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Structure–Function Relationships in Heme-Proteins

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ABSTRACT

Biological systems rely on heme-proteins to carry out a number of basic functions essential for their survival. Hemes, or iron–porphyrin complexes, are the versatile and ubiquitous active centers of these proteins. In the past decade, discovery of new heme-proteins, together with functional and structural research, provided a wealth of information on these diverse and biologically important molecules. Structure determination work has shown that nature has used a variety of different scaffolds and architectures to bind heme and modulate functions such as redox properties. Structural data have also provided insights into the heme-linked protein conformational changes required in many regulatory heme-proteins. Remarkable efforts have been made towards the understanding of factors governing redox potentials. Site-directed mutagenesis studies and theoretical calculations on heme environments investigated the roles of hydrophobic and electrostatic residues, and analyzed the effect of heme solvent accessibility. This review focuses on the structure–function relationships underlying the association of heme in signaling and iron metabolism proteins. In addition, an account is given about molecular features affecting heme's redox properties; this briefly revisits previous conclusions in the light of some more recent reports.

INTRODUCTION

HEMES, OR IRON–PORPHYRIN COMPLEXES are prosthetic groups widely used in nature. Heme-containing proteins include molecules with quite distinct activities, ranging from electron transfer, catalysis, oxygen transport and storage, ligand binding, signal transduction, and control of gene expression. This diversity of functions originates from the versatility of the heme group and the variety of interactions with protein scaffolds that generate different heme environments. Because of these reasons, heme-proteins have been the focus of a great deal of work over the last half a century and still retain a key place of interest in biochemical research. In addition to their distinct functions, known heme-proteins feature a fascinating range of different folding architectures. The phylogenetic widespread occurrence of hemes originates from the use of porphyrins in photosynthesis and cellular respiration for energy transduction reactions supporting the proton motive force (PMF). Oxygen transport and storage by hemoglobin and myoglobin account for the ubiquitous presence of heme throughout vertebrates and invertebrates.

Historically, heme-proteins stimulated special attention because of the pioneering work on the structure determination of myoglobin and hemoglobin (Kendrew *et al.*, 1958; Perutz *et al.*, 1960). However, heme-proteins continue to be of central scientific interest due to the wealth of new and very diverse structural data, as well as the recent discoveries of heme-containing molecules with novel functions, including guanylate cyclase (Bredt and Snyder, 1994), CcmE (Schultz *et al.*, 1998), Irr (Qi *et al.*, 1999), and Hem-AT (Hou *et al.*, 2000). In guanylate cyclase, the binding of the small regulatory molecule nitric oxide to the heme-iron activates the enzyme. This leads to cyclic GMP formation, and sets in motion a signaling cascade. The *Escherichia coli* protein CcmE acts as a heme-chaperone by binding heme for transfer to apo-cytochrome *c* in the intermembrane space of mitochondria, participating in cytochrome biosynthesis. Another bacterial protein, Irr, or iron-response regulator, is involved in the regulation of heme biosynthesis through a heme-dependent process of destabilization and degradation. Irr interacts with ferrochelatase and thereby may regulate heme biosynthesis in mammalian cells. Hem-AT is the first example of a heme-protein in Archea, where it acts as an aero-

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taxis transducer by sensing atmospheric oxygen through its myoglobin-like N-terminal domain, which controls the activity of the signaling C-terminal domain. The latter has significant sequence similarity with the signaling MCP bacterial chemoreceptors involved in chemotaxis. These molecules are examples of the most newly discovered heme-proteins. The determination of their structures will hopefully provide more insights into their mechanism of action.

To date, a remarkable number of heme-proteins featuring distinct folds have been structurally characterized. Recently reported structures include the heme–hemopexin complex (Paoli *et al.*, 1999), heme-HasA (Arnoux *et al.*, 1999), the nine-heme cytochrome *c* (Matias *et al.*, 1999), the cytochrome domain of fungal cellobiose dehydrogenase (Hallberg *et al.*, 2000), and ubiquinol oxidase (Abramson *et al.*, 2000). The range of different folding topologies displayed by these proteins shows that during evolution multiple structural solutions were found for the task of binding the same chemical species. From a functional perspective, the delocalized π-electron system of the porphyrin ring, the redox properties of the central iron atom and the spectrum of interactions provided by the different protein scaffolds all contribute to the ubiquitous versatility of heme. They allow the heme to support activities as diverse as redox reactions in electron transfer and catalysis, as well as signaling and the control of gene expression.

HEME-MEDIATED SIGNALING

Hemoglobin provides the most classical example of heme signaling to the surrounding protein environment. Its mechanism of heme–heme interactions relies on structural changes, triggered by the shift of the iron atom into the heme plane upon oxygen binding. These events give rise to signals communicated to another heme across the subunit interface, resulting in a heme environment with enhanced ligand affinity. The extensive research carried out in the past decade or so on the cooperative effects in hemoglobin has recently been reviewed (Perutz *et al.*, 1998).

A surprisingly large number of heme-proteins are presently known, including some involved in regulatory cascades and in the control of gene expression. Guanylate cyclase, FixL, and CooA are three such proteins that function through an effector ligand (nitric oxide, oxygen, and carbon monoxide, respectively) binding to the heme–ferrous iron and signaling through heme-linked conformational changes. In the case of guanylate cyclase only biochemical and spectroscopic data are available, but in the case of FixL and CooA structural studies have provided insights into their mechanism of action (Gong *et al.*, 1998; Lanzilotta *et al.*, 2000). FixL acts in nitrogen fixation by *Rhizobium* species as part of a two-component signaling system. This is one of the simplest signaling strategies, commonly employed by prokaryotes. FixL is a sensor that protects the nitrogenase enzyme from oxygen, so that gene expression is activated only in anaerobic conditions. In the absence of oxygen, the PAS heme domain of FixL activates the neighboring kinase domain, leading to phosphorylation of the transcription factor FixJ, which then binds to its cognate DNA. Comparison of the crystal structures of the PAS domain in ligand-bound and ligand-free forms has given the first clues into the heme-dependent signaling mechanism. This is effected through ligand-induced steric and stereochemical changes at the heme site; in particular, oxygen arrival results in shifts of hydrophobic groups that hinder the binding site, with the concomitant displacement of a loop positioned over two pyrrole rings (Perutz *et al.*, 1999). Detailed understanding of how these conformational signals are transduced to the kinase domain will require further structural analysis on the whole FixL molecule.

In CooA, the heme-dependent signals directly affect DNA binding for the control of gene expression of the CO oxidation pathway in *Rhodospirillum rubrum*. The protein CooA is a transcription factor that belongs to the catabolite activator protein (CAP) family and activates gene expression upon binding of the CO effector to its heme site. Structure determination of CooA revealed a homodimeric molecule with a similar fold to the CAP protein (Lanzilotta *et al.*, 2000). Dimerization probably contributes to the allosteric switch between the active, CO-bound and the inactive, ligand-free forms. Two heme groups, positioned across the dimer interface, are thought to trigger a change in conformation at the heme environment, involving a rearrangement of the monomers relative to each other with the ultimate repositioning of the DNA recognition helices. The requirement of a structural transition is apparent from the binding site and the iron ligation observed in the structure of the unbound state. In fact, one of the heme ligands must be displaced upon CO binding. The heme ligands are a histidine and the N-terminal proline; the latter coordinates the iron through its nitrogen atom. This kind of ligation is unprecedented. Both the proline and other amino acids neighboring the binding site have been implicated in the dynamic changes involved in the cooperative activation of this bacterial transcription factor. Elucidation of the ligand-bound CooA structure will allow comparisons with the ligand-free form to be made, helping to decipher the molecular mechanism.

Another protein in which biological evolution has adapted heme to function as a signaling trigger is HAP1, a transcriptional activator in yeast (Pfeifer *et al.*, 1989; Fytlovich *et al.*, 1993; Zhang and Guarente, 1994). Heme synthesis in the mitochondria requires oxygen, and as the heme concentration rises, heme binds to HAP1 activating its transcriptional functions. This results in the expression of many enzymes for oxidative phosphorylation. HAP1 is a large molecule (1483 residues) that contains a zinc finger domain, a dimerization domain, a heme-responsive domain, and additional domains probably involved with transcriptional activation. Six repeats of a conserved motif R/K-C-P-I/V are believed to be involved in the association with heme. However, no structural information on the binding site is available yet.

REVERSIBLE HEME-PROTEIN INTERACTIONS FOR TRANSPORT AND IRON HOMEOSTASIS

As a vital cofactor to aerobic life, heme is a key metabolite in iron homeostasis both in eukaryotes and prokaryotes, particularly in pathogenicity. Ferrous protoporphyrin IX is a reactive species leading to oxidation of cellular components and consequent toxic effects to tissues. There has been, therefore, an evolutionary need for a molecule that is able to bind and se-

quester heme, for example, the heme lost during hemoglobin turnover, or released into the circulation by trauma and hemolysis. In mammalian organisms, the protein hemopexin (HPX) has the dual function of protecting cells from oxidative damage and of preserving cellular iron by transport to hepatocytes for receptor-mediated endocytosis. Protection from oxidative damage of free heme is particularly important for cells of barrier tissues serving the brain, the peripheral nervous system, and neural retina. A basal level of intravascular hemolysis exists in blood, as a result of normal cell breakdown, which accounts for more than 10% of all erythrocyte deaths. Hemolysis is more severe in pathologic conditions like hemolytic anemia, thalassemias, some bacterial and viral infections, and crush injuries. In addition, HPX is implicated in the control of expressions of genes for heme and iron metabolism, such as HO-1 (Alam and Smith, 1989), and for the protection against oxidative stress, such as metallothionein-1 (Alam and Smith, 1992).

HPX, which is one of the four most abundant proteins in blood serum, binds heme with the highest affinity known for a heme-protein ($K_d < 1$ pM; Wu and Morgan, 1998). The evolution of such a high affinity is a functional/structural response to the necessity of sequestering free heme within the blood stream with great efficiency. While the heme–HPX form is a tight, stable complex, the ligand is released upon interaction with a specific receptor on the liver cells. Insights into the nature of these seemingly opposite actions of tight binding and receptor-induced release were provided by the structure of the heme–HPX complex (Paoli *et al.*, 1999). The ligand is bound in a surface pocket formed at the interface between two structurally similar β-propeller domains, and embraced by an exposed flexible loop (Fig. 1a). This heme environment is unique among heme-proteins. High-affinity binding is achieved by anchoring the propionate groups inside the complex into the domain–domain interface through extensive electrostatic interactions and by packing a number of aromatic side chains around one of the pyrrole rings. One of the histidines of the iron's bis-histidyl coordination is relatively labile, and may be involved in the mechanism of heme release. This is also likely to be helped by the location of the ligand, sandwiched between the two domains. The interface presumably plays a role in opening upon interacting with the receptor. Given the presence of 14 ordered solvent molecules locked in the complex, it is tempting to picture a water-aided unzipping of the interface during ligand release.

The heme–HPX complex clearly provides a fascinating example of structure–function relationships, where specific and extensive interactions have resulted in a novel site for high-affinity, yet reversible, heme binding. Interestingly, bacterial pathogens have also evolved proteins for iron acquisition in vertebrate hosts, where the levels of free heme, or free iron, are kept at a minimum by sequestration through HPX, transferrin, and lactoferrin. For instance, *Haemophilus influenzae* secrets HxuA, a large protein able to associate with the heme–HPX complex (Hanson *et al.*, 1992). However, how this leads to iron extraction for cell growth has not yet been elucidated. Another pathogen, *Serratia marcescens*, has evolved a protein called HasA, which can circumvent HPX by allowing heme release from hemoglobin and seizing the ligand (Létoffé *et al.*, 1994). This hemophore releases the heme to a plasma membrane receptor, HasR, for subsequent internalization into the bacterial cell. So, HasA, like hemopexin, acts both as a binder and a

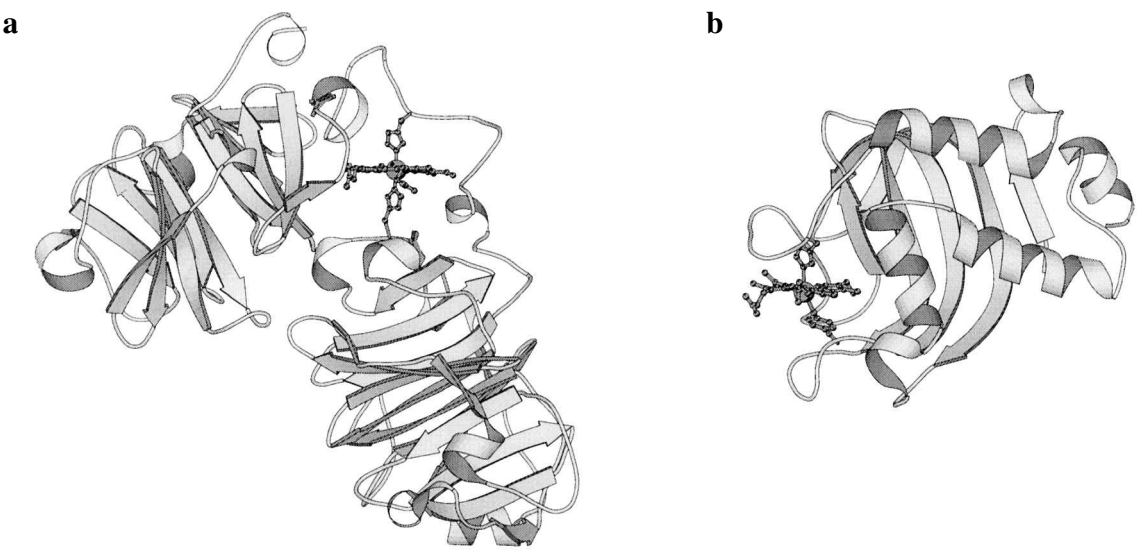

FIG. 1. Schematic representation of the folding topologies and heme binding sites in HPX and HasA. (**a**) In HPX, the heme (shown in darker gray in both diagrams) is bound between two β-propeller domains in a bis-histidyl ligation. Both the domain interface and the flexible loop region involved in heme binding are likely to play a role in the mechanisms of ligand association and dissociation (Paoli *et al.*, 1999; PDB 1QHU). (**b**) In HasA, the heme-binding site is formed by loop regions that extend from the $\alpha\beta$ fold. The heme iron is coordinated by a histidine and a tyrosine. Structural analysis suggests that control of affinity for ligand binding and release is effected via a strong hydrogen bond to the tyrosine axial ligand (Arnoux *et al.*, 1999; PDB 1DKH). In both HPX and HasA the hemes are relatively exposed to solvent (exposed surface area: 190 Å^2 in HPX and 186 Å^2 in HasA). However, the folds, heme environments and binding modes are distinctively different. The figure was drawn with MOLSCRIPT (Kraulis, 1991).

transporter of heme. However, HasA bears no structural relationship with hemopexin. A novel α/β fold in HasA associates with heme in a unique manner, bound with a tyrosine–histidyl coordination in a crevice at the surface of the molecule (Arnoux *et al.*, 1999). This heme-binding site is formed mainly by two extended loops that connect the α and β portions of the structure (Fig. 1b). Structurally, the bulk of the protein, specifically its hydrophobic core, is not affected by, nor involved with, heme binding. Despite the presence of numerous hydrophobic and stacking interactions, the ligand is only partially buried. The mechanism of binding and release has been proposed to rely on the interplay between hydrogen-bonded side chains involving the tyrosine and histidine axial ligands. Such a hydrogen-bonded network modulates the affinity for the heme by changing the reactivity of the axial ligands. This would determine not only the stability of the heme-HasA complex ($K_d < 10^{-18}$ M) formed upon sequestration of heme from blood plasma or hemoglobin, but also the ability of HasA to deliver the ligand to HasR for internalization and bacterial iron metabolism. Given the similarities between the heme binding and transporting functions of HPX and HasA, it is fascinating to see how evolution has produced two proteins with very different structures and distinct mechanisms of action (Fig. 1).

CYTOCHROMES AND OTHER REDOX HEME-PROTEINS: CONTROL OF REDOX PROPERTIES

At least half of known heme-containing proteins are cytochromes. These molecules are ubiquitous throughout living organisms, for they carry out electron transfer reactions in energy-related cellular processes. These reactions take place along a potential gradient, the extremes of which are determined by the reduction potentials of the cofactors. In catalysis, redox properties of heme are exploited by oxygenases and peroxidases. Mono-oxygenases, such as cytochrome P-450 and secondary amine mono-oxygenase, extract oxygen atoms from O_2 to oxidize organic substrates. Heme-proteins with peroxidase activity, for example, chloroperoxidase, horseradish peroxidase, and catalases, carry out substrate oxidation using H_2O_2 and other organoperoxides. These groups of enzymes highlight the many diverse reactions supported by a single type of prosthetic group. The versatility of the heme redox center has been recently reviewed (Chapman *et al.*, 1997).

The redox functions of heme-proteins are mainly governed by the precise protein–heme interactions that modulate and direct the chemistry of the iron center. In addition, changes in the substituent groups at the periphery of the porphyrin ring give rise to different types of iron–porphyrin derivatives. Probably the best-known heme-type is the b-heme, or iron–protoporphyrin IX, found in the globins, catalases, and peroxidases. This heme is also present in *b*-type cytochromes, such as cytochrome b_5, although most of the proteins involved in electron transfer reactions contain *c*-hemes that are covalently bound to the protein. The linkage is via two thioether bonds between cysteine residues and the 1′-carbon atoms of the vinyl groups (in pyrrole rings 2 and 4). This covalent association involves a conserved CxxCH motif, referred to as the "fingerprint peptide" (Fig. 2). In some rare cases, as for two of the four hemes of cytochrome c_3, the covalent attachment is effected through a CxxxxCH motif. Other heme types are known in vital proteins such as cytochrome *c* oxidase, the terminal enzyme of most respiratory chains. The crystal structure of this four-subunit, mem-

Yeast Cyt c		...TR**C**LQ**CH**TV...
Cyt f		...IV**C**AN**CH**LA...
Cyt c3	heme I	...VK**C**GD**CH**HP...
	heme III	...KS**C**VG**CH**VE...
Photosynthetic	heme I	...EG**C**TY**CH**DE...
Reaction	heme II	...VT**C**YT**CH**RG...
Centre	heme III	...TN**C**TF**CH**NA...
R.viridis	heme IV	...AD**C**RT**CH**QG...
Hydroxylamine	heme I	...KD**C**VE**CH**SD...
Oxidoreductase	heme II	...VG**C**ID**CH**VD...
	heme III	...DT**C**GT**CH**LR...
	heme P460	...EG**C**TM**CH**TN...
	heme V	...NK**C**DN**CH**TR...
	heme VI	...EA**C**AT**CH**SG...
	heme VII	...PT**C**AA**CH**ME...
	heme VIII	...LT**C**TQ**CH**SE...

FIG. 2. Alignment of the CxxCH fingerprint sequences in cytochromes with distinct folds and different heme environments. The alignment highlights the exact conservation of the heme-binding motif. Two of the four hemes of cytochrome c_3 are not listed, because they are bound by a CxxxxCH motif. In cytochrome c_3 the fold gives rise to a crossed tetra-heme arrangement, while in the photosynthetic reaction centre cytochrome (*Rhodopseudomonas viridis*) the fold positions the four hemes in a horizontal array. The 9-heme cytochrome *c* from sulphate-reducing bacteria (*Desulfovibrio desulfuricans*) is not included because its structure is made up of multiple copies of the tetra-heme cytochrome c_3 fold. The tetra-heme cytochrome c_{554} is also excluded because its folding topology is like the one of hydroxylamine oxidoreductase. Despite their lack of overall significant sequence identity, these proteins have the same secondary elements and similar tertiary structures around the hemes (Iverson *et al.*, 1998).

brane-spanning protein gave insights into the involvement of two *a*-type hemes in the reduction of molecular oxygen to water with the concomitant electrogenic transfer of protons across the membrane (Iwata *et al.*, 1995). These a-type hemes are characterized by the presence of hydrophobic hydroxyethylfarnesyl chain (on pyrrole ring III) and a formyl group (on pyrrole ring IV). A classification of cofactors and prosthetic group-containing proteins, including proteins with heme types not mentioned above, is available in the PROMISE database (http://bioinf.leeds.ac.uk/promise) (Degtyarenko *et al.*, 1998).

The *c*-type cytochromes form a large set of phylogenetically ubiquitous proteins, which have been extensively studied both biochemically and structurally (Moore and Pettigrew, 1990; Scott and Mauk, 1996). Heme is bound with the same, canonical cytochrome *c* folding topology, consisting of a simple α-helical globular fold (Fig. 3a). In chloroplast cytochrome *f*, a redox partner of cytochrome b_6 and electron donor to plastocyanin, the *c*-type heme is covalently linked, in the usual way, to an unusual fold (Fig. 3b), known as the type III fibronectin domain (FnIII) (Martinez *et al.*, 1995). The core of this fold is similar to an immunoglobulin β-sandwich, where two β-sheets pack on top of each other. There are several proteins, unrelated to cytochrome *f*, that fold with this topology, including: PapD, a bacterial chaperone; the D2 domain of human cell-surface CD4; two extracellular domains of the human growth hormone receptor and the FnIII domain of the extracellular matrix protein, tenascin. Martinez and colleagues (1995) argued that the evolution of this fold in cytochrome *f* might have been independent from the FnIII architectures mentioned above (Martinez *et al.*, 1995). In cytochrome *f*, in addition to the unusual fold, the *c*-type heme iron is coordinated at its sixth ligand position by the N-terminal amino group of the protein (Fig. 3b). Amino ligation had been predicted from spectroscopic data; involvement of the amino terminus was totally unprecedented among all known heme-proteins.

Cytochrome *f* is not the only intriguing *c*-type protein. Cytochrome c_3 is a molecule with both an unusual fold and a multiheme arrangement, known since the late '70s (Haser *et al.*, 1979). In the past 4 years, crystallographic work has unveiled several structures of other multiheme-proteins, including hydroxylamine oxidoreductase, with seven c-type hemes and a novel P460 heme (Igarashi *et al.*, 1997); cytochrome c_{554}, a tetra-heme molecule with a fold different from cytochrome c_3 (Iverson *et al.*, 1998); and a 9-heme cytochrome *c*, in which multiple copies of the cytochrome c_3-type fold associate with nine hemes in a single polypeptide chain (Matias *et al.*, 1999). Both hydroxylamine oxidoreductase and cytochrome c_{554} are part of the oxidation pathway of ammonia to nitrite in the biological nitrification carried out by the bacterium *Nitrosomonas europea*. These proteins are so abundant in the periplasm that the bacteria are distinctively red in color. In the second step of ammonia oxidation, catalyzed by hydroxylamine oxidoreductase, electrons are transferred to cytochrome c_{554} along an electron transport chain. In hydroxylamine oxidoreductase ligand binding takes place at the catalytic P460 heme, while the remaining seven c-hemes serve as a sink to move electrons away from the active site. This enzyme functions as a trimer, and therefore forms a striking arrangement of 24 hemes. Such a construction provides the redox power for the reduction of oxygen to water in both oxidation of ammonia as well as for ATP synthesis. The complexity of this nitrification process is not unique. In fact, in sulphate-reducing bacteria most cytochromes *c* have multiple hemes. The 9-heme cytochrome *c* of *Desulfovibrio desulfuricans* (Matias *et al.*, 1999) may not be the extreme case: a 16-heme high molecular-weight cytochrome (Hmc) exists in *Desulfovibrio vulgaris* (Higuchi *et al.*, 1987; Pollack *et al.*, 1991), although it is still structurally uncharacterized. The exquisite protein–heme frameworks of these multiheme molecules indicate molecular pathways used for the controlled transfer of electrons along the steps of precisely positioned prosthetic groups, with the concomitant release of free energy.

All *c*-type cytochromes bind heme through a CxxCH motif (Fig. 2). This unifying theme is used repeatedly in the structures of the multiheme cytochromes. Structural comparisons be-

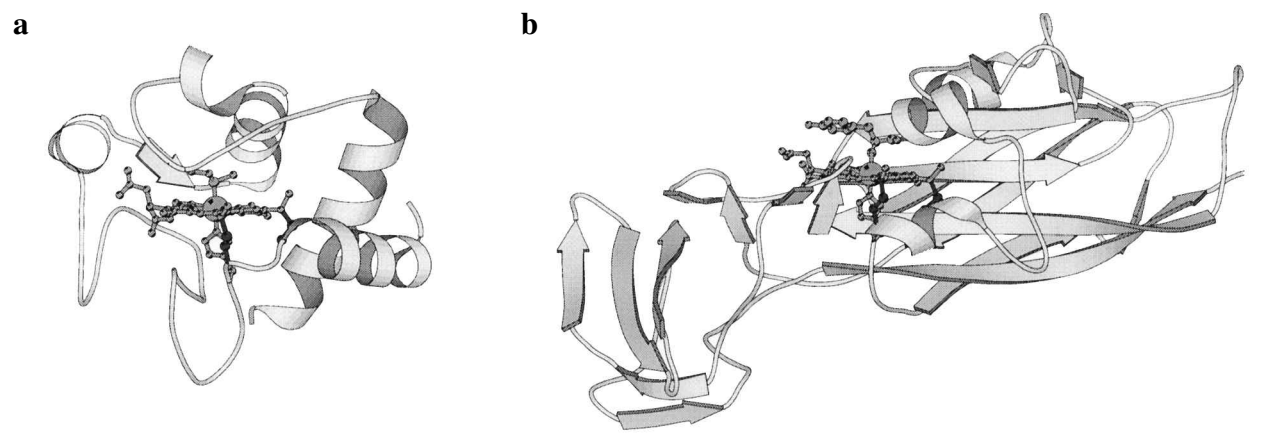

FIG. 3. Schematic representation of the folding topologies and heme-binding sites in yeast cytochrome *c* and chloroplast cytochrome *f*. (**a**) In cytochrome *c*, the heme is bound by a methionine and a histidine, and is associated with a simple, predominantly α-helical fold (Louie and Brayer, 1990; PDB 1YCC). The thioether covalent bonds are highlighted in very dark gray. (**b**) Cytochrome *f* features ligation of the heme iron by the amino terminus of the protein. The β-structure of the FnIII fold, behind the heme pocket, is apparent (Martinez *et al.*, 1994; PDB 1CTM). The figure was drawn with MOLSCRIPT (Kraulis, 1991).

tween the fingerprint peptides in cytochromes *f* and *c* show that their heme environments differ markedly although the regions of the heme-proteins linkage are identical. Formation of these covalent protein–heme thioether bonds requires specific enzymes, such as cytochrome *c* and c_1 heme-lyases (Gonzales and Neupert, 1990). These enzymes are also involved in the import of the apo-forms of mitochondrial cytochromes from the cytosol, where they are synthesized. Heme lyases contain one to three conserved Cys-Pro-Val (CPV) motifs, which are believed to be involved in heme binding (Steiner *et al.*, 1996). Why *c*-type proteins require a covalent protein–heme linkage is unknown. Perhaps the heme molecule is more stably anchored, thus conferring an evolutionary advantage because mutations in the heme pocket can be better tolerated, and allowing these proteins to fine-tune and optimize their redox properties.

The redox potential of the heme is determined mainly by the nature of its molecular environment and the axial ligands of the iron. The influence of the heme environment is apparent in cytochromes where the axial ligation of the heme iron is the same, and yet significant differences in the midpoint potentials are observed, as demonstrated by the two striking examples presented below. In multiheme cytochromes the iron is generally coordinated by histidines, in contrast to most *c*-type mono-heme cytochromes where histidine and methionine ligands are used. While all *c*-type hemes in hydroxylamine oxidoreductase have a bis-histidyl ligation, their redox midpoint potentials vary from +290 mV to −410 mV (Collins *et al.*, 1993). In cytochrome c_3 from sulfur-reducing bacteria, all four hemes have histidine ligands to the iron atoms, but their midpoint potentials range from −140 mV to −380 mV (Morais *et al.*, 1995). Comparison of different *c*-type cytochromes shows that the heme redox potential in these molecules cover a range over 1 V, from +640 mV in cytochrome c_{552} of *Thiobacillus ferrooxidans* on one extreme, to −400 mV in cytochrome c_3 of *Desulfovibrio* on another extreme (Mus-Veteau *et al.*, 1992) (Fig. 4).

Structure–function relationships on the control of redox properties have been investigated for over 30 years with the greatest efforts centered on cytochrome *c* (Moore *et al.*, 1984; Pielak *et al.*, 1985; Cutler *et al.*, 1989; Brayer and Murphy, 1996; Rafferty *et al.*, 1996). Nevertheless, and despite substantial structural data from numerous *c*-type cytochromes, including, for example, c_2, c_5, c_6, c_{550}, c_{551}, c_{552}, c_{553}, the attempts to accurately predict the redox potentials have had limited success. The reactivity of hemes varies according to the

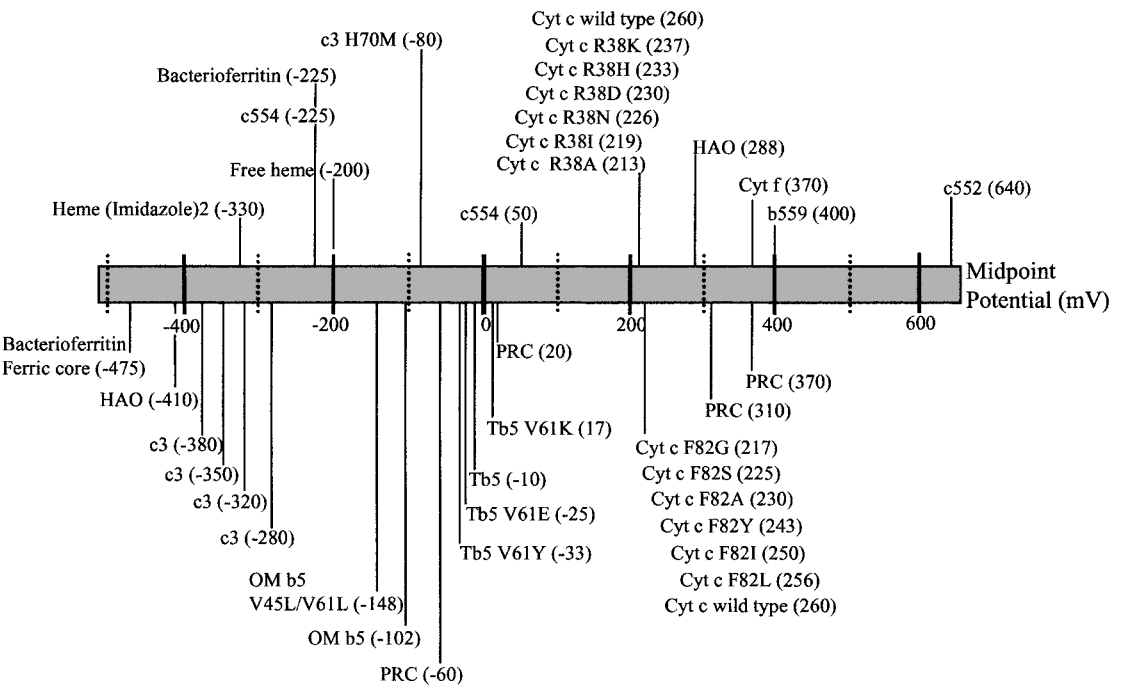

FIG. 4. Diagram reporting the redox potentials for a selection of cytochromes, including both *c*- and *b*-type cytochromes. Using the same heme prosthetic group can give rise to different midpoint potentials ranging over 1 V, depending mainly on the nature of its environment in the protein and on the axial ligands of the iron. At the positive extreme are cytochrome c_{552} from *Thiobacillus ferrooxidans* (Mus-Veteau *et al.*, 1992), cytochrome b_{559} (Shifman *et al.*, 2000), and chloroplast cytochrome *f* (Ponamarev *et al.*, 2000). At the negative extreme is one of the eight hemes of hydroxylamine oxidoreductase (Prince and George, 1997), together with bacterioferritin (Chapman *et al.*, 1997). Differences between the redox potentials of the hemes of cytochrome c_{554} (Prince and George, 1997), of the *Rhodopseudomonas viridis* photosynthetic reaction center (PRC) (Gunner and Honig, 1991) and of cytochrome c_3 are apparent (Mus-Veteau *et al.*, 1992). Mutagenesis of the histidine ligand 70 to methionine in cytochrome c_3 causes a change of at least 200 mV in one of the midpoint potentials (Mus-Veteau *et al.*, 1992). The changes in redox potentials caused by mutations in other proteins are reported: the outer membrane (OM b5) cytochrome b_5 (Rivera *et al.*, 1998), the trypsin fragment (Tb5) of bovine liver cytochrome b_5 (Wu and Xia, 1999), and yeast iso-I cytochrome *c* (Mauk and Moore, 1997). Finally, free heme (Shifman *et al.*, 2000) and heme complexed with two imidazole groups (Gibney *et al.*, 1998) are included for comparison.

following factors: (i) substituent groups on the porphyrin ring, (ii) axial ligands to the iron, (iii) the hydrophobic environment and electrostatic effects, and (iv) the heme exposure (i.e., solvent accessibility). A selective account of these factors follows, with the exception of the effects of different heme derivatives, because *c*-type cytochromes use the same heme.

LIGATION OF THE HEME IRON

The axial ligands do play a major role in controlling redox potentials due to their electronic effects on the metal center. The influences of the proximal ligand and its environment have been extensively investigated (Dawson, 1988; Mauk, 1991; Mus-Veteau *et al*., 1992; Poulos, 1996), prompted partly by the determination of the structures of several heme-proteins together with the data from site-directed mutagenesis. Hydrogen bonding to the iron's proximal ligand affects the distribution of charge as well as the strength of the ligand–metal bond. In heme-proteins, three residues are used as proximal ligands: histidine in globins and peroxidases; tyrosine in catalases; and cysteine in chloroperoxidases and cytochrome P450 mono-oxygenases. In globins, the proximal histidine donates a hydrogen bond to a main-chain carbonyl oxygen, whereas in peroxidases it interacts with an aspartate. The interaction with the negatively charged aspartate leads to a stronger hydrogen bond than in the globins, and consequently, to better electron donation to the metal center. This is likely to contribute to the ability of peroxidases to stabilize higher oxidation states of the iron during catalysis. Dawson has described these effects in terms of a "push-pull" concept (Dawson, 1988). The push comes from the electron donation by the proximal ligand. The pull, on the other hand, is the consequence of the nature of distal groups, such as the arginine in heme–peroxidases, where it is involved in polarizing the peroxide O—O bond.

In catalase, the proximal tyrosine accepts a hydrogen bond from an arginine. In chloroperoxidase and cytochrome P450, the proximal cysteine is engaged in hydrogen-bonding interactions with two main-chain amide groups. Interestingly, despite their different folds, both catalase and chloroperoxidase have an electropositive proximal environment in which interactions decrease the negative charge on the phenolate and thiolate ligands. This contributes to the high redox potential of these proteins. Here, as in the case of peroxidases and globins, electronic arguments in terms of the "push–pull" concept have been proposed (Dawson, 1988), highlighting the interplay of electronic effects between the axial ligands and the heme iron.

Mutation of one histidine of the four bis-histidyl coordinated hemes of the tetra-heme cytochrome c_3 to methionine (Mus-Veteau *et al*., 1992), as found in most mono-heme *c*-type cytochromes, caused a large increase of about 200 mV in one of the four redox potentials. When the methionine–histidyl ligation of mitochondrial cytochrome *c* was changed to a bis-histidyl ligation (Raphael and Gray, 1989), a decrease of about 200 mV in midpoint potential was observed. As a general rule, the bis-histidyl coordination in *c*-type cytochromes may be associated with lower redox potentials than the methionine–histidyl coordination (Chapman *et al*., 1997). However, exceptions exist, and data from other mutagenesis studies also indicate that there are other factors that affect the redox properties of the heme.

HYDROPHOBIC ENVIRONMENT AND ELECTROSTATIC EFFECTS

Kassner first proposed that heme-proteins decrease their midpoint potential by increasing the polarity of the heme environment, because this tends to stabilize a more highly charged oxidized state (Kassner, 1972, 1973). Theoretical calculations and site-directed mutagenesis (e.g., Varadarajan *et al*., 1989; Gunner and Honig, 1991; Mauk and Moore, 1997) have provided more insights into how the relationship between electrostatic versus hydrophobic features in the heme pocket affects redox potentials. A striking example is provided by the mutagenesis experiment of two cytochrome *f* molecules from different species. In cytochromes *f* from different species there is a high conservation of most residues in the heme pocket expect at a position where amino acids such as tryptophan, phenylalanine, leucine, and valine are observed. Tryptophan 4 was mutated to phenylalanine in cytochrome *f* from cyanobacterium *Phormidium laminosum*, while the phenylalanine at the same position in cytochrome *f* from the green alga *Chlamydomonas reinhardtii* was changed to tryptophan (Ponamerev *et al*., 2000). The structure of the wild type shows the indole ring (or benzyl ring in the green alga cytochrome) extending over the tetrapyrrole, positioned perpendicular to the heme plane. In the tryptophan to phenylalanine mutant the redox potential increases from 297 to 323 mV. In the phenylalanine to tryptophan mutant the redox potential decreases from 370 to 336 mV. In both cases the changes are about one-half of the 70 mV difference between the two species. The authors propose that the decreased midpoint potential induced by tryptophan is caused by the indole ring's π interactions with the porphyrin, which would stabilize the oxidized form of the heme through electrostatic repulsion with the Fe orbitals.

The multiheme cytochromes are obviously more complex and the redox properties of a given heme are governed not only by the polarity of the surrounding amino acid residues, but also by the variable electrostatic field from the neighboring hemes. Studies on the tetraheme cytochrome c_3 indicate that the electrostatic effects from these heme–heme interactions can result in changes of up to 60 mV (Santos *et al*., 1984; Fan *et al*., 1990).

HEME EXPOSURE AND SOLVENT ACCESSIBILITY

It is well established that the level of heme exposure to solvent is also an important factor governing redox potentials. Solvent accessibility calculations on atomic coordinates of a number of protein structures were used to show that as exposure to water increases, i.e., the polarity of the heme environment increases, midpoint potentials decrease (Stellwagen, 1978). However, there are several examples in which this correlation seems to fall short. For instance, when the phenylalanine at position 82 of cytochrome *c* is mutated to smaller amino acids (leucine, isoleucine, alanine, and glycine), the redox potential of the mu-

tants becomes more negative without significant changes in heme exposure as shown by the crystal structures of these mutants (Louie and Brayer, 1989). Interestingly, the structure in the mutants adopt a different conformation from the wild type, to fill the void formed by the removal of the bulky side chain. These mutants may exhibit increased dynamics and flexibility owing to a possibly looser packing in the molecule. This work indicates that conformational changes and dynamic properties can sometimes make rationalizations of redox behavior more complicated. In general, the effect of water on the redox properties of the heme has been shown in numerous calculations and experiments (e.g., Warshel *et al.*, 1997; Rivera *et al.*, 1998). A recent theoretical study focused on an overall consideration of porphyrin π-electron polarisation as well as pH, ionic strength, and other electrostatic-related factors (Edholm *et al.*, 2000). This work underlines that redox control is the result of complex additive effects. An interesting case, illustrating this point, is the tyrosine-to-phenylalanine mutation at position 67 in cytochrome *c* (Berghuis *et al.*, 1994). Structure determination of the mutant reveals a rearrangement of the hydrogen-bonding network together with some shifts in atomic positions. Furthermore, a water molecule is found bound internally, in addition to the water molecule already present in the wild type. In this example, the simple loss of one hydroxyl group increases the polarity of the local heme environment resulting in a marked decrease in redox potential.

PERSPECTIVES

The wide spectrum of functions observed in heme-containing proteins is a consequence of the versatility of the heme group whose properties are modulated by its molecular environment. Structural studies have helped define the interactions in the heme pocket of many proteins. This has provided insights into the strategies of ligand binding and release by HPX and HasA, and into the mechanisms of signal transduction in FixL and CooA. Further studies are needed to establish the details of how heme-linked conformational changes are related to the action of these proteins. In the cytochromes, catalases, and peroxidases, diverse chemical reactions are sustained by the same heme prosthetic group. Comparison of different cytochromes shows that the protein scaffold controls redox properties of heme over a range of more than 1 V. Research over the past three decades has produced a wealth of structural and functional data leading to qualitative principles to understand midpoint potentials. Theoretical calculations of midpoint potentials of heme-proteins have shown great promise, although they are often insufficient to account for the number of correlated parameters affecting the properties of the heme. In particular, protein flexibility can result in structural rearrangements that are not possible to predict *a priori*, posing a problem for theoretical calculations of redox potentials. The complex interplay of multiple factors that influence the properties of heme and its environment make quantitative predictive descriptions of redox properties inaccurate. There is no doubt that future structural and functional studies on both redox and nonredox heme-proteins will reveal more interesting aspects of the multipurpose, widespread prosthetic group heme.

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