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Midpoint reduction potentials and heme binding stoichiometries of de novo proteins from designed combinatorial libraries

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Abstract

We previously reported the de novo design of combinatorial libraries of proteins targeted to fold into four-helix bundles. The sequences of these proteins were designed using a binary code strategy in which each position in the linear sequence is designated as either polar or nonpolar, but the exact identity of the amino acid at each position is varied combinatorially. We subsequently reported that approximately half of these binary coded proteins were capable of binding heme. These de novo heme-binding proteins showed CO binding characteristics similar to natural heme proteins, and several were active as peroxidases. Here we analyze the midpoint reduction potentials and heme binding stoichiometries of several of these de novo heme proteins. All the proteins bound heme with a 1:1 stoichiometry. The reduction potentials ranged from −112 to −176 mV. We suggest that this represents an estimate of the default range of potentials for heme proteins that have neither been prejudiced by rational design nor selected by evolution.
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Keywords: Protein design; Binary patterning; De novo proteins; Heme proteins; Protein reduction potential

1. Introduction

The pioneering work of Walter Kauzmann laid the foundation for much of our current understanding of protein structure, stability and function. Kauzmann described the crucial role of the 'hydrophobic bond' in protein stability [1,2]. While many forces are involved in specifying protein structure, the 'hydrophobic bond' is considered a major contributor to the overall free energy stabilizing the folded structures of globular proteins. The importance of sequestering hydrophobic residues from water has also proven invaluable for the de novo design of proteins.

We previously reported the design and construction of several combinatorial libraries of proteins using a binary code strategy [3,4]. In this strategy, each position in an amino acid sequence is designed to be either polar or nonpolar; however, the exact identity of each polar and nonpolar residue is not specified, and is varied combinatorially. Combinatorial diversity is made possible by the organization of the genetic code: five nonpolar

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amino acids (Met, Leu, Ile, Val and Phe) are encoded by the degenerate DNA codon NTN and six polar amino acids (Lys, His, Glu, Gln, Asp and Asn) are encoded by the degenerate DNA codon XAN (N represents any of the DNA bases A, G, C or T, while X represents A, G or C) [3].

The binary code strategy is based on the premise that the periodicity of polar and nonpolar amino acids in the linear sequence of a protein is sufficient to direct the formation of amphiphilic elements of secondary structure, such as α-helices and β-strands. These amphiphilic helices and strands then self-assemble into globular folds that minimize exposure of the hydrophobic side chains to solvent. For the construction of de novo four-helix bundles, the binary patterning of polar and nonpolar amino acids was designed to match the structural periodicity of 3.6 residues per turn found in α-helical secondary structure. For the construction of β-sheet proteins, the binary patterning was designed to match the alternating periodicity found in β-strand secondary structure.

The sequences from the first binary code library were targeted to fold into 74-residue four-helix bundles [3]. Characterization of >50 proteins from the initial library demonstrated that virtually all of them folded into compact α-helical structures [3,5,6], and a few of them adopted structures with some native-like properties [5–7].

We subsequently reported that approximately half the proteins in the four-helix bundle library were capable of binding heme with micromolar affinity [8]. When ligating heme, these solutions displayed the typical red color associated with natural heme proteins. Moreover, the designed heme-binding proteins displayed the typical UV–Vis spectra associated with natural heme proteins. The de novo heme proteins have Soret absorbance maxima at ~412 and ~426 nm in the oxidized and reduced states, respectively. The reduced heme proteins also show the characteristic Q-band absorbances at ~530 and ~560 nm [8].

A second-generation library of four-helix bundle proteins was recently designed and characterized [27]. This second-generation library was prepared as an iterative redesign of the first library with the aim of producing proteins that are more stable and more native-like than those in the original 74-residue library. The second-generation sequences are 102 amino acids long and were designed to have helices approximately 50% longer than those in the original library. The elongated sequences were constructed using a first-generation sequence, #86, as the ‘parent’ sequence. Protein 86 was chosen because (i) it was straightforward to express and purify, (ii) remains monomeric at high concentrations, and (iii) binds heme. Protein 86—like many of the proteins from the original library—formed a fluctional structure with properties reminiscent of molten-globule folding intermediates. A major goal of the redesign was to devise a second-generation library of proteins with structures that were well-ordered rather than ‘molten’. Five proteins from the second-generation library were expressed and purified. Characterization of these proteins demonstrated that four of them were dramatically more stable and more native-like than those in the original 74-residue library (Wei et al., submitted for publication). They denature cooperatively and show well-dispersed NMR spectra, trademarks of well-folded native-like structures. All the sequences examined from this second library bind heme with micromolar affinity.

The sequences from these two libraries (the original and the second generation) were designed to fold into four-helix bundle proteins. They were not explicitly designed to bind heme. While histidine and methionine, known heme-ligating residues in natural heme proteins, were incorporated into the combinatorial mix, no attempts were made to build a heme-binding pocket into the proteins. Moreover, the sequences were not subjected to any selection, either in vivo or in vitro, for heme binding or heme-based functionality. Therefore, these sequences provide a unique opportunity for an unbiased assessment of the functional potential of heme proteins that have neither been prejudiced by rational design nor selected by evolution.

The functions of natural heme proteins can be grouped into three general categories: (i) catalysis, (ii) binding of small molecules (e.g. O_2, CO, CN and NO) and (iii) electron transfer. Each of these functions can be assessed for the de novo proteins from our binary code libraries. We have already established the catalytic potential of the novel

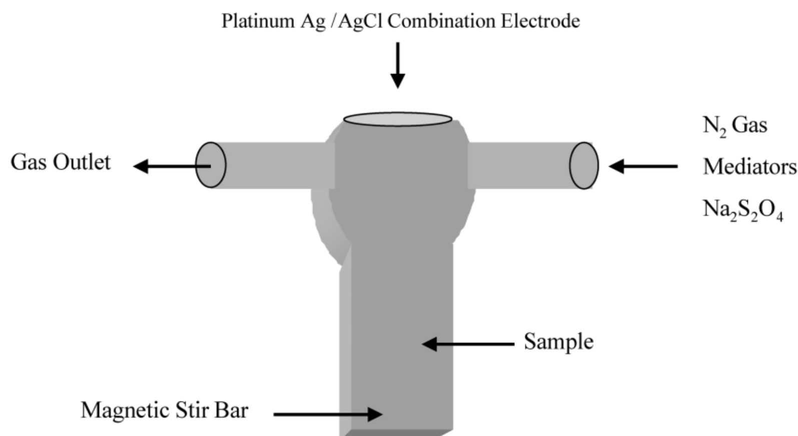

Fig. 1. Schematic diagram of the 1-cm quartz cuvette used for the redox titrations.

heme proteins by demonstrating that several of them have significant peroxidase activity, including one heme protein with a catalytic turnover rate only ~3.5-fold slower than that of horseradish peroxidase [9]. We have also studied the carbon monoxide binding characteristics of several of the heme proteins and shown that the CO binding kinetics and resonance Raman spectral features are similar to those of natural heme proteins [10]. In the current study, we assess the reduction potentials and heme binding stoichiometries for several proteins from our binary pattered libraries of α-helical proteins.

2. Materials and methods

2.1. Redox titrations

Proteins were overexpressed in *E. coli* strain X90(DE3) grown in 2xYT as described previously [3]. The proteins were extracted from cells using a freeze–thaw protocol [11] and then solubilized in 100 mM $MgCl_2$ [5–7]. Cellular contaminants were removed by acid precipitation in 50 mM sodium acetate buffer (pH 4.0). The resulting supernatant was loaded onto a POROS HS cation exchange column (PerSeptive Biosystems), and eluted using a gradient of NaCl from 0 to 1.5 M. Purified proteins were concentrated, and buffer exchanged to 50 mM Tris, 50 mM NaCl, pH 8.0 using Centricon Plus-20 filters (Millipore).

Incorporation of heme into the purified proteins was accomplished using a freshly prepared stock solution of 1–2 M hemin chloride (Sigma) in 0.1 M NaOH. Aliquots of this stock solution were added to the protein samples until a light red solution was observed. Typical heme protein solutions were 10–15 μM, with Soret peaks between 0.8 and 1.6 absorbance units. Protein concentrations were estimated to be approximately 10-fold greater than total heme concentration, thereby minimizing the amount of unbound heme. Typical preparation of a heme protein was as follows: 10 mg of hemin chloride (Sigma) was dissolved in 10 ml of 0.1 M NaOH. Thirty microliters of this stock heme solution was added to 800 μl of concentrated protein in 50 mM Tris, 50 mM NaCl (pH 8.0) buffer. The resulting solution was always lightly red. The solution was then diluted with Tris buffer to a final volume of 3.5 ml.

The heme protein sample was placed in a specially designed bulbous 1 cm path length quartz cuvette (Fig. 1). The cuvette had a large septum through which a platinum Ag/AgCl combination electrode (Accumet) with an attached Accumet AB15 digital display was placed for measuring the solution potential. The cuvette was constructed with two glass side arms for introducing inert gas (N_2 or Ar) over the sample. The reducing agent ($Na_2S_2O_4$) and the redox mediators (Table 1) were also introduced to the sample through the side

Table 1
Midpoint potentials of the mediators used for redox titrations

Mediators	E_m (pH 7.0) (mV)	Concentration used
2-Hydroxy-1,4-naphthoquinone	−152	25 μM
Pyocyanine	−34	20 μM
Duroquinone	5	70 μM
Phenazine ethosulfate	55	20 μM
Phenazine methosulfate	80	20 μM
Quinhydrone[a]	280	Saturated

[a] Not added to the sample. Used to calibrate the electrode for each titration.

arms of the cuvette. A small magnetic stirring bar was placed at the bottom of the cuvette to mix the reagents.

The heme protein sample and magnetic stir bar were placed in the cuvette. The electrode was calibrated before sealing it into the cuvette. The electrode was calibrated by placing it into a saturated solution of quinhydrone in 1.0 M phosphate buffer pH 7.0. The resulting potential should be 280 mV relative to the standard hydrogen electrode (SHE). The difference between 280 and the measurement taken from the quinhydrone-saturated sample is the amount added to each measurement throughout the titration. For example, if the quinhydrone-immersed electrode showed a solution potential of 80 mV, every measurement would require an additional 200 mV added to the reading to be standardized against SHE.

The glass side arms were sealed with rubber septa. Syringe needles were inserted into the side arm septa and the system was deoxygenated for at least 1 h with either N_2 or Ar. A series of mediators were added through the septum-covered side arm (Table 1). These mediators are electron buffering agents helping to stabilize the potential of the solution. The reduction potential of each mediator is listed in Table 1. Stock solutions of each mediator were prepared in DMSO. All the mediators were purchased from Sigma except Pyocyanin, which was prepared by the photoactivation of phenazine methosulfate in water [12].

After fully deoxygenating the sample, a UV–Vis spectrum of the initial sample was recorded using a Hewlett Packard 1042 diode array spectrophotometer. The background for the spectra was air. A stock solution of saturated sodium hydrosulfite in water was used as the reducing agent. Aliquots of sodium hydrosulfite less than 1 μl were added to the sample per injection. The sample was stirred and the system was allowed to equilibrate for several minutes. Both the UV–Vis spectrum and solution potential were recorded after each injection of hydrosulfite. The titration continued until the sample solution was maximally reduced by the hydrosulfite (≈ −400 mV vs. SHE).

Data were analyzed using Kaleidagraph, and titration curves were fit to the Nernst equation:

$$\%R = \frac{1}{10^{(E_h - E_m)/(RT/nF)} + 1} \times 100$$

where $\%R$ is the percentage of reduced metalloprotein, E_h is the solution potential, E_m is the midpoint reduction potential of the heme, and n is the number of electrons involved in the reaction ($n = 1$). The equation was entered into Kaleidagraph as:

$$Y = m7 + \{m2/10\hat{}((m0 - m1)/(59.15{*}n) + 1)\}$$

This is the equation for a single electron reaction where $n = 1$. The $m0$ term represents the values for the X-axis. The $m7$ term represents the 100% oxidized signal at the bottom of the plot (−0.015 in Fig. 3). The $m2$ term represents the total amplitude of the plot (0.065 absorbance units in Fig. 3). The $m1$ term is the midpoint reduction potential of the heme protein.

2.2. Heme binding stoichiometry

Two stock solutions were prepared: the first solution contained 17 μM protein in 50 mM Tris, 50 mM NaCl, pH 8.0. The second solution was an equivalent concentration (17 μM) heme solution in Tris buffer. Different ratios of the two solutions were mixed, but the total volume of the combined samples was held constant at 500 μl. UV–Vis spectra were recorded for each mixed sample. The amount of heme/protein complex was measured by recording the Soret peak at 412 nm, and subtracting away the absorbance at 356 nm, which

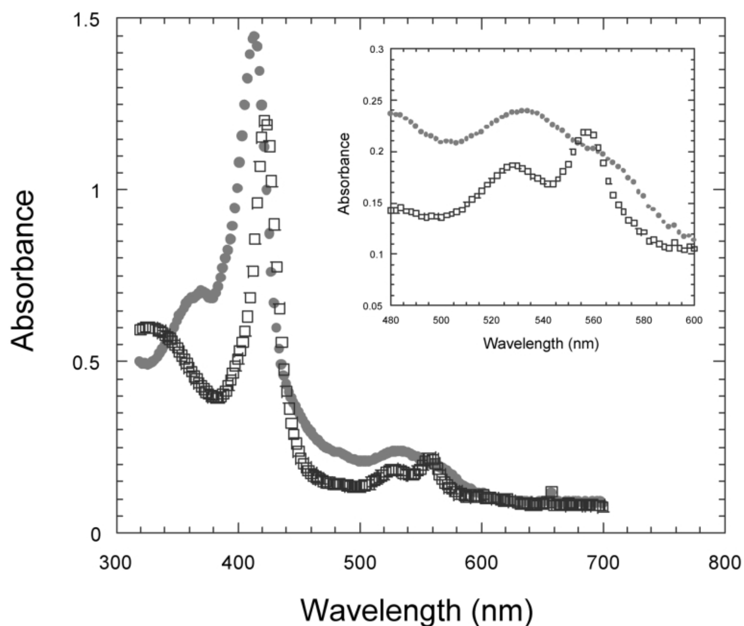

Fig. 2. UV–Vis spectra of oxidized (filled circles) and reduced (open squares) heme protein S-824. *Inset:* Magnification of the Q-band region of the oxidized and reduced heme protein S-824.

is due to any residual free heme remaining in solution. This difference was then plotted against the molar percentage of heme in the sample (Fig. 4).

Heme solutions were prepared fresh daily. The heme concentration was determined by measuring the UV–Vis spectra of the solution ($\varepsilon_{385\ \mathrm{nm}} = 58.4$ $\mathrm{mM}^{-1}\ \mathrm{cm}^{-1}$). The protein concentrations were determined using standard amino acid analysis. The analyses were performed by the W.M. Keck Foundation Biotechnology Resource Laboratory of Yale University.

3. Results

3.1. Midpoint reduction potentials

The midpoint reduction potential (E'_o) is the solution potential at which the concentration of oxidized heme protein equals the concentration of reduced heme protein. The midpoint potentials of our heme proteins were determined by redox titrations [13]. Samples of heme protein were reduced gradually using the electron donating agent sodium hydrosulfite ($Na_2S_2O_4$). The extent of heme-protein reduction for each sample was monitored spectroscopically. As shown in Fig. 2, the reduced and oxidized states of the heme proteins are spectroscopically different, presenting an easy means for quantifying the relative populations of each species during a titration. As shown in the inset of Fig. 2, an absorbance maximum at 558 nm and a minimum at 544 nm exist for the reduced heme protein. The reduced heme protein also shows a large absorbance maximum at 422 nm while the oxidized species has a maximum at 414 nm. Any of these spectral differences could be used to monitor the change in population from the oxidized (Fe^{3+}) to the reduced (Fe^{2+}) state. The Soret absorbance (414 nm for the oxidized and 422 nm for the reduced) was not used because several of the mediators absorb in this region. Therefore, the Q-bands (absorbance maxima at ~530 and ~560 nm), which do not overlap with the mediators, were used to monitor the titrations. Fig. 3 shows the redox titration for protein S-824. Table 2 lists the midpoint reduction potentials for

eight of the binary code heme proteins. The midpoint potentials ranged from −112 to −176 mV.

3.2. Heme binding stoichiometry

To determine the heme to protein stoichiometries for the designed proteins, we used the continuous variation method (also called Job plots) [14]. Two solutions of equal concentration were prepared. The first solution contains the protein in a non-intrusive buffer. The second solution contains the ligand of interest, heme in these studies. The solutions were mixed in various ratios, while keeping the total volume of the mixtures constant, to vary the relative mole fractions of the protein and the ligand.

For each heme/protein mixture a UV–Vis spectrum was recorded. In these spectra, the absorbance at 412 nm measures the concentration of the heme protein, while the absorbance at 356 mm measures the concentration of free heme. The difference between these values ($A_{412}-A_{356}$) was plotted as a function of the mole fraction of heme (Fig. 4). This difference achieves its maximal value at the molar ratio of heme to protein that produces the most heme protein and the least free heme. The Job plot shown in Fig. 4 shows that for protein S-285, the value of $A_{412}-A_{356}$ peaks when the mole

Table 2
Midpoint reduction potentials and heme:protein binding stoichiometries for several designed heme-binding proteins

Protein	E_m vs. SHE (mV)	Heme:protein
F	−130	1:1
G	−161	ND
76	−176	1:1
86	−112	1:1
90	−165	1:1
S-213	−132	1:1
S-285	−154	1:1
S-824	−174	1:1

Proteins with an 'S' are second generation library sequences having elongated helices.

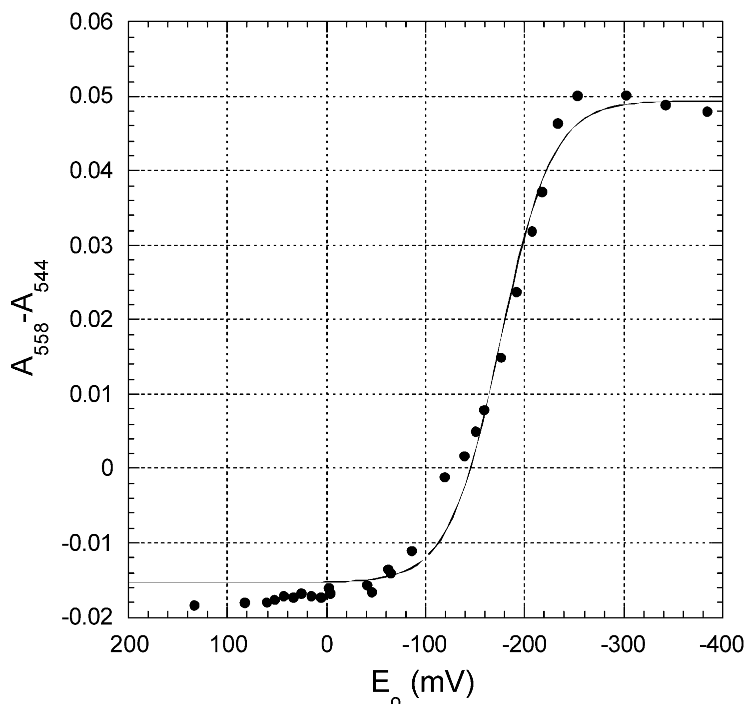

Fig. 3. Redox titration curve for heme protein S-824. Line represents a best fit to the Nernst equation for a single electron reaction.

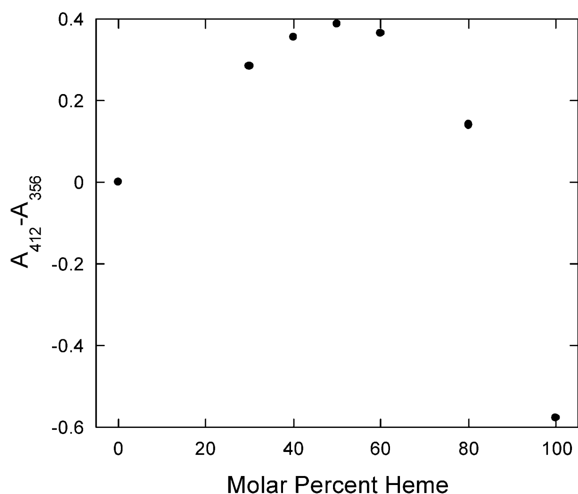

Fig. 4. Job plot used to determine the stoichiometry of heme binding to protein S-285.

percent of heme is 50%. Formation of the complex is maximized when protein and heme are present in a 1 to 1 ratio. The results of Job plots for the de novo proteins, summarized in Table 2, indicate that the proteins characterized in this study bind heme with a 1:1 stoichiometry.

4. Discussion

The midpoint reduction potentials for the designed heme-binding proteins range from −112 to −176 mV. This 64 mV range was found in a very small sampling of this library (compared to the theoretical diversity of $>10^{41}$ sequences in the initial library [3]). This range of midpoint potentials can be compared with the values measured for other designed four-helix bundle heme proteins, as shown in Table 3. Those proteins, designed to ligate heme between two histidine residues, have midpoint reduction potentials ranging from −90 to −220 mV. Included in Table 3 are the nearly 400 four-helix bundle proteins constructed by Haehnel and coworkers, which had midpoint potentials that ranged from −90 to −150 mV [15].

Heme alone has a reduction potential of approximately −220 mV. Binding to a protein can shift this potential considerably. Thus, the midpoint

Table 3
Midpoint reduction potentials of several designed four-helix bundle heme-binding proteins

Protein	E_m (mV)	Reference
MOP1[a]	−110/−170	[23]
H10A24[a]	−170/−265	[24]
H11A24	−166	[24]
$VAVH_{25}$	−170	[24]
Retro(S–S)	−220	[25]
Haehnel's 400 sequences	−150 to −90	[15]
F	−130	This work
G	−161	This work
76	−176	This work
86	−112	This work
90	−165	This work
S-213	−132	This work
S-285	−154	This work
S-824	−174	This work

[a] Binds 2 hemes per protein.

reduction potentials of natural heme proteins span a range of ~800 mV (Fig. 5). The potential of a protein-bound heme is influenced by several factors including the electronic nature of the substituents attached to the heme, the identities of the amino acids ligating the heme, the solvent accessibility of the heme, and the electrostatic interactions with residues surrounding the heme. These factors have been analyzed and discussed both for natural, and for designed heme proteins [16–19].

In recent years, several of these factors have been altered in studies aimed at engineering the midpoint potential of heme proteins. For example, Dutton and coworkers used several complementary strategies to modulate the potential of their de novo four-helix bundle H10A24 [20]: (i) using different heme groups with different ring substit-

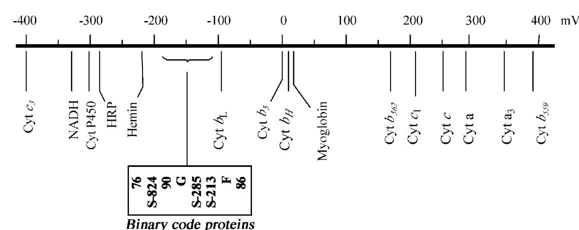

Fig. 5. Midpoint reduction potentials of heme and various heme-binding proteins [18,20,21,26]. Potentials are under standard conditions at pH 7.0.

uents, they produced a range of potentials spanning 300 mV. (ii) By altering the pH, and thereby protonating a nearby glutamate, they produced a range of 160 mV. (iii) Finally, by incorporating charged residues into the heme pocket they produced a range of 120 mV. By combining all three factors, Dutton and coworkers were able to construct a family of de novo heme proteins with midpoint reduction potentials spanning 435 mV.

Recently, McLendon and coworkers explored the range of potentials that can be achieved by modifying one or two amino acids surrounding the heme pocket of a naturally evolved four-helix bundle [21,22]. By constructing a library of mutations in cytochrome b_{562}, they showed that even modest changes in residues surrounding the heme significantly alter the reduction potential. Through several generations of mutagenesis, McLendon and coworkers lowered the reduction potential of cytochrome b_{562} by 160 mV [22]. With one exception, all the mutants in their libraries had lower potentials than wild type cytochrome b_{562} (which has a midpoint potential of $+167$ mV). The large drop in reduction potential for variants in these libraries was achieved while keeping the heme ligands (His and Met) and the solution conditions constant. These studies suggest that natural heme proteins evolved to achieve reduction potentials that may be quite different from unevolved 'default' potentials.

What then, is the default midpoint potential for a heme protein that has not been optimized by selection? The midpoint reduction potentials for the binary patterned heme-binding proteins studied herein range from -112 to -176 mV. Since this 64-mV range was found in a very small sampling of our binary code library, the range in the entire library would probably be slightly larger. Indeed, comparison of our results with those of other workers suggests a slightly larger range: as shown in Table 3, the midpoint potential of iron protoporphyrin IX in de novo α-helical proteins ranges from -90 to -220 mV.

5. Conclusions

The binary code proteins were not explicitly designed to bind heme. Nor were they subjected to genetic selections (either in vivo or in vitro) for heme binding. Therefore, these libraries of de novo proteins provide an unusual opportunity for an unbiased assessment of the default reduction potential of heme proteins that have neither been prejudiced by rational design nor selected by evolution. Characterization of these proteins shows that the reduction potentials span a much narrower range of values (-112 to -176 mV) than that observed for natural heme proteins (Fig. 5). We propose that this narrower range be considered as a 'default' range for reduction potentials for α-helical heme proteins. Natural proteins with potentials that deviate significantly from these default values were likely selected by evolution for specific biological properties. Likewise, future designs of de novo heme proteins with desired properties that differ from these default values presumably will require optimization by explicit design and/or evolution in vitro.

Acknowledgments

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