, redox catalysis, gas sensing, signaling, and gas transport.[1,2] The protein environment tunes the properties of this cofactor to enable this wide range of functions, but precisely how interactions between the polypeptide and heme tune its physical and electronic structure remains a question of interest. Addressing this question is important for developing our understanding of structure−function relationships in the ever-growing class of heme-binding proteins known in biology. These efforts also form an important basis for the engineering of heme proteins with properties of interest. In some heme proteins, the heme cofactor is covalently bound to the polypeptide, usually in the

Received: February 26, 2015
Published: June 17, 2015

DOI: 10.1021/acs.accounts.5b00106
Acc. Chem. Res. 2015, 48, 1845−1852

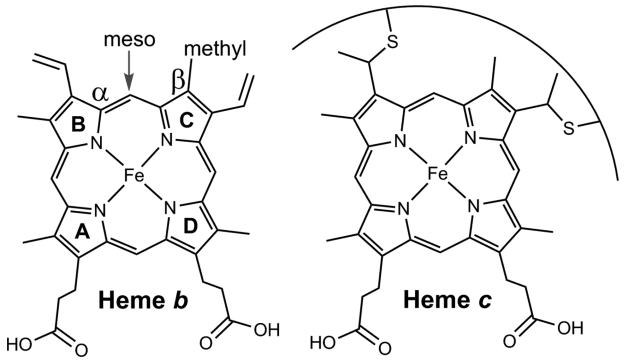


Figure 1. The two most frequently occurring hemes in biology. On heme *b* (left), four types of heme carbons are labeled: *α*-pyrrole, *β*-pyrrole, heme meso, and heme methyl.

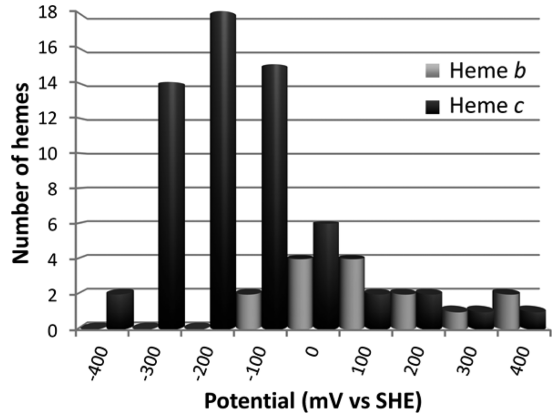


Figure 2. Histogram showing the distribution of redox potentials (in mV vs SHE) for heme *b* (gray bars) and heme *c* (black bars) with bis-His ligation. The bin size used was 100 mV. The data set was compiled with the assistance of the Heme Protein Database.[7] The full data set that was used can be found in Table S1 in the Supporting Information.

form of heme *c* (Figure 1). The resulting stable heme−protein conjugates are attractive targets for engineering heme peptides and proteins for a range of applications. The implications of heme covalent attachment for its electronic structure, reactivity, and biological function, however, are not fully understood.

This Account will focus on the application of spectroscopic techniques in combination with protein engineering to determine structure−function relationships in heme proteins with covalently bound heme *c* (Figure 1), known as cytochromes *c* (cyts *c*). In addition, the development of novel systems containing heme *c* and its derivatives will be described. Section 2 provides context in the form of a comparison of the properties of heme *c* and heme *b*. Section 3 covers the effects of the predominant nonplanar distortion of heme *c*, known as ruffling, on its electronic structure and electron transfer activity, and section 4 describes studies on the vibrational dynamics of heme *c*. Section 5 discusses functional implications of heme covalent attachment for cyt *c* biology. In section 6, recent advances in the use of engineered cyts *c*, heme *c* peptides, and related derivatives will be summarized.

2. COMPARISON OF HEME *B* AND HEME *C* IN PROTEINS

Heme *c* is formed from heme *b* through covalent attachment to the protein backbone in which the heme *b* vinyl groups are replaced by thioether groups (Figure 1). Although variations exist,[3] the most common peptide sequence for heme attachment is Cys-Xaa-Xaa-Cys-His (CXXCH), where Xaa can be any amino acid. The fact that heme *c* is found in all domains of life invites the question as to the functional role of heme *c* covalent attachment, particularly when the organism must expend additional energy after heme synthesis to carry out heme *c* biogenesis. A number of insightful discussions are available,[4−6] but an inorganic chemistry perspective on the effects of covalent attachment on heme electronic structure and reactivity has been lacking. Spectroscopic studies of the influence of heme *c* covalent attachment on electronic structure have provided new insight into our understanding of its functional role.

A striking contrast between heme *b* and heme *c* is the range of redox potentials. Considering the most frequently found axial ligand set common to these hemes, bis-His, heme *b* potentials range from approximately −130 to +390 mV vs standard hydrogen electrode (SHE), whereas heme *c* accesses a much wider range, particularly at the lower end, from approximately −400 to +400 mV (Figure 2).[7,8] One suggested basis for this difference is that heme *c* can be more exposed to solvent than heme *b* while remaining bound to the polypeptide. Although solvent exposure has been found to be an important factor influencing potential, the correlation to redox potential is modest.[8] Furthermore, in a study using multiconformational continuum electrostatics to calculate redox potentials of 141 unique hemes in proteins, it was found that desolvation energy ranges from 3 to 305 meV for the set of proteins with an 800 mV range of potentials. Thus, it is clear that differences in heme solvation alone cannot account for differences in potential.[8] A possible effect on potential that has received less attention is how covalent attachment influences electronic structure. Furthermore, differences in electronic structure are expected to have an influence on reactivity (usually electron transfer rates) by affecting electron distribution on the porphyrin.[9] These electronic structure effects are the focus of the following sections.

3. HEME RUFFLING AND ELECTRONIC STRUCTURE

Hemes in proteins rarely adopt a planar conformation, and nonplanar distortions are generally conserved among protein classes, implying a link to function.[10] Hemes can take on different out-of-plane distortions with doming, saddling, and ruffling being the most predominant. Heme distortions are most reliably measured by analysis of high-resolution crystal structures through a normal coordinate structural decomposition procedure that determines the contribution of each of six low-frequency normal modes corresponding to these out-of-plane distortions.[11] Ruffling is the predominant nonplanar distortion seen in heme *c* and it involves twisting of the pyrrole rings about the Fe−$N_{pyrrole}$ bonds, consequently moving the meso carbons alternately up and down out of the plane (Figure 3). Analyses of heme *c* peptides have suggested that the CXXCH heme attachment itself induces heme ruffling.[12] Furthermore, comparison of heme geometries in crystal structures of proteins containing heme *b* and heme *c* available in the Protein Data Bank shows a strong bias toward ruffled conformations for heme *c* (Figure 4). It is important to note that ruffling is also observed in His-X ligated heme *b*, sometimes to a large extent,[13,14] demonstrating that polypeptide−heme interactions can promote ruffling without covalent

DOI: 10.1021/acs.accounts.5b00106
Acc. Chem. Res. 2015, 48, 1845−1852

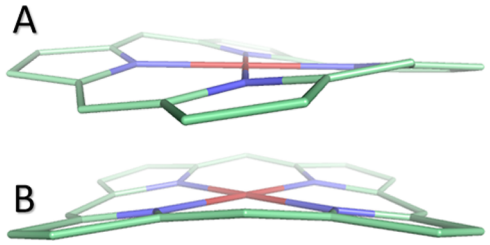


Figure 3. A ruffled heme shown from two angles: (A) looking down the Fe−N(pyrrole) bond and (B) centered on a bridging meso carbon. The Fe porphyrin model has a ruffling distortion of 1.0 Å. The structures were generated using Cartesian coordinates of a symmetric, planar iron porphyrin with typical bond lengths and angles. An idealized 1.0 Å ruffling distortion was applied using a quantitative description of the ruffling normal mode previously published, where the square root of the sum of the out-of-plane displacements of the core porphyrin atoms is equal to 1.0 Å.[11]

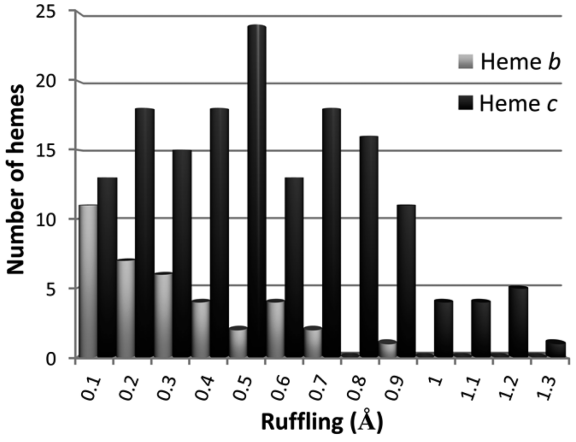


Figure 4. Histogram showing the distribution of measured ruffling distortions found in both bis-His heme *b* (gray) and bis-His heme *c* (black) for heme proteins in the Protein Data Bank. Ruffling was measured using a normal-coordinate structural decomposition procedure.[11] Analysis was restricted to heme proteins with X-ray crystal structures at a resolution of 2.0 Å or better. In multiheme proteins, results are reported for each individual heme. When more than one molecule was found in the asymmetric unit, the values were averaged. The bin size used was 0.1 Å ruffling. The data used can be found in the Supporting Information.

attachment and that noncovalent interactions with heme also play an important role in determining heme conformation.

A number of studies of heme proteins have linked ruffling to function. For example, nonplanar distortions of heme in bacterial heme oxygenases have been found to activate the heme for cleavage,[13] and the high amount of ruffling of NO-carrying nitrophorins has been implicated in the ability of these sites to reversibly bind NO.[14] However, what has not been fully appreciated is how heme conformation affects electronic structure and function in low-spin cytochromes. It has been observed that ruffling lowers heme reduction potential,[14,15] yet a clear relationship between the extent of ruffling and potential has been difficult to establish as a result of the many contributing factors. Aiding this effort was the preparation of a series of variants of small bacterial cyts *c* with altered amounts of heme ruffling.[16−18] The mutations involved residues in the proximal (axial His side) heme pocket and/or within the CXXCH motif. The basis for the selection of some of these residues was the X-ray crystal structure of the F7A mutant (*Pa*-F7A) of the low-spin His/Met-ligated *Pseudomonas aeruginosa* cyt c_{551} (*Pa c*-551).[19] This mutation influences hydrogen bonding with the first Cys residue in the CXXCH motif and results in enhanced heme ruffling (Figure 5). A homologous

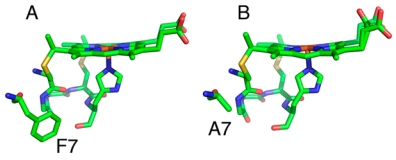


Figure 5. Structures of active sites of (A) *Pa c*-551[20] and (B) *Pa*-F7A[21] showing the heme, CXXCH motif, and residue 7. The F7A mutation increases heme ruffling relative to wild-type *Pa c*-551.

small low-spin cyt c_{552} from the bacterium *Hydrogenobacter thermophilus* (*Ht c*-552) has an Ala at position 7, and thus the *Ht*-A7F mutant was also prepared and predicted to have a heme that is relaxed into a more planar conformation than wild-type. The *Ht c*-552/*Ht*-A7F pair was the subject of spectroscopic and computational analysis of ruffling that provided details relating the extent of ruffling to both reduction potential and electronic structure.[16] In the ferric state, this protein has one unpaired electron that is delocalized over the porphyrin, giving rise to nuclear hyperfine shifts (HFSs) observable by NMR. Measured HFSs for wild-type and *Ht*-A7F were compared to DFT-calculated HFSs for a series of model hemes with varied extents of ruffling. The trend in calculated HFSs followed the observed changes for the less-ruffled *Ht*-A7F compared to wild type, validating the calculations. This analysis also revealed that the delocalization of the orbital hole to the porphyrin edge is decreased with ruffling, which would be expected to decrease coupling to redox partners and thus electron transfer rates. Furthermore, the results supported the hypothesis that the decrease in reduction potential is caused by an increase in ruffling. The basis for this relationship is that ruffling destabilizes the t_{2g} orbital set for both oxidation states, but because the ferrous state has one more electron in these orbitals than the ferric state, ferrous heme is more destabilized by ruffling, leading to a lowering of the reduction potential.[16] A follow-up study of *Pa c*-551 and *Pa*-F7A went into more depth by examining the chemical shifts of the heme core carbons as well as to provide a detailed view of how heme ruffling influences spin delocalization (Figure 6).[17]

To better understand the effect of ruffling on electron transfer rates, photoreduction kinetics were measured in *Pa c*-551 and *Pa*-F7A by transient absorption spectroscopy.[24] The donor was identified as an aromatic amino acid near the heme, and the results were compared to horse heart cyt *c*, which has a more ruffled heme than both variants. Interestingly, the photoreduction cross section was found to decrease as heme ruffling increased with the F7A mutation. Furthermore, the photoreduction cross section for the variant with the most planar heme is nearly two orders of magnitude larger than that for the most ruffled heme. The trend reveals that ruffling modulates the electron transfer rate exponentially, which would be consistent with modulation of the tunneling matrix element associated with overlap of the donor and the iron acceptor orbitals. This study provides the first robust test of the effect of heme ruffling on electron transfer rates and confirms predictions from NMR studies that ruffling tends to localize the ferric iron d(π) hole on the iron atom, which effectively decreases the overlap between the d(π) hole and the electron

DOI: 10.1021/acs.accounts.5b00106
Acc. Chem. Res. 2015, 48, 1845−1852

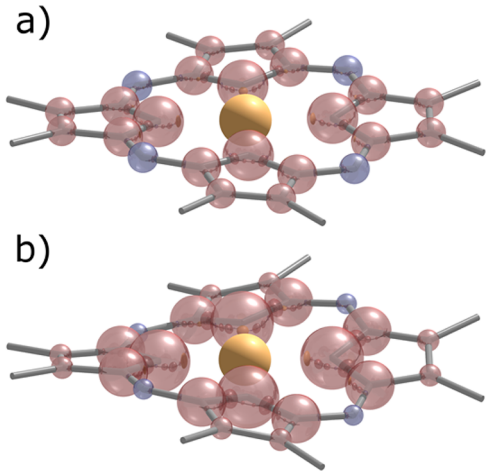


Figure 6. Graphical representation of the relative average spin densities on the α-pyrrole carbons, β-pyrrole carbons, meso carbons, and pyrrole nitrogens determined from analysis of HFSs.[17] Red and blue represent positive and negative spin densities, respectively. The volumes of the spheres are proportional to the empirically calculated spin densities in (a) *Pa* *c*-551 and (b) the more ruffled *Pa* F7A variant. A more accurate depiction of pyrrole nitrogen spin densities, based on pulsed EPR and DFT studies, would show a mixture of positive and negative spin density with an excess of negative spin density.[22,23]

donor orbital.[16] Consequences for function are discussed further in section 5.

To test the generality of the conclusions relating ruffling and electronic structure, another series of mutations at positions 13 and 22 on the proximal side of the porphyrin were found to increase ruffling in *Ht* *c*-552 and also to lower reduction potential.[17,18,25,26] EPR was used to determine ligand-field parameters of these more-ruffled mutants, and as ruffling was increased, the axial ligand field term (Δ/λ) decreased,[25] as predicted in the prior NMR and DFT study of *Ht*-A7F and wild-type (Figure 7).[16] Another effect observed in this series of

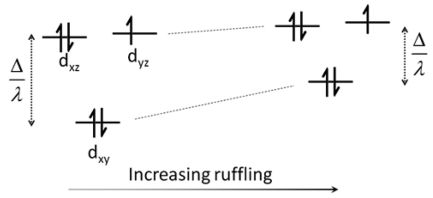


Figure 7. Influence of heme ruffling on orbital energies that determine the EPR ligand field parameter Δ/λ. An increase in ruffling decreases Δ/λ by preferentially raising the energy of the d_{xy} orbital.

mutants by NMR through the analysis of Fermi contact shifts was an increase in the Fe(III)−His bond strength as ruffling increased.[17,26] Earlier studies of model systems had shown that more tightly tethered axial imidazole ligands yield a stronger imidazole−iron interaction as revealed by an increased ligand-field strength.[27] Relating this finding to ruffling, it has been observed in NMR studies of *Pa* *c*-551, *Ht* *c*-552, and their mutants that variants in which the structure of the heme pocket favors more ruffling have a more rigid CXXCH motif, which may also enhance His−Fe bonding.[18]

In summary, the presence of heme attachment to a polypeptide in heme *c* has been shown to promote ruffling, but noncovalent interactions also play a role. The hypothesis that ruffling lowers redox potential has been difficult to test because of the lack of appropriate model systems, but studies of judiciously chosen cyt *c* variants provide strong support for this hypothesis. Furthermore, combining DFT calculations and spectroscopic studies has lent insight into the effects of heme ruffling on electronic structure, and variations in electronic structure that result from ruffling have been shown to strongly influence electron transfer rates.

4. HEME *C* VIBRATIONAL DYNAMICS

Heme is dynamic and samples a range of conformations, and the structure observed in the experiments described above represents an average structure. The vibrational modes associated with the common out-of-plane heme distortions occur at energies below kT, indicating that they will be significantly populated at room temperature and may play a role in function. The detection of these modes by infrared or resonance Raman spectroscopy is difficult because of technical issues. However, femtosecond vibrational coherence spectroscopy (VCS) can be used to observe these modes.[24] VCS showed that a mode (γ_a) ~45−60 cm^{-1} is associated with ruffling in cyts *c* with a frequency correlating inversely with the amount of ruffling. Furthermore, this band becomes more prominent in spectra of cyt *c* variants with greater amounts of ruffling with an amplitude corresponding to the square of the magnitude of the ruffling distortion.[24]

Studies of heme iron vibrational dynamics have also been carried out using nuclear resonance vibrational spectroscopy (NRVS), a technique that provides the complete vibrational spectrum of a Mössbauer-active nucleus, in this case, ^{57}Fe. The selection rule for NRVS is that motion of the probe nucleus must be involved for the mode to be observed. An NRVS study of *Ht* *c*-552 made use of a series of isotope-labeled variants (on top of ^{57}Fe enrichment) to facilitate a detailed assignment of the NRVS spectrum. A quantum chemistry-centered normal coordinate analysis of the NRVS data considering the isotope-labeled derivatives allowed a detailed assignment of the NRVS spectra.[28] The most striking result of this work was the finding that iron vibrations were strongly coupled to the vibrations of polypeptide residues on the protein surface. Residues in and near the CXXCH motif are known to play a role in protein−protein interactions, and these residues are strongly vibrationally coupled to iron via the covalent linkages (Figure 8). This finding provides a tantalizing hint that biologically relevant protein−protein interactions may influence the vibrational dynamics of iron as well as heme conformation.

A follow-up NRVS study interrogated the vibrational dynamics of a series of variants of *Ht* *c*-552 noted above that show a known trend in the extent of ruffling: *Ht*-M13V, M13V/K22M, and A7F.[30] Heme ruffling increases in the series *Ht*-A7F < wild-type < *Ht*-M13V < *Ht*-M13V/K22M. This study revealed that features in the 350−400 cm^{-1} region of the NRVS spectrum were influenced by ruffling. The modes that responded to changes in ruffling reflect the degree of CXXCH loop stiffness and Fe(III)−His bonding properties. Furthermore, porphyrin modes that can be detected by NRVS were affected by ruffling. These results provide support for previous interpretations of how heme ruffling is related to the CXXCH loop properties and to Fe(III)−His bonding and also reveal the influence of ruffling on iron vibrations.

DOI: 10.1021/acs.accounts.5b00106
Acc. Chem. Res. 2015, 48, 1845−1852

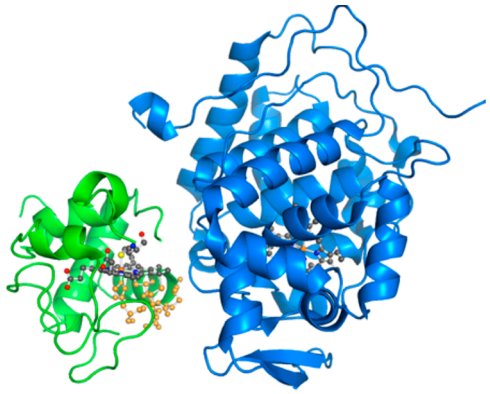


Figure 8. *Saccharomyces cerevisiae* cytochrome *c* peroxidase (blue): cyt *c* (green) complex (PDB: 2PCB).[29] The active sites of the proteins are shown as ball-and-stick representations, and the CXXCH residues in cyt *c* are highlighted in orange.[28] Reproduced with permission from ref 28. Copyright (2012) National Academy of Sciences of the United States of America.

5. SIGNIFICANCE OF HEME COVALENT ATTACHMENT FOR CYT *C* BIOLOGY

The wide distribution of *c*-type heme in biology suggests that heme covalent attachment plays a biological role. The studies summarized in this Account underline the physicochemical effects of heme covalent attachment that influence electron transfer thermodynamics and kinetics. One finding is that heme covalent attachment contributes to determining the heme reduction potential by influencing heme conformation and the Fe(III)−His bonding interaction. The higher ligand-field strength seen in heme *c* relative to heme *b* may also be important for stabilization of low-spin Fe, which is necessary for minimizing Marcus reorganization energy. Because most biological electron transfers occur at low driving force, a low reorganization energy will increase rates.[31] Although bis-His hemes are typically low-spin in both the ferric and ferrous states, heme *b* with His/Met axial ligation has some high-spin character in the ferric state, whereas His/Met cyts *c* are low-spin.[27] There is also compelling evidence that increased ruffling decreases electronic coupling to redox partners, leading to the proposal that modulation of ruffling regulates electron transfer.[24] In addition, NRVS studies reveal that the protein surface is vibrationally coupled to the iron, indicating a means by which the binding of cyt *c* to other proteins or to membranes may influence iron vibrations along with heme ruffling.[28] Indeed, studies of mitochondrial cyt *c* bound to the mitochondrial membrane have shown that the heme relaxes to a more planar conformation relative to cyt *c* that is freely diffusing in the cytosol.[32] The resulting change in heme conformation would increase electronic coupling to redox partners and thus electron transfer rates.

Another functional basis for covalent attachment is specific to the role of mitochondrial cyts *c* in programmed cell death (apoptosis) (Figure 9). In response to pro-apoptotic signals, mitochondrial cyt *c* undergoes a significant conformational change to become a peroxidase.[33] As a peroxidase, cyt *c* oxidizes the mitochondrial lipid cardiolipin, increasing mitochondrial permeability. Cyt *c* is then released from the mitochondrial intermembrane space into the cytosol, where it binds apoptosis protease activating factor 1 (Apaf-1), which is a key triggering event in the pathway. This process is illustrated in Figure 9. The multifaceted role of cyt *c* in apoptosis suggests two reasons why the heme must be covalently bound in mitochondrial cyt *c*. The first relates to the large structural change and conformational dynamics observed[34] as cyt *c* becomes a peroxidase. Covalent attachment of the heme to the protein may be necessary to keep heme bound in the peroxidase-competent conformation of the protein. The second role of covalent heme attachment for mitochondrial cyt *c* could be to prevent the presence of holo-cyt *c* or of an apo-cyt *c* variant with a nativelike fold in the cytosol during its synthesis, which would trigger apoptosis via Apaf-1 binding even at very low cytosolic cyt *c* concentrations.[35] Because apo-cyt *c* is largely unfolded and only folds after covalent heme attachment,[36] by attaching heme covalently within the mitochondria, the presence of cytosolic folded cyt *c* is avoided during its synthesis. It is possible that noncovalent heme binding within the mitochondria could provide this control because heme synthesis occurs in mitochondria. However, the presence of cytosolic heme *b*

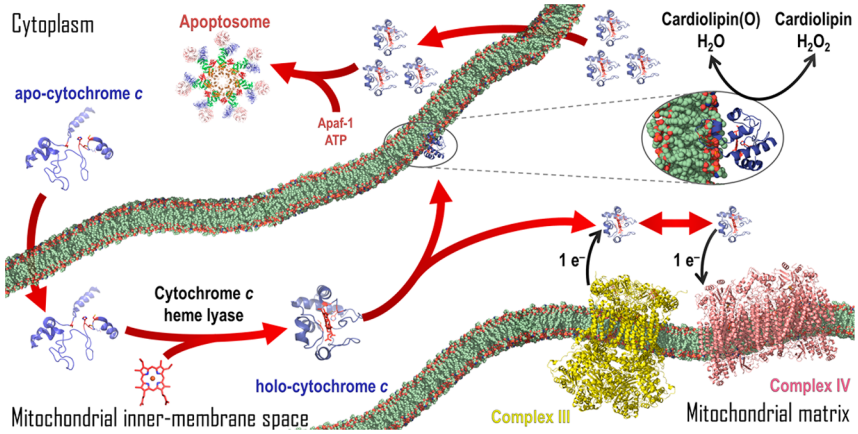


Figure 9. Schematic illustration of the locations of cyt *c* during maturation and apoptosis triggering. The red arrows provide guidance on translocations and modifications of cyt *c* associated with its functions. Starting on the left side of the figure, apo-cyt *c* is expressed in the cytoplasm and transported to the mitochondrial intermembrane space where cyt *c* heme lyase catalyzes heme attachment. In oxidative phosphorylation, cyt *c* shuttles electrons from Complex III to Complex IV. In apoptosis triggering, cyt *c* undergoes a structural rearrangement and catalyzes H_2O_2-dependent oxidation of cardiolipin. Cyt *c* is released into the cytoplasm where association with Apaf-1 forms the apoptosome, eventually leading to cell death.

DOI: 10.1021/acs.accounts.5b00106
Acc. Chem. Res. 2015, 48, 1845−1852

proteins[37,38] indicates that heme can be made available in the cytosol. Thus, requiring covalent attachment for folding and forming holocyt *c* within mitochondria may be necessary to ensure against triggering apoptosis. Finally, heme covalent attachment is also important for the formation of multiheme cytochromes that perform long-range electron transfer.[39] Assembly of stable structures with a high heme-to-protein ratio and with hemes at distances and orientations optimized for rapid electron transfer would be difficult without covalent heme attachment.

6. APPLICATIONS OF HEME *C* PEPTIDES AND ARTIFICIAL CYTOCHROMES

The covalent attachment of heme *c* to peptides and to proteins has been used in a number of applications, including aiding protein purification and the development of catalysts. Small heme *c* peptides, known as microperoxidases (MPs) (Figure 10), have been used as model systems of heme enzymes and as

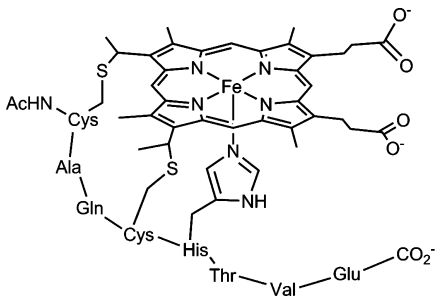


Figure 10. Microperoxidase-8 (MP-8) derived from horse heart cytochrome *c* contains two covalent linkages to a heme through a CAQCH motif. Three other residues (TVE) are present on the C-terminus. For MP-11, an additional three residues (VQK) are present on the N-terminus.

catalysts. MPs are usually prepared by proteolysis of cyt *c*, generating a peptide containing the heme-linked CXXCH peptide. Advantages of MPs over full-length cytochromes in some applications are (1) their small size, providing a higher density of active sites when immobilized on a chip or electrode, (2) their lack of tertiary structure, meaning that folding and denaturation is not a concern, and (3) the fact that their metal site is solvent exposed, allowing ligand and substrate access. MP-11 and MP-8 are the most popular MPs and are generated from proteolysis of horse cyt *c*. MPs can also be produced through expression of novel peptide sequences that include a CXXCH motif to which heme can be attached. The peptide can be expressed directly[40] or as a tag on a carrier protein, which increases yields.[41−43]

Microperoxidases have been widely employed as peroxide biosensors, and a low detection limit of 1 nM H_2O_2 has been achieved.[44] They have also also been used as oxidation catalysts. By raising catalytic antibodies against MP-8, a substrate binding pocket has been engineered to achieve regioselective nitration of phenols[45] or the stereoselective oxidation of sulfides.[46] Although most catalysis performed by MPs has been oxidation reactions, cobalt-substituted acetylated MP-11 (CoMP11-Ac) has been shown to act as a catalyst for the reduction of protons to hydrogen (H_2). CoMP11-Ac provides over 20,000 turnovers electrocatalytically and functions at neutral pH and in the presence of oxygen.[47] Interestingly, this catalyst performs significantly better than synthetic cobalt-substituted water-soluble porphyrins, which give less than 1 turnover in 20 min of electrolysis in an acidic solution.[48] Although the basis for this difference in activity is not fully understood, one hypothesis is that the presence of a tethered axial base in CoMP11-Ac enhances its activity.

Artificial microperoxidases expressed as fusion tags with a carrier protein have been used in protein affinity purification.[42,49] The tagged protein is visibly colored and therefore easily tracked by eye. The use of these heme-tagged proteins or artificial cytochromes in other applications can also be envisioned. In just one example, Zn-substituted heme *c* tags have been proposed as endogenous chromophores for fluorescence resonance energy transfer experiments in which they can serve as a donor or an acceptor.[50,51]

7. CONCLUSION

Covalent attachment of heme *c* to proteins is widely found in nature. Functional implications of this covalent attachment have been proposed as a result of advances in our understanding of cyt *c* electronic structure and vibrational dynamics. In particular, covalent heme binding induces the ruffling distortion and increases the strength of the His−Fe(III) interaction. These factors play a role in determining reduction potential and electronic structure with consequences for function. Furthermore, covalent attachment enhances vibrational coupling between the iron and protein surface, where interactions with other proteins and with membranes take place. Heme *c* peptides and derivatives have seen a wide range of uses in biosensors, fuel cells, and for hydrogen production from water. Also, heme *c* fusion tags have provided an elegant new affinity purification protocol for proteins. Looking forward, our understanding of how interactions between heme and polypeptides influence heme (or metalloporphyrin) reactivity promises to facilitate the design and preparation of artificial cyts *c* for use in a wide range of applications.

■ ASSOCIATED CONTENT

Supporting Information

Compiled data on heme *b* and heme *c* with bis-His axial ligation. Reduction potentials are provided in Table S1 and measurements of heme ruffling are provided in Table S2. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.accounts.5b00106.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: bren@chem.rochester.edu.

Notes

The authors declare no competing financial interest.

Biographies

Jesse G. Kleingardner received his B.S. in chemistry from Ithaca College in 2005 and his Ph.D. in bioinorganic chemistry from the University of Rochester in 2013. He is currently a Postdoctoral Teaching Fellow in the Department of Chemistry at Ithaca College. He will be starting as an Assistant Professor of Chemistry at Messiah College in the summer of 2015.

Kara L. Bren received her B.S. in chemistry from Carleton College in 1991 and her Ph.D. in inorganic chemistry from California Institute of Technology in 1996. She is currently a Professor in the Department of Chemistry at the University of Rochester. Her research interests are

DOI: 10.1021/acs.accounts.5b00106
Acc. Chem. Res. 2015, 48, 1845−1852

focused on engineering cytochromes and metallopeptides to introduce novel functions and on understanding structure−function relationships in heme proteins.

■ ACKNOWLEDGMENTS

This work on the development of catalysts based on cytochromes is supported by the Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, Office of Science, U.S. Department of Energy, Grant No. DE-FG02-09ER16121.

■ REFERENCES

(1) Poulos, T. L. Heme enzyme structure and function. *Chem. Rev.* **2014**, *114*, 3919−3962.
(2) Bowman, S. E. J.; Bren, K. L. The chemistry and biochemistry of heme *c*: Functional bases for covalent attachment. *Nat. Prod. Rep.* **2008**, *25*, 1118−1130.
(3) Kleingardner, J. G.; Bren, K. L. Comparing substrate specificity between cytochrome *c* maturation and cytochrome *c* heme lyase systems for cytochrome *c* biogenesis. *Metallomics* **2011**, *3*, 396−403.
(4) Barker, P. D.; Ferguson, S. J. Still a puzzle: Why is haem covalently attached in *c*-type cytochromes? *Structure* **1999**, *7*, R281−R290.
(5) Stevens, J. M.; Daltrop, O.; Allen, J. W. A.; Ferguson, S. J. *C*-type cytochrome formation: Chemical and biological enigmas. *Acc. Chem. Res.* **2004**, *37*, 999−1007.
(6) Allen, J. W. A.; Barker, P. D.; Daltrop, O.; Stevens, J. M.; Tomlinson, E. J.; Sinha, N.; Sambongi, Y.; Ferguson, S. J. Why isn't "standard" heme good enough for *c*-type and *d*(1)-type cytochromes? *Dalton T.* **2005**, 3410−3418.
(7) Reedy, C. J.; Elvekrog, M. M.; Gibney, B. R. Development of a heme protein structure−electrochemical function database. *Nucleic Acids Res.* **2008**, *36*, D307−D313.
(8) Zheng, Z.; Gunner, M. R. Analysis of the electrochemistry of hemes with E(m)s spanning 800 mV. *Proteins* **2009**, *75*, 719−734.
(9) Liptak, M. D.; Fagerlund, R. D.; Ledgerwood, E. C.; Wilbanks, S. M.; Bren, K. L. The proapoptotic G41S mutation of human cytochrome *c* alters the heme electronic structure and increases the electron self-exchange rate. *J. Am. Chem. Soc.* **2011**, *133*, 1153−1155.
(10) Shelnutt, J. A.; Song, X. Z.; Ma, J. G.; Jia, S. L.; Jentzen, W.; Medforth, C. J. Nonplanar porphyrins and their significance in proteins. *Chem. Soc. Rev.* **1998**, *27*, 31−41.
(11) Jentzen, W.; Song, X. Z.; Shelnutt, J. A. Structural characterization of synthetic and protein-bound porphyrins in terms of the lowest-frequency normal coordinates of the macrocycle. *J. Phys. Chem. B* **1997**, *101*, 1684−1699.
(12) Ma, J. G.; Vanderkooi, J. M.; Zhang, J.; Jia, S. L.; Shelnutt, J. A. Resonance raman investigation of nickel microperoxidase-11. *Biochemistry* **1999**, *38*, 2787−2795.
(13) Caignan, G. A.; Deshmukh, R.; Zeng, Y. H.; Wilks, A.; Bunce, R. A.; Rivera, M. The hydroxide complex of *Pseudomonas aeruginosa* heme oxygenase as a model of the low-spin iron(III) hydroperoxide intermediate in heme catabolism: C-13 NMR spectroscopic studies suggest the active participation of the heme in macrocycle hydroxylation. *J. Am. Chem. Soc.* **2003**, *125*, 11842−11852.
(14) Shokhireva, T. K.; Berry, R. E.; Uno, E.; Balfour, C. A.; Zhang, H. J.; Walker, F. A. Electrochemical and NMR spectroscopic studies of distal pocket mutants of nitrophorin 2: Stability, structure, and dynamics of axial ligand complexes. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3778−3783.
(15) Safo, M. K.; Nesset, M. J. M.; Walker, F. A.; Debrunner, P. G.; Scheidt, W. R. Models of the cytochromes. Axial ligand orientation and complex stability in iron(II) porphyrinates: The case of the noninteracting d(π) orbitals. *J. Am. Chem. Soc.* **1997**, *119*, 9438−9448.
(16) Liptak, M. D.; Wen, X.; Bren, K. L. NMR and DFT investigation of heme ruffling: Functional implications for cytochrome *c*. *J. Am. Chem. Soc.* **2010**, *132*, 9753−9763.
(17) Kleingardner, J. G.; Bowman, S. E. J.; Bren, K. L. The influence of heme ruffling on spin densities in ferricytochromes *c* probed by heme core (13)C NMR. *Inorg. Chem.* **2013**, *52*, 12933−12946.
(18) Michel, L. V.; Ye, T.; Bowman, S. E. J.; Levin, B. D.; Hahn, M. A.; Russell, B. S.; Elliott, S. J.; Bren, K. L. Heme attachment motif mobility tunes cytochrome *c* redox potential. *Biochemistry* **2007**, *46*, 11753−11760.
(19) Borgia, A.; Bonivento, D.; Travaglini-Allocatelli, C.; Di Matteo, A.; Brunori, M. Unveiling a hidden folding intermediate in *c*-type cytochromes by protein engineering. *J. Biol. Chem.* **2006**, *281*, 9331−9336.
(20) Matsuura, Y.; Takano, T.; Dickerson, R. E. Structure of cytochrome *c*-551 from *Pseudomonas aeruginosa* refined at 1.6-Å resolution and comparison of the 2 redox forms. *J. Mol. Biol.* **1982**, *156*, 389−409.
(21) Borgia, A.; Gianni, S.; Brunori, M.; Travaglini-Allocatelli, C. Fast folding kinetics and stabilization of apo-cytochrome *c*. *FEBS Lett.* **2008**, *582*, 1003−1007.
(22) Van Doorslaer, S.; Tilleman, L.; Verrept, B.; Desmet, F.; Maurelli, S.; Trandafir, F.; Moens, L.; Dewilde, S. Marked difference in the electronic structure of cyanide-ligated ferric protoglobins and myoglobin due to heme ruffling. *Inorg. Chem.* **2012**, *51*, 8834−8841.
(23) Johansson, M. P.; Sundholm, D.; Gerfen, G.; Wikstrom, M. The spin distribution in low-spin iron porphyrins. *J. Am. Chem. Soc.* **2002**, *124*, 11771−11780.
(24) Sun, Y.; Benabbas, A.; Zeng, W.; Kleingardner, J. G.; Bren, K. L.; Champion, P. M. Investigations of heme distortion, low-frequency vibrational excitations, and electron transfer in cytochrome *c*. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111*, 6570−6575.
(25) Can, M.; Zoppellaro, G.; Andersson, K. K.; Bren, K. L. Modulation of ligand-field parameters by heme ruffling in cytochromes *c* revealed by EPR spectroscopy. *Inorg. Chem.* **2011**, *50*, 12018−12024.
(26) Bowman, S. E. J.; Bren, K. L. Variation and analysis of second-sphere interactions and axial histidinate character in *c*-type cytochromes. *Inorg. Chem.* **2010**, *49*, 7890−7897.
(27) Cowley, A. B.; Lukat-Rodgers, G. S.; Rodgers, K. R.; Benson, D. R. A possible role for the covalent heme-protein linkage in cytochrome *c* revealed via comparison of *N*-acetylmicroperoxidase-8 and a synthetic, monohistidine-coordinated heme peptide. *Biochemistry* **2004**, *43*, 1656−1666.
(28) Galinato, M. G. I.; Kleingardner, J. G.; Bowman, S. E. J.; Alp, E. E.; Zhao, J.; Bren, K. L.; Lehnert, N. Heme-protein vibrational couplings in cytochrome *c* provide a dynamic link that connects the heme-iron and the protein surface. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 8896−8900.
(29) Pelletier, H.; Kraut, J. Crystal structure of a complex between electron-transfer partners, cytochrome-*c* peroxidase and cytochrome *c*. *Science* **1992**, *258*, 1748−1755.
(30) Galinato, M. G. I.; Bowman, S. E. J.; Kleingardner, J. G.; Martin, S.; Zhao, J.; Sturhahn, W.; Alp, E. E.; Bren, K. L.; Lehnert, N. Effects of protein structure on iron-polypeptide vibrational dynamic coupling in cytochrome *c*. *Biochemistry* **2015**, *54*, 1064−1076.
(31) Marcus, R. A.; Sutin, N. Electron transfers in chemistry and biology. *Biochim. Biophys. Acta* **1985**, *811*, 265−322.
(32) Berezhna, S.; Wohlrab, H.; Champion, P. M. Resonance Raman investigations of cytochrome *c* conformational change upon interaction with the membranes of intact and Ca^{2+}-exposed mitochondria. *Biochemistry* **2003**, *42*, 6149−6158.
(33) Kagan, V. E.; Tyurin, V. A.; Jiang, J. F.; Tyurina, Y. Y.; Ritov, V. B.; Amoscato, A. A.; Osipov, A. N.; Belikova, N. A.; Kapralov, A. A.; Kini, V.; Vlasova, I. I.; Zhao, Q.; Zou, M. M.; Di, P.; Svistunenko, D. A.; Kurnikov, I. V.; Borisenko, G. G. Cytochrome *c* acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nat. Chem. Biol.* **2005**, *1*, 223−232.
(34) Hanske, J.; Toffey, J. R.; Morenz, A. M.; Bonilla, A. J.; Schiavoni, K. H.; Pletneva, E. V. Conformational properties of cardiolipin-bound cytochrome *c*. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 125−130.

DOI: 10.1021/acs.accounts.5b00106
Acc. Chem. Res. 2015, 48, 1845−1852

(35) Purring, C.; Zou, H.; Wang, X. D.; McLendon, G. Stoichiometry, free energy, and kinetic aspects of cytochrome *c*: Apaf-1 binding in apoptosis. *J. Am. Chem. Soc.* **1999**, *121*, 7435−7436.
(36) Dumont, M. E.; Corin, A. F.; Campbell, G. A. Noncovalent binding of heme induces a compact apocytochrome *c* structure. *Biochemistry* **1994**, *33*, 7368−7378.
(37) Kabil, O.; Zhou, Y.; Banerjee, R. Human cystathionine *β*-synthase is a target for sumoylation. *Biochemistry* **2006**, *45*, 13528−13536.
(38) Lynch, J.; Fukuda, Y.; Krishnamurthy, P.; Du, G. Q.; Schuetz, J. D. Cell survival under stress is enhanced by a mitochondrial ATP-binding cassette transporter that regulates hemoproteins. *Cancer Res.* **2009**, *69*, 5560−5567.
(39) Bewley, K. D.; Ellis, K. E.; Firer-Sherwood, M. A.; Elliott, S. J. Multi-heme proteins: Nature's electronic multi-purpose tool. *Biochim. Biophys. Acta* **2013**, *1827*, 938−948.
(40) Braun, M.; Thöny-Meyer, L. Biosynthesis of artificial micro-peroxidases by exploiting the secretion and cytochrome *c* maturation apparatuses of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 12830−12835.
(41) Braun, M.; Rubio, I. G.; Thöny-Meyer, L. A heme tag for in vivo synthesis of artificial cytochromes. *Appl. Microbiol. Biotechnol.* **2005**, *67*, 234−239.
(42) Asher, W. B.; Bren, K. L. Use of a heme fusion tag for protein purification and quantification. *Protein Sci.* **2010**, *19*, 1830−1839.
(43) Asher, W. B.; Bren, K. L. Cytochrome *c* heme lyase can mature a fusion peptide composed of the amino-terminal residues of horse cytochrome *c*. *Chem. Commun. (Cambridge, U.K.)* **2012**, *48*, 8344−8346.
(44) Korri-Youssoufi, H.; Desbenoit, N.; Ricoux, R.; Mahy, J. P.; Lecomte, S. Elaboration of a new hydrogen peroxide biosensor using microperoxidase 8 (MP8) immobilized on a polypyrrole coated electrode. *Mater. Sci. Eng., C* **2008**, *28*, 855−860.
(45) Ricoux, R.; Raffy, Q.; Mahy, J.-P. New biocatalysts mimicking oxidative hemoproteins: Hemoabzymes. *C. R. Chim.* **2007**, *10*, 684−702.
(46) Ricoux, R.; Lukowska, E.; Pezzotti, F.; Mahy, J. P. New activities of a catalytic antibody with a peroxidase activity - formation of Fe(II)-RNO complexes and stereoselective oxidation of sulfides. *Eur. J. Biochem.* **2004**, *271*, 1277−1283.
(47) Kleingardner, J. G.; Kandemir, B.; Bren, K. L. Hydrogen evolution from neutral water under aerobic conditions catalyzed by cobalt microperoxidase-11. *J. Am. Chem. Soc.* **2014**, *136*, 4−7.
(48) Kellett, R. M.; Spiro, T. G. Cobalt(I) porphyrin catalysts of hydrogen production from water. *Inorg. Chem.* **1985**, *24*, 2373−2377.
(49) Asher, W. B.; Bren, K. L. Affinity purification of heme-tagged proteins. *Methods Mol. Biol.* **2014**, *1177*, 12−33.
(50) Ensign, A. A.; Jo, I.; Yildirim, I.; Krauss, T. D.; Bren, K. L. Zinc porphyrin: A fluorescent acceptor in studies of Zn-cytochrome *c* unfolding by fluorescence resonance energy transfer. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 10779−10784.
(51) Lee, A. J.; Ensign, A. A.; Krauss, T. D.; Bren, K. L. Zinc porphyrin as a donor for FRET in Zn(II)cytochrome c. *J. Am. Chem. Soc.* **2010**, *132*, 1752−1753.

DOI: 10.1021/acs.accounts.5b00106
Acc. Chem. Res. 2015, 48, 1845−1852