Design, synthesis, and characterization of a novel hemoprotein

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Abstract

Here we describe a synthetic protein (6H7H) designed to bind four heme groups via bis-histidine axial ligation. The hemes are designed to bind perpendicular to another in an orientation that mimics the relative geometry of the two heme *a* groups in the active site of cytochrome *c* oxidase. Our newly developed protein-design program, called *CORE*, was implemented in the design of this novel hemoprotein. Heme titration studies resolved four distinct K_D values ($K_{D1} = 80$ nM, $K_{D2} = 18$ nM, $K_{D3} \geq 3$ mM, $K_{D4} \leq 570$ nM, with $K_{D3} \times K_{D4} = 1700$); positive cooperativity in binding between the first and second heme, as well as substantial positive cooperativity between the third and forth heme, was observed. Chemical and thermal denaturation studies reveal a stable protein with native-like properties. Visible circular dichroism spectroscopy of holo-6H7H indicates excitonic coupling between heme groups. Further electrochemical and spectroscopic characterization of the holo-protein support a structure that is consistent with the predefined target structure.

Keywords: de novo proteins; heme; hemoprotein; protein design

Supplemental material: See www.proteinscience.org.

Recent advances in theoretical, experimental, and computational protein design have led to the synthesis of metalloproteins with targeted function (Mutz et al. 1996, 1999; Pinto et al., 1997; Benson et al. 1998; Rau et al. 1998). A class of designed metalloproteins receiving considerable attention is hemoproteins (Sasaki and Kaiser 1989; Choma et al. 1994; Robertson et al. 1994; Rabanal et al. 1996; Gibney et al. 1997; Rojas et al. 1997a, 1997b; Rau and Haehnel 1998). Native hemoproteins such as cytochromes, globins, and peroxidases perform a wide range of cellular function including electron transfer, oxygen transport, and catalysis. It is this diversity of reactivity that has been a driving force for the de novo design of functional hemoproteins. These designed proteins offer the potential for robust, highly tunable structures that are likely to provide ideal systems in

which native hemoproteins can be better understood. An understanding of the role that ligands and local heme environment play in controlling the redox and catalytic reactivity of bound heme groups will ultimately lead to the construction of synthetic hemoproteins with novel targeted functionality.

Combinatorial and so-called rational design methods have shown promise in producing synthetic proteins with predefined structures. Development of computational methods (Hellenga and Richards 1994; Desjarlais and Handel 1995a,b; Dahiyat and Mayo 1996, 1997; Dahiyat et al. 1997; Lazar et al. 1997; Su and Mayo 1997; Street and Mayo 1999; Jiang et al. 2000) has further expanded the ability to design synthetic proteins with targeted structure and function. However, despite the available tools, there still remains only a small number of uniquely folded designed hemoproteins. In many examples, the flexibility often associated with synthetic proteins precludes detailed characterization of structure-function relationships.

We have implemented a computational approach in the design of a protein that binds heme in predefined binding

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sites. The designed hemoprotein is a highly stable four- α -helical structure with native-like properties that binds up to four low-spin heme groups via bis-histidine axial ligation. The heme groups are designed to bind in a perpendicular orientation relative to another. Inspiration for the design is drawn from the diheme (heme aa_3) active site of cytochrome c oxidase (CcO; Iwata et al. 1995; Tsukihara et al. 1995). Electron transfer from heme a to heme a_3 is a key step in the catalytic cycle of CcO that leads to reduction of O_2 to H_2O and proton translocation across the membrane (Babcock and Varotsis 1993; Hill 1993; Einarsson et al. 1995). The critical component in the hemoprotein design described in this article is the implementation of *CORE*, our newly developed protein-design program (Jiang et al. 2000).

Results and discussion

Protein design

To accommodate perpendicular heme groups in a simple protein structure, an all-parallel four- α -helix motif was selected. The design, shown in Figure 1, incorporates four

Fig. 1. Model of designed hemoprotein showing the all-parallel backbone structure and four perpendicular bis-histidine ligated heme groups. The two loops at the bottom represent C-terminal disulfide links between parallel α -helices.

helical segments of identical sequence with each of the two pairs of helices linked by a disulfide bond. The resulting noncovalent dimer of helix-link-helix peptides contains a pseudofourfold symmetry axis parallel to the helix dipoles. The design accommodates four heme groups oriented perpendicular to one another.

The amphiphilic α -helical units are 27 amino acids long with a C-terminal Gly-Cys sequence that, when oxidized, forms the flexible disulfide link between two parallel helices. Each helix contains two histidine residues at positions 6 and 7, yielding four pairs of ligands that are designed to bind the four low-spin heme groups via bis-histidine axial ligation. The design requires that a histidine at position 6 on one helix and a histidine at position 7 from an adjacent helix serve as the pair of ligands that binds each heme.

The backbone structure of the protein, called 6H7H, was modeled by starting with four identical 27 amino acid helices with histidines at positions 6 and 7. The backbone dihedral angles of the helices were adjusted to yield 3.6 residues/turn (see below). Four heme groups were then attached to the four pairs of histidine ligands, fixing each $N_{\text{His6}}\text{-Fe}_{\text{Heme}}\text{-N}_{\text{His7}}$ angle to 180° and each $\text{Fe}_{\text{Heme}}\text{-N}_{\text{His}}$ bond distance to 2.0 Å. While maintaining these parameters and the necessary fourfold symmetry axis, the histidine side chain dihedral angles (χ_1 and χ_2) were adjusted until the C-terminal end of the helices reached an appropriate distance to allow formation of the disulfide bond between C-terminal cysteine residues. The result is the V-shaped structure evident in Figure 1.

Positioning of hydrophobic and hydrophilic residues that constitute the helical domain was determined using a method that expands on the repeating heptapeptide method often used to design two-helix coiled coils and four-helix peptides. The heptad method assumes 3.5 residues/turn and, therefore, seven unique positions. Typically, hydrophobic residues are assigned to positions a and d , while positions b , c , e , f , and g are assigned as hydrophilic residues (DeGrado et al. 1989; Myszkowski and Chaiken 1994). It is common to assign opposite charges to residues at positions e and g to maximize favorable interchain electrostatic interactions (Zhou et al. 1994). Although the heptad method has been somewhat successful in yielding α -helical proteins of predefined structure, it is more realistic to characterize α -helices in a four-helix protein by 3.6 residues/turn. A simple yet relevant survey of the α -helices in three highly helical natural proteins (ROP, Mb, cyt b_{562}) reveals that the average value for the sum of the backbone dihedral angles (Φ and Ψ) is -104° . This value translates to 3.57 residues/turn yielding 25 unique positions. For simplicity, this was rounded to 3.6 residues/turn, yielding 18 (a - r) unique positions that define an α -helix, instead of the seven in the heptad method. As indicated in Figure 2, the a - r method assigns positions a , d , e , h , k , l , and o to hydrophobic interior residues defining nearly 40% of the helix face. Positions

Fig. 2. Helical wheel projection showing the assignment of hydrophobic and hydrophilic positions using the *a-r* method described in the text. The C β carbons of the side chains are labeled *a* through *r*. Hydrophobic positions (*e*, *l*, *a*, *h*, *o*, *d*, *k*) are at the bottom of the projection. Hydrophilic positions at the interface between helices (*b*, *i*, *p*, and *n*, *g*, *r*) are on the left and right. Exterior hydrophilic positions (*m*, *f*, *q*, *j*, *c*) are at the top of the projection.

i, *b*, and *p* and *g*, *n*, and *r* define opposite sides of the interhelical interface and are assigned as hydrophilic residues of opposite charge to assure optimal interhelical electrostatic interactions. The remaining positions (*c*, *f*, *j*, *m*, and *q*) are opposite the hydrophobic face and are assigned to hydrophilic residues with the potential to only form intrahelical contacts. The *a-r* method will, of course, assign hydrophobic residues to positions different from what would have been assigned by the heptad method; however, it yields sequences with hydrophobic positions that match more closely with that of natural four-helix proteins (Z. Xu, R.S. Farid, unpubl.).

The sequence of the 27-amino-acid peptide is presented below:

As described previously, positions *a*, *d*, *e*, *h*, *k*, *l*, and *o* are hydrophobic residues (with the exception of the histidine ligands at positions *d* and *e*). The identity of the hydropho-

bic residues was determined using *CORE*, our newly developed protein-design program (Jiang et al. 2000). Three separate runs were initiated starting with Ala at each core position. This yielded three distinct families of sequences; however, the top-ranked sequence from each run was identical. Therefore, this sequence was chosen as the one most likely to produce a uniquely folded protein with high thermal stability.

Slices through the helices (Fig. 3) of the resulting structure show the computer-designed hydrophobic core residues. Also highlighted are potential inter- and intrahelical electrostatic interactions among interface and exterior hydrophilic residues. Four tryptophan residues at positions 3 and eight phenylalanine residues at positions 10 and 13 effectively fill the interior space of the V-shaped backbone structure while defining the top and bottom of the heme binding sites, respectively. Lysine residues at positions 2 and 4 are designed to form favorable electrostatic interactions with the heme propionate side chains.

The relative geometry of adjacent perpendicular heme groups in the model of 6H7H is similar to that found for the two perpendicular heme *a* groups in cytochrome *c* oxidase (CcO). The angle between planes that define heme *a* and *a*₃ in native CcO is 108°; this value is similar to the 90° angle between adjacent hemes in 6H7H. The shortest edge-to-edge distance between heme groups in CcO is 7 Å as compared to 6 Å in the model of 6H7H.

Peptide synthesis and size-exclusion chromatography

The 27-amino-acid peptide that 6H7H is derived from was synthesized, purified, and allowed to oxidize in air, yielding a disulfide-linked di- α -helical peptide that was purified again by reversed-phase HPLC. The identity of the di- α -helical peptide was confirmed by electrospray/ionization mass spectrometry (MW: 6738). The molecular weight of the protein in solution as assessed by size exclusion chromatography is 11 ± 2 kD, a value consistent with formation of the designed four- α -helix protein (MW_{calc} = 13.5 kD).

Circular dichroism spectroscopy

The circular dichroism (CD) spectrum of apo-6H7H (see Supplemental Material) is characteristic of a protein with a high degree of helicity. The apo-protein has a mean residue ellipticity at 222 nm ($[\theta]_{222}$) of $-24,800$ deg cm²/dmol and a $[\theta]_{222} : [\theta]_{208}$ ratio of 0.93, indicating $\approx 90\%$ α -helical content (Greenfield 1996). Fitting the CD spectrum using the Brahms basis set spectra yields a value of $90\% \pm 8\%$ helicity, in close agreement with the value determined from the $[\theta]_{222}$ value.

Heme binding

Detailed titration studies were undertaken to determine whether 6H7H binds four heme groups via bis-histidine

Fig. 3. Four slices through the helices of holo-6H7H highlighting residues that define the perpendicular *bis*-histidine bound heme groups, packing of interior hydrophobic residues, and potential charged interactions between exterior and interface residues. The heme edges including peripheral groups are represented by thick lines in A and B. The heme propionate side groups are shown explicitly extending from the heme edge.

axial ligation as designed. Hemin from DMSO or 100 mM NaOH solution was incrementally added to a 23.5 μ M protein solution. An absorption spectrum was recorded following each addition. Binding is considered complete when difference spectra match that of free hemin. At the protein concentration used, this required addition of ~ 215 μ M of hemin or ~ 9 equivalents.

To determine the concentration of bound and free heme at all concentrations of added heme, each of the 36 spectra

collected during the heme titration were fit to a linear combination of component spectra using a program written in Mathematica (Wolfram Research; see Supplemental Material). The same three component spectra were sufficient to fit each of the 36 spectra. The first component spectrum is simply that of free heme obtained from difference spectra at the end of the titration, where the concentration of added heme far exceeds the concentration of protein. The second component spectrum corresponds to difference spectra ob-

tained early in the titration, below 1.8 equivalents of added heme. These difference spectra are nearly identical to one another and, therefore, represent a basis set spectrum. A third component spectrum was needed to fit the spectra at heme concentrations above 2 equivalents. The three component spectra corresponding to free heme, bound heme below 2 equivalents, and bound heme above 2 equivalents are shown in Figure 4. The two component spectra that correspond to bound heme are clearly similar to one another and are consistent with low-spin bis-histidine bound-heme groups. Indeed, the component spectrum corresponding to the first hemes that bind to 6H7H is nearly superimposable with that of native cytochrome b_{561} , a natural protein that binds a single heme via bis-histidine ligation. Figure 5 compares the synthetic and natural hemoprotein spectra revealing remarkable similarity, including spectral widths and extinction coefficients for both the oxidized and reduced states. The component spectrum that corresponds to hemes that bind above 2 equivalents, although distinct from that of the first hemes, is also clearly consistent with bis-histidine ligation. The Soret band maximum is centered at 410 nm, compared to 416 nm for the first hemes. This 6 nm difference is consistent with differences in λ_{max} among native cytochrome b proteins.

Having established that two distinct types of hemes bind to 6H7H, it remains to determine heme binding stoichiometry and dissociation constants. To do this, the precise con-

Fig. 4. Component absorption spectra used to fit spectra from heme titration experiment: (A) first and second hemes; (B) third and fourth hemes; (C) free heme.

Fig. 5. Superimposition of absorption spectra of holo-6H7H (0.1 equivalents initial hemin concentration) and native cyt b_{561} in both oxidized and reduced states. Thick line: holo-6H7H $\lambda_{\text{max}}(\text{ox}) = 414$ nm, $\text{FWHM}(\text{ox}) = 31$ nm, $\lambda_{\text{max}}(\text{red}) = 426$ nm, $\text{FWHM}(\text{red}) = 17$ nm. Thin line: cyt b_{561} $\lambda_{\text{max}}(\text{ox}) = 415$ nm, $\text{FWHM}(\text{ox}) = 29$ nm, $\lambda_{\text{max}}(\text{red}) = 429$ nm, $\text{FWHM}(\text{red}) = 18$ nm.

centration of bound heme and free heme at each point in the titration must be known. This was made possible by noting that at low initial hemin concentrations, only one of the component spectra was needed to fit the difference spectra and that at very high initial hemin concentrations, the difference spectra correspond to free hemin. Therefore, it was possible to determine the extinction coefficients of the three component spectra presented in Figure 4. Accordingly, at all heme concentrations, the fit of the difference spectra yields the concentration of heme bound to the protein or free in solution. A plot of these concentrations as a function of the initial hemin concentration is shown in Figure 6. The plot shows that below 2 equivalents of added heme, two hemes bind tightly, as evidenced by the minimal concentration of free heme observed. The first component spectrum in Figure 4 corresponds to these two heme groups. Above 2 equivalents, the concentration of the two tightly bound hemes remains constant, while the concentration of the other two spectrally distinct bound hemes increases. These two hemes bind more loosely than the first two hemes; an additional 7 equivalents of heme is needed to fully fill the third and fourth binding sites. These results clearly demonstrate that, as designed, 6H7H binds four bis-histidine ligated heme groups.

Fig. 6. Plot showing the concentration of bound hemes and free heme that correspond to the component spectra in Figure 3 as a function of initial hemin concentration. The first and second bound hemes are represented by black circles, third and fourth bound hemes by gray circles, and free heme by white circles. Heme concentrations are divided by the initial protein concentration (23.5 μM) to indicate equivalents of bound and added heme. The plot shows that two equivalents of heme bind tightly to 6H7H and that a second 2 equivalents of heme, distinct from the first two, bind more loosely. The data were fit to a model that incorporates individual K_D values for each of the four hemes.

Binding of the first two hemes is clearly associated with submicromolar dissociation constants, as evidenced by the nearly linear increase in bound heme concentration with addition of heme (Fig. 6). To gain greater insight into the nature of the binding of these hemes, careful analysis of the difference spectra below 2 equivalents of added heme was conducted. This revealed a slight red shift of the heme Soret band on heme addition (Fig. 7). This result implies that the λ_{max} of the monoheme and diheme proteins differ slightly. The data were fit to two K_D values, revealing positive cooperativity in heme binding with a K_{D2}/K_{D1} ratio of 0.22, a λ_{max} of the monoheme protein of 414.3 nm, and a λ_{max} of the diheme protein of 415.5 nm. It is not possible from these data to determine individual values for K_{D1} and K_{D2} ; only the ratio of K_D values can be determined. To obtain accurate values for the individual dissociation constants, heme titration at lower protein concentration (0.95 μM) was conducted (Fig. 8). At this protein concentration, complications from binding of the third and fourth heme are essentially eliminated. Fixing K_{D2}/K_{D1} to a value of 0.22 and fitting the data in Figure 8 to a model that incorporates two K_D values yields $K_{D1} = 80 \pm 10$ nM and $K_{D2} = 18 \pm 2$ nM.

Analysis of the shift in λ_{max} above 2 equivalents was not possible because of the presence of relatively high concen-

Fig. 7. Shift in the wavelength maximum (λ_{max}) of the heme Soret band on addition of up to 2 equivalents of hemin. The data was fit to a model in which two distinct heme groups, with different λ_{max} values, bind to 6H7H (see text for details).-

tration of free heme, precluding accurate determination of any λ_{max} shift that may exist. However, as the first two heme binding sites are nearly 100% filled at 2 equivalents of added heme, it is possible to determine the dissociation

Fig. 8. Plot of bound and free heme as a function of initial hemin concentration, similar to Figure 5, but for a much lower protein concentration (1.09 μM). The first and second bound hemes are represented by black circles, third and fourth bound hemes by gray circles, and free heme by white circles. The plot shows that even at low protein concentration, 2 equivalents of heme bind to 6H7H. The second two heme groups do not bind because the protein concentration is lower than their K_D values. The data were fit to a model that incorporates individual K_D values for first two tightly bound hemes.

constants for the third and fourth hemes (K_{D3} , K_{D4}) by fitting the data in Figure 6 above 2 equivalents of added heme. K_{D1} and K_{D2} were simply assumed to be zero, which is reasonable given that their values are approximately three orders of magnitude lower than the initial protein concentration. Fitting the data to a model that incorporates two K_D values cannot yield individual values for K_{D3} and K_{D4} ; however, the fit does yield $K_{D3} \times K_{D4} = 1700 \pm 50$ and $K_{D3} \geq 3$ mM. The quality of the fit is maintained for values of $K_{D3} \geq 3$ mM as long as the product of K_{D3} and K_{D4} is 1700. For example, if K_{D3} is assumed to be 3 mM (the lower limit), K_{D4} must be 570 nM (the upper limit for K_{D4}), and if K_{D3} is set to 10 mM, K_{D4} is 170 nM, and so forth. Although only a lower limit for K_{D3} and an upper limit for K_{D4} could be determined, it is clear that substantial positive cooperativity in heme binding exists between the third and fourth hemes.

As a consequence of the substantial negative cooperativity in heme binding between the second and third heme and the low (nM) K_D values for the first and second heme, it is possible to isolate nearly pure diheme-6H7H. If the protein concentration is as low as 1.5 μ M and 2 equivalents of heme are added, 90% of the protein species in solution is diheme-6H7H. The remaining 10% exists as monoheme-6H7H (7%) and apo-6H7H (3%). At a higher initial protein concentration of 20 μ M, addition of 2 equivalents of heme gives diheme-6H7H in 97% yield. At the same concentration of protein and assuming the lower limit for K_{D3} (3 mM), addition of ~ 10 equivalents of heme is required to give a 90% yield of tetraheme-6H7H. Because of the positive cooperativity between the third and fourth heme, the remaining 10% exists as diheme-6H7H, not fourth triheme-6H7H. The yield of tetraheme-6H7H is not dependent on the value chosen for K_{D3} , as the product of K_{D3} and K_{D4} is constant.

Mono-histidine variant of 6H7H

The above results suggest that, as designed, holo-6H7H adopts an all-parallel topology in which His6/His7 pairs bind heme. To further support the designed structure, a variant that replaces His 7 with Ser was synthesized, yielding a four-helix protein (6H7S) with only four His residues. If the precise designed backbone structure and His side chain orientation of 6H7H is maintained in the 6H7S variant, the four His7 residues would be orthogonal to one another, thereby precluding incorporation of heme groups via axial bis-histidine ligation.

Surprisingly, 6H7S does bind heme. Figure 9 shows the heme titration data that indicates binding of a single heme group with a K_D of 12 ± 5 μ M. The absorption spectrum of holo-6H7S (not shown) is superimposable with that of diheme-6H7H, showing that the heme is bound via bis-histidine ligation. The K_D is, however, significantly larger than that of the first two hemes that bind to 6H7H. Size exclusion

Fig. 9. Plot of the concentration of bound heme and free heme as a function of initial heme concentration for 6H7S, showing that only one equivalent of heme binds. The initial protein concentration was 38 μ M. The data was fit using a single dissociation constant yielding $K_D = 12 \pm 5$ μ M.

chromatography reveals a molecular weight for holo-6H7S that is consistent with a four-helix structure (data not shown). Given these unexpected results, it is necessary to address possible structures for 6H7S that could lead to binding of single low-spin heme group. An antiparallel four-helix structure is ruled out because the two-fold symmetry axis would yield two identical heme binding pockets at opposite ends of the protein. It is very unlikely that in such a structure only one heme group would bind. Therefore, the most plausible explanation for the heme binding data is that 6H7S maintains an all-parallel topology with slight changes in the structure that allow a pair of His 7 residues to orient in such a way as to allow bis-histidine ligation of a single heme. Furthermore, the incorporation of a single heme group must somehow preclude binding of a second heme. This idea was tested by attempting to generate a model for 6H7S in which a single heme group is bound to two His7 residues from diagonally disposed helices. In such a structure, the bound heme would entirely block incorporation of a second heme. It was possible to model such a structure by a slight rotation of the helices and modification of the His side-chain torsion angles (χ_1 and χ_2). Although a reasonable model could be generated, several unfavorable van der Waals interactions were identified between the bis-histidine ligated heme group and hydrophobic amino acid side chains, thus providing a plausible explanation for the relatively large K_D value for heme binding to 6H7S.

Far-UV/visible circular dichroism spectroscopy of holoprotein

The far-UV CD spectrum of 6H7H is only slightly altered on addition of heme, suggesting that there is little change in

the secondary structure on heme binding. $[\theta]_{222}$ for the tetraheme protein is only 7% greater than that of the apo-protein. The $[\theta]_{222} : [\theta]_{208}$ ratio increases slightly from 0.93 for the apo-protein to 1.04 for the tetraheme protein. The change in the $[\theta]_{222} : [\theta]_{208}$ ratio coincides with the change in concentration of bound heme as a function of added heme (Fig. 10), suggesting that minor changes in the secondary structure are directly associated with heme binding. This result is consistent with the observed positive cooperativity in heme binding; it is conceivable that heme binding induces a subtle change in the protein structure that facilitates binding of subsequent heme groups.

Size-exclusion chromatography of the holoprotein

To confirm that heme binding does not alter the aggregation state of 6H7H, the molecular weight of diheme-6H7H was determined by size exclusion chromatography. Using a calibrated column, the molecular weight of holo-6H7H was determined to be 15.5 ± 2.0 kD, a value consistent with the theoretical molecular weight of 14.8 kD. The third and fourth hemes do not bind tightly enough to allow molecular weight determination by this method. However, as the heme titration data shows that the first and second heme spectra are unaltered above 2 equivalents of added heme, it is reasonable to assume that the third and fourth hemes do not change the aggregation state and that the four- α -helix structure is maintained at all heme concentrations.

Chemical denaturation

Chemical denaturation with GdmCl at 25°C of apo- and holo-6H7H was performed to assess the degree of coopera-

Fig. 10. Plot showing the similar dependence of the $\epsilon_{222}/\epsilon_{208}$ ratio (from far-UV CD spectra) and total bound heme concentration as a function of initial hemin concentration.

Fig. 11. Temperature melt of apo-6H7H in 1.9 M GdmCl (squares) and of diheme-6H7H in 3 M GdmCl.

tivity and free energy of unfolding. At a protein concentration of 20 μ M, apo-6H7H exhibits a cooperative denaturation transition with an m value of -2.3 kcal/(mol M) and ΔG_u of 13.8 kcal/mol. As expected, diheme-6H7H exhibits a more cooperative denaturation transition and a larger free energy of unfolding: $m = -3.1$ kcal/(mol M), $\Delta G_u = 18$ kcal/mol. The large m value for the holoprotein is indicative of a cooperatively folded protein, a key property that characterizes native proteins.

Thermal denaturation

Thermal denaturation of apo- and holo-6H7H in buffer did not yield any detectable melting transition; at 95°C, apo- and holo-6H7H are only 40% and 34% unfolded, respectively. Therefore, thermal denaturation was conducted in the presence of GdmCl. Apo-6H7H was thermally denatured in buffer containing 1.9 M GdmCl, yielding a relatively narrow denaturation profile with a T_m of $75.9 \pm 0.4^\circ\text{C}$ (Fig. 11). Van't Hoff analysis of the data gives a ΔH_m of 12.0 ± 0.4 kcal/mol and a ΔC_p of 123 ± 14 cal/(mol K). The thermal denaturation profile of diheme-6H7H (Fig. 11) was obtained in the presence of 3 M GdmCl, yielding a T_m of $67.9 \pm 0.5^\circ\text{C}$. Fitting the data gives a ΔH_m of 19.3 ± 1.0 kcal/mol and a ΔC_p of 405 ± 19 cal/(mol K).

ANS binding

ANS is a hydrophobic probe molecule that has been used extensively to probe the “molten globule” and native state

of synthetic and natural proteins (Ku wajima 1989; Semisotnov et al. 1991; Quinn et al. 1994; Poklar et al. 1997). ANS has also been used to determine the hydrophobicity of the heme cavity of apocytochrome b_5 (Falzone et al. 1996). Since it was observed that apocyt b_5 has no apparent affinity for ANS, it was concluded that the heme pocket is not especially hydrophobic.

Addition of 10 μM apo-6H7H to a solution containing 0.5 μM ANS results in a significant increase and blue shift of ANS fluorescence (Fig. 12), indicating that the probe molecule binds to the hydrophobic interior of apo-6H7H, presumably in the designed heme binding pocket. A plot of the fluorescence intensity as a function of protein concentration (not shown) yields a K_D of 8.2 μM . In contrast to apo-6H7H, di heme-6H7H does not exhibit any appreciable binding of ANS (Fig. 12), consistent with a compact structure that precludes incorporation of the hydrophobic probe molecule.

Electrochemistry

Redox potentials for holo-6H7H were measured using standard spectroelectrochemical methods. The data, shown in Figure 13A, was initially fit to a single Nernst (see Supplemental Material); however, the residuals indicated the strong possibility that an additional redox couple was present. Therefore, the data for di heme-6H7H was fit to a double Nernst yielding two potentials of equal amplitude: $E_1^\circ = -91$ and $E_2^\circ = -133$ mV. These potentials presumably correspond to the two distinct bound heme groups. This is confirmed by the observation that λ_{max} of the α -band corresponding to reduced heme blue shifts with decreasing potential (Fig. 13A). If the potentials of the two hemes were identical, no shift in λ_{max} would have been observed. Indeed, the monoheme variant 6H7S does not exhibit any

detectable shift in λ_{max} , suggesting that the shift observed for 6H7H is not an artifact of the experiment. The λ_{max} data for di heme-6H7H can in principle be fit to four parameters: two electrochemical potentials, (E_1° and E_2°) and two λ_{max} values (λ_1 and λ_2), corresponding to the two heme groups. However, the level of noise in the data precludes a four-parameter fit. Instead, the data was fit fixing E_1° and E_2° to the values determined above while allowing λ_1 and λ_2 to vary. This fit yielded the curve shown in Figure 13A, and values for λ_1 and λ_2 of 560.6 and 556.6 nm, respectively. The excellent fit to the data strongly suggests that indeed the hemes in di heme-6H7H have distinct electrochemical potentials with a ΔE° of -42 mV.

It is tempting, but not entirely correct, to assign the origin of this ΔE° to electrostatic interaction between the two bound hemes. In a molecule with two noninteracting redox centers, the difference in redox potentials, ΔE° , is -35.6 mV at 25°C (Bard and Faulkner 1980). The difference is calculated from $-(2RT/F)\ln 2$ and arises from statistical considerations; the E° of the second heme is more negative (harder to reduce) than the first because there is only one heme rather than two available for reduction. The experimental ΔE° value of -42 mV is close to the theoretical value of -35.6 mV, suggesting that there is little, if any, electronic interaction between the hemes in di heme-6H7H.

Characterization of the redox properties of tetraheme-6H7H was also conducted. Because large concentrations of free heme interfere with the spectroelectrochemical experiment, only 4 equivalents of hemin was used. At this initial concentration of hemin and given the initial protein concentration of 20 μM , the distribution of species in solution is the following: concentration of free heme = 28 μM (35%), di heme-6H7H = 13.7 μM (34%), and tetraheme-6H7H = 6.1 μM (31%). Note that nearly one-third of the added hemin is incorporated into tetraheme-6H7H. Essentially no tri heme-6H7H exists in solution as a result of the substantial positive cooperativity in heme binding between the third and fourth heme. The redox data for this mixture of free heme, di heme-, and tetraheme-6H7H are presented in Figure 13B. The data fit well to the sum of two Nernst equations yielding potentials of -110 mV and -195 mV. The high potential reduction is nearly identical to the average of the two redox potentials for di heme-6H7H. The lower potential matches that of free heme (Fig. 13C). Attempts to fit the data to more than two Nernst equations were unsuccessful because of the complexity of the mixture of holoproteins and free heme. However, it is clear that the reduction potential of the hemes in tetraheme-6H7H is very similar to that measured for di heme-6H7H. The apparent similarity in heme redox potentials is consistent with the pseudo fourfold symmetry of the designed protein. It suggests that the hemes are bound in pockets with similar local dielectric constants and solvent exposure (Stellwagen 1978).

Fig. 12. Fluorescence spectra of 0.5 μM ANS in the presence of 10 μM apo-6H7H (solid line), 10 μM di heme-6H6H (dotted), and free in buffer (dashed), indicating binding of ANS to apo-, but not holo-6H7H.

Fig. 13. (A) Redox titration data and shift in the wavelength maximum ($\lambda_{\max}$) of the reduced heme α -band for di-heme-6H7H. (B) Redox data for 6H7H with 4 equivalents of added hemin. (C) Redox data for free hemin in buffer. The black lines through the data are fits to one or the sum of two Nernst equations, and the gray lines are the component Nernsts in the cases where the data was fit to the sum of two Nernst equations. Arrows indicate midpoint potentials.

CO binding

Although the redox potential of the bound heme groups in 6H7H is not high enough to allow binding of dioxygen, it was possible to investigate CO binding. Figure 14 shows the reduced plus CO minus reduced spectrum obtained for di-heme-6H7H. The normalized difference spectrum for 6H7H with 4 equivalents of added hemin is superimposable with that of di-heme-6H7H. The difference spectrum shows very similar features to that obtained for low-spin myoglobin (Fig. 14), prepared by addition of excess pyridine to a solution of myoglobin at pH 13 (Wood 1984). The striking similarities in these spectra further confirm the low-spin bis-histidine ligation state of our designed hemoprotein.

Tryptophan fluorescence

The four tryptophan residues at position 3 on each helix of 6H7H define one edge of the heme binding site. To probe the local protein environment in the vicinity of the Trp residues for apo- and holo-6H7H, fluorescence spectroscopy was employed. Tryptophan fluorescence has been used extensively as a probe of local protein environment in natural proteins (Chetverin et al. 1980; Lee et al. 1989; Viguera et al. 1992; Genov et al. 1993; Gilardi et al. 1994; Chen and Sanyal 1999). In particular, the tryptophan fluorescence maximum, λ_F , and full width at half maximum (FWHM) are used as a probe for the extent of solvent exposure: λ_F for a buried Trp is 330–332 nm, for a Trp with limited exposure λ_F is 340–342 nm, and λ_F is 350–353 nm for a Trp exposed to water (Burststein et al. 1973). The tryptophan fluorescence maximum of apo-6H7H is 342.7 nm, and the FWHM is 56.7 nm. These values are indicative of a side chain immobilized at the surface of the protein with

limited solvent exposure (Burststein et al. 1973), consistent with the model presented in Figures 1 and 3. Titration with heme results in concomitant blue shift in λ_F as well as significant quenching. Addition of 1.5 equivalents of heme shifts λ_F to 340.1 nm (FWHM = 56.1 nm). On addition of 2.0 equivalents of heme, the tryptophan fluorescence is no longer observable. The λ_F for di-heme-6H7H, calculated by extrapolation, is 339.4 ± 0.2 nm. This value is similar to that of rabbit pyruvate kinase ($\lambda_F = 339$ nm), which has three partially exposed Trp residues near the surface of the protein (Burststein et al. 1973). These results indicate that, as designed, the bound heme groups are proximal to the tryptophan residues, allowing efficient quenching via energy transfer and that binding of the hydrophobic heme groups results in diminished exposure of the Trp residues to water. This may occur as the result of either direct shielding by heme or through a more closely packed protein structure in which solvent is more effectively excluded from the interior of the protein. Indeed, the calculated solvent exposure of Trp in 6H7H decreases from 85% to 62% on heme binding assuming no gross change in the backbone structure. (Solvent exposure was calculated using the program DSSP [Kabsch and Sander 1983].)

Visible circular dichroism

Hemin is an achiral molecule and, therefore, in solution does not exhibit a CD signal. However, when hemin is incorporated into the highly asymmetric environment of a protein, pronounced dichroism in the Soret region is often observed. Heme CD has been attributed to coupled oscillator interactions between heme transitions and allowed $\pi\pi^*$ transition on nearby aromatic residues (Hsu and Woody 1971). It has also been shown that CD can be the result of coupled oscillator interactions with peptide $\pi\pi^*$ transitions and high-energy peptide and thioether sulfur transitions (Blauer et al. 1993). In addition, inherent heme chirality arising from nonplanar distortions can contribute to the Soret CD (Blauer et al. 1993). More recently, it has been shown in studies with hemoglobin that heme–heme excitonic interactions also induce CD in the Soret band (Goldbeck et al. 1997). This has also been supported by theoretical work (Woody 1985).

To investigate any potential heme–heme excitonic coupling in holo-6H7H, CD spectra in the Soret region were collected for solutions in which 1, 2, and 3 equivalents of hemin were added to 55.6 μ M of apo-6H7H. Figure 15 shows these CD spectra and for comparison the CD spectrum of the monoheme 6H7S variant. Holo-6H7S displays classic negative and positive Cotton effects at 403 and 424 nm, respectively. Furthermore, the normalized CD spectra for holo-6H7S are identical at all heme concentrations up to 1 equivalent (data not shown). The CD spectra for holo-6H7H are more complex, and in contrast to 6H7S, display

Fig. 14. Reduced plus CO minus reduced difference spectra for di-heme-6H7H (thick line) and low-spin Mb (thin line).

Fig. 15. (A) Circular dichroism spectra in the Soret region for holo-6H7H in which 1, 2, and 3 equivalents of hemin was added. (B) Soret circular dichroism spectrum for the monoheme variant holo-6H7S.

dramatic spectral modulation as a function of initial hemin concentration. To better understand the spectra, it is important to recognize the distribution of holo-species at each initial hemin concentration. Using the previously determined K_D values, 1 equivalent of added hemin yields ~20% monoheme-6H7H, 40% diheme-6H7H, and 20% apo-6H7H. At 2 equivalents of hemin, 98% is diheme-6H7H. At 3 equivalents, 72% is diheme-6H7H and 27% is tetraheme-6H7H. The change in the CD spectra on going from 1 to 2 equivalents is presumably the result of a higher concentration of diheme-6H7H relative to monoheme-6H7H. As major changes in the protein structure have been ruled out from far-UV CD studies, the most likely explanation for the spectral differences is heme–heme excitonic coupling in the diheme protein that is necessarily absent in monoheme-6H7H. Because of the positive cooperativity in heme binding, it was not possible to obtain a spectrum corresponding to pure monoheme-6H7H. Differences between the spectrum corresponding to 3 equivalents of added heme and that of diheme-6H7H are tentatively assigned to the presence of tet-

raheme-6H7H in solution. Again, the spectral differences most likely arise from heme–heme excitonic coupling in tetraheme-6H7H distinct from that present in diheme-6H7H. Although not electrostatically coupled, these results strongly suggest that the hemes in holo-6H7H are electronically coupled.

Concluding remarks

The synthetic hemoprotein presented here is a rare example of a de novo–designed native-like protein with a structure that appears to coincide with the predefined target structure. The bis-histidine ligated heme groups in tetraheme-6H7H appear to bind in precisely the orientation dictated by the target structure. The successful design is the result of implementing *CORE* in selection of the hydrophobic residues that define the core of the protein and the heme binding sites. The design methodologies established in this article have recently led to the successful design of a second-generation hemoprotein that binds only two heme groups oriented perpendicular to one another (Z. Xu and R.S. Farid, in prep.). Research will now focus on design of metalloproteins with targeted function such as electron-transfer and oxygen binding and activation.

Materials and methods

Materials

FMOC-protected amino acids, PAL–PEG–PS solid-support resin, and reagents used for solid-phase peptide synthesis were purchased from Perceptive Biosystems. Hemin was obtained from Acros and used without further purification. HPLC grade CH_3CN was purchased from Fisher Scientific.

Protein design and visualization

Proteins were modeled using Sybyl 6.3 (Tripos) on an RM5200 300 MHz SGI O2 workstation.

Sequence selection using *CORE* was conducted on an IBM SP2 workstation or an R10000 180 MHz SGI Onyx workstation. A detailed description of how the *CORE* is implemented in protein design is described elsewhere (Jiang et al. 2000); however, a brief outline of the procedure used in designing 6H7H is presented here. The backbone structure with bound heme groups and exterior hydrophilic residues was held fixed while core hydrophobic residues were targeted for design. The identity of these residues at positions 3, 10, 13, 14, 17, 21, and 24 were selected from a subset of 7 hydrophobic residues (Ala, Val, Ile, Leu, Phe, Trp). Selection of sequences predicted to yield optimally stable protein structures was accomplished using Metropolis-driven simulated annealing and low-temperature Monte Carlo sampling.

Peptide synthesis and purification

Peptides were synthesized on a Millipore Model 9050Plus solid-phase peptide synthesizer using FMOC-protected amino acids on a

PAL-PEG-PS resin. Peptides were cleaved by stirring the dry resin for 2 h under nitrogen at room temperature with 8.8 : 0.5 : 0.2 : 0.5 TFA : phenol : triisopropylsilane : water followed by precipitation with cold diethylether. Crude peptides were purified by reversed-phase HPLC using a 2.5 × 25-cm preparative Vydac C₁₈ column and a linear H₂O/CH₃CN (0.1% TFA) gradient at a flow rate of 7 mL/min. Purified peptides were then lyophilized and stored at 4°C. Formation of the disulfide linked di- α -helical peptides was accomplished by stirring monomer peptides overnight in a 250 mM Tris buffer solution at pH 8.5. The disulfide-linked peptides were purified by reversed-phase HPLC as described above. Electrospray/ionization mass spectrometry (PeptideGenic Research) was used to confirm the identity of the peptides.

Heme titration

Heme titrations were conducted at pH 8.8 in 250 mM Tris-acetate buffer. Peptide concentrations were determined by absorbance at 280 nm using an extinction coefficient (ϵ) of 5600 M⁻¹ cm⁻¹. The ϵ was determined from the spectrum of apo-6H7H in 6 M guanidinium chloride (GdmCl). Stock solutions of hemin (1–2 mM) were freshly prepared in DMSO or in 0.1 M NaOH. Incremental amounts (typically 1–2 μ L) of hemin were added to protein solutions (1–50 μ M) via a 10- μ L Hamilton syringe. Following each injection of hemin, the solution was stirred and allowed to equilibrate for 5 min before acquiring spectra.

Redox titration

Spectroelectrochemical experiments were conducted using a homemade redox cuvette fitted with two side arms (one for nitrogen gas inlet and one with a septum for dithionite injections). Stirring was accomplished by vertically agitating an internal magnetic stir bar with an exterior magnet. An anaerobic atmosphere in the cuvette was maintained by a stream of nitrogen presaturated with water to prevent evaporation. Redox potentials were measured with a Platinum/Ag/AgCl combination electrode calibrated with a saturated solution of quinhydrone in 100 mM phosphate buffer (pH 7.0) before and after each redox titration. Mediation between the electrode and protein-bound heme groups was achieved using a range of redox mediators chosen for their electrochemical potentials and their lack of optical interference in the 500–600-nm region. The following mediators were employed that allow mediation from approximately –200 to +100 mV: 20 μ M phenazine, 20 μ M 2-hydroxy-1,4-naphthoquinone, 20 μ M phenazine methosulphate, 70 μ M duroquinone, and 5 μ M pyocyanine. Protein concentrations were in the range of 8–20 μ M in 250 mM Tris-acetate buffer (pH 8.8). Absorption spectra from 450 to 700 nm were collected on an HP 8452A diode array spectrophotometer following incremental addition of dithionite, the chemical reductant. The percentage of reduced heme was determined from the α -band intensity at around 560 nm minus absorption at 750 nm to correct for baseline drift. Data were fitted to the Nernst equation using Kaleidagraph 3.0.5 (Abelbeck Software).

Size-exclusion chromatography

Size-exclusion chromatography was performed by HPLC using a Pharmacia Superdex 75 HR 10/30 column. The column was eluted at a flow rate of 0.8 mL/min with 100 mM phosphate, 100 mM NaCl (pH 8.0) and calibrated with ribonuclease A (13.7 kDa), chy-

motrypsinogen A (25.0 kDa), ovalbumin (43.0 kDa), and BSA (67.0 kDa).

Spectroscopy

Absorption spectra were obtained on a Perkin Elmer Lambda2S spectrophotometer, using 0.5-nm wavelength steps at a scan rate of 240 nm/min. Fluorescence spectra were obtained on an SLM 8100 Fluorometer, using a bandwidth of 4 nm on both excitation and emission monochrometers, 1 nm wavelength steps, and 5 sec average time. For tryptophan emission spectra, protein solutions at pH 8 (100 mM Tris buffer) were excited at 280 nm. Spectra were collected from 290 to 450 nm. Circular dichroism (CD) spectra were obtained in 0.1-cm cuvettes on an AVIV Model 202 CD instrument, using a 1 sec average time, a 1 nm bandwidth, and a wavelength step size of 1 nm. Three spectra were scanned and averaged to yield the reported CD spectra.

ANS binding

Binding of 1-anilinonaphthalene-8-sulfonate (ANS) to apo-6H7H and holo-6H7H was monitored by fluorescence spectroscopy. The excitation wavelength was 370 nm, and emission spectra were recorded from 390 to 600 nm. Samples were 0.5 μ M in ANS (pH 8; 100 mM phosphate buffer), and protein concentrations ranged from 1 to 40 μ M. Bovine serum albumin, to which ANS is known to bind (Pal and Patra 1994), was used as a control protein. Emission intensities were corrected for the internal filter effect arising from competing heme absorption using the following equation:

$$E_c = E_o(1 - 10^{-A_H}) \frac{A_o}{A_H}$$

E_c is the corrected emission intensity, E_o is the measured emission intensity, A_o is the optical density at the excitation wavelength for the apoprotein, and A_H is the optical density at the excitation wavelength of the sample.

CO binding

Carbon monoxide was bubbled through the solution containing holo-6H7H for 5 min before acquiring absorption spectra. Saturation of the solution with CO was conducted directly in a 1 × 1-cm quartz cuvette fitted with a rubber septum using two syringe needles, one for the CO inlet and the one as an outlet.

Supplemental material

Circular dichroism spectrum of apo-6H7H, Mathematica program used to fit heme titration spectra, and the fit of the redox data shown in Figure 13A to a single Nernst equation (3 pages).

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