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**ARTICLE** *in* BIOCHEMISTRY · SEPTEMBER 2009

Impact Factor: 3.02 · DOI: 10.1021/bi900518q · Source: PubMed



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DOI: 10.1021/bi900518q

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# Design of a Novel Heme Protein with a Non-Heme Globin Scaffold†

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*Received March 26, 2009; Revised Manuscript Received June 2, 2009*

ABSTRACT: A binding site for iron protoporphyrin IX (heme) was designed and embedded in a photosynthetic non-heme protein, phycocyanin, which forms a globin-like backbone structure, called a globin fold, but lacks sequence similarity to the globin family containing myoglobins and hemoglobins. Based on the structural alignment of the phycocyanin and myoglobin molecules, the proximal and distal His residues were repositioned in the phycocyanin sequence for heme ligation. The heme-binding pocket was created around the His residues by several residue replacements in the phycocyanin core. The synthesized phycocyanin variant, designated as HPY, bound one heme per protein molecule and showed spectroscopic features characteristic of six-coordinated heme proteins. The heme-binding HPY exhibited redox activity with an electrochemical midpoint potential of −130 mV against the standard hydrogen electrode, which was approximately 200 mV lower than the potential of natural myoglobins but 50 mV higher than the typical values of designed heme proteins with four-helix bundle or globin scaffolds. HPY also displayed native-like folding properties, in contrast to these designed heme proteins. However, the bound ferrous heme of HPY was quickly reoxidized by air and did not stably bind $O_2$, unlike the natural globins. The present results demonstrated that the globin fold of a non-globin protein is suitable for binding heme but is not sufficient for the reversible $O_2$ binding and myoglobin functions. The comparison of HPY with the natural globins may yield new insights into the essential features for realizing the natural heme protein functions.



Phycobiliproteins or phycocyanins covalently bind a linear tetrapyrrole chromophore (phycobilin) and function as light-absorbing antennas in cyanobacteria and red algae to funnel excitation energy into the photosynthetic reaction center, located in the outer surface of the thylakoid membranes (*1*). The X-ray crystallographic structures of several phycocyanins revealed close similarity to the tertiary structures of globin-family proteins, despite the lack of significant sequence similarity (see Figure 1A) (*2*−*5*). Globins containing myoglobins (Mbs) and hemoglobins (Hbs) are found in a wide variety of organisms, including bacteria, insects, vertebrates, and plants, and are one of the most widely investigated proteins (*6*, *7*). Globins have a noncovalently bound iron protoporphyrin IX (protoheme or heme *b*) as the prosthetic group, which reversibly binds molecular oxygen ($O_2$). Thus, these proteins preserve a common protein fold, but differ in their prosthetic groups and functions, and exhibit no detectable sequence homology. Furthermore, the two different prosthetic groups, heme and phycobilin, are chemically and metabolically related (Figure 1B,C). These observations have raised profound interests in the origin and evolution of these two protein families.

Many mutagenesis studies on natural heme proteins including globins, cytochromes, and heme-containing enzymes have been performed to understand their structure−function relationships and molecular evolution, from basic perspectives, and to produce novel molecules with useful functions, for industrial purposes. Recently, a *de novo* design approach was also adopted in studies of heme proteins, mainly for the former basic aspects (*8*−*16*). A variety of *de novo* heme-binding proteins with the four-helix bundle structure have been synthesized. *De novo* heme proteins with two to four protohemes bound in a four-helix bundle exhibited redox potentials ($E_m$) ranging from −100 to −200 mV vs the standard hydrogen electrode (SHE) at pH 7−8 (*8*, *9*), and *de novo* heme proteins with a single protoheme exhibited $E_m$ values typically around −200 mV vs SHE. The heme redox potential of artificial four-helix bundles can be modulated by changing pH, heme types, and the protein sequence (*10*−*13*). These $E_m$ values of the bound protoheme are generally lower than those of the natural *b*-type cytochrome, which normally range from +100 to −100 mV vs SHE. Springs et al. constructed libraries of redox variants of cytochrome $b_{562}$, a naturally occurring protoheme-binding four-helix bundle, and demonstrated that the redox potential of the wild type is at the high-potential extremum of the $E_m$ distribution of the variant cytochromes (*17*). We synthesized *de novo* *c*-type cytochromes with the four-helix bundle scaffold and discovered that the redox potentials of the *de novo* cytochromes *c* were much lower than those of the natural *c*-type cytochromes, which generally have higher $E_m$ values than those of natural *b*-type cytochromes. Furthermore, we found that the replacement of protoheme with covalently attached heme *c*, with identical axial ligands, only minimally affected the redox potentials (*13*). These results indicate that the natural heme proteins evolved to differentially stabilize (or destabilize) either the reduced or oxidized forms and that the selective stabilization is required to control the redox properties of *de novo* proteins. Redox control is essential for designing heme-containing enzymes with useful

†This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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 Published on Web 07/14/2009 

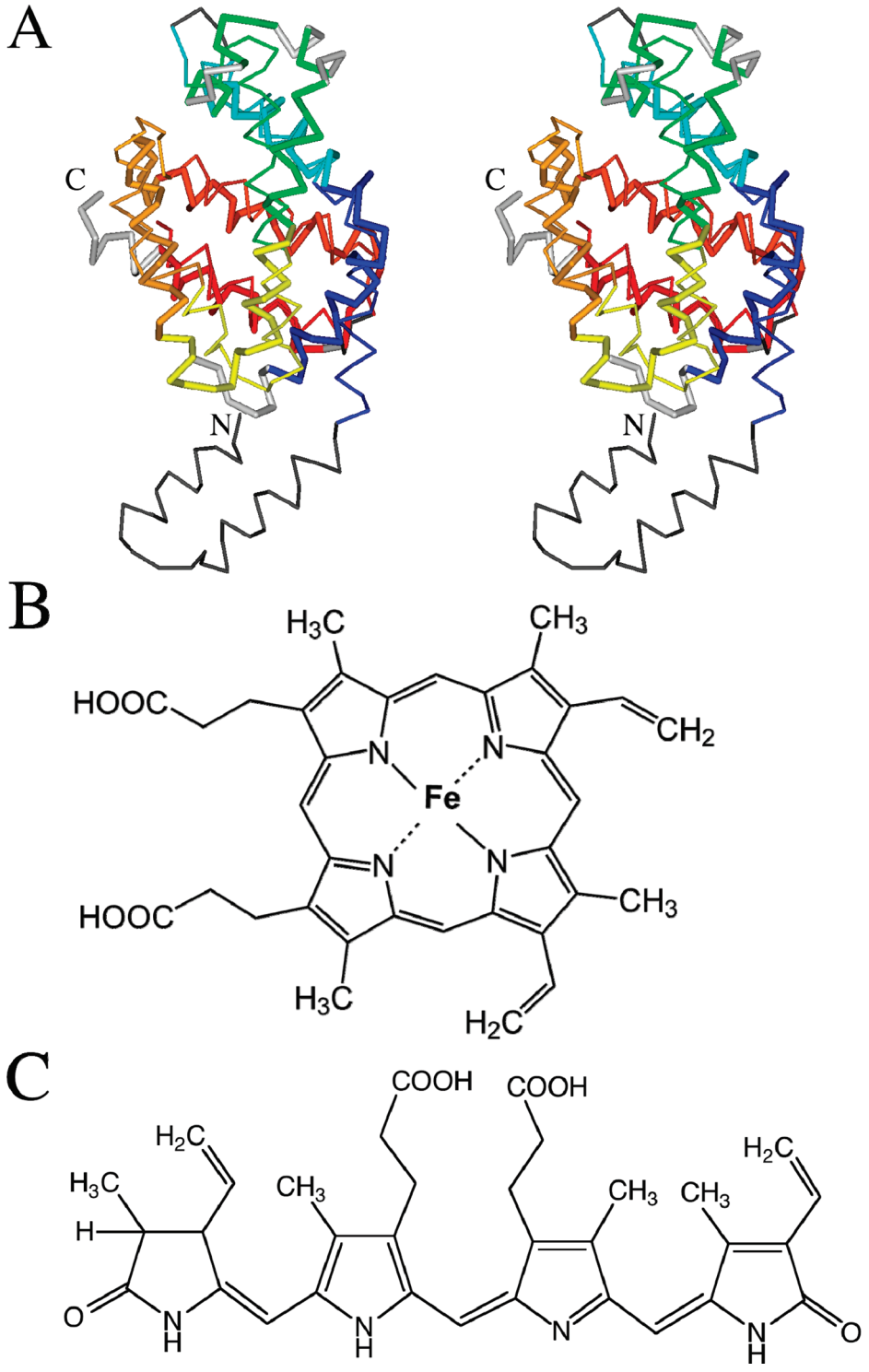


FIGURE 1: Comparison of protein backbone structures and of prosthetic groups between cyanobacterial phycocyanin and aplysia myoglobin. (A) Stereo representation of the structural alignment of phycocyanin with myoglobin by MATRAS (*18*). The phycocyanin and myoglobin $C_\alpha$ traces, represented by thin and bold sticks, respectively, are superimposed with the minimal rmsd of 3.7 Å. The backbone segments that are aligned with each other are indicated by the same colors of blue, cyan, green, yellow, orange, and red from the N to C termini. The regions that could not be aligned are colored gray. (B, C) Chemical structures of the myoglobin and phycocyanin prosthetic groups, iron protoporphyrin IX (protoheme) and phycobilin, respectively.

catalytic functions for industrial applications but remains to be achieved.

We have computationally designed *de novo* heme proteins with a globin fold (*14*, *15*). The synthesized globins bound one protoheme per protein molecule and exhibited redox potentials between −200 and −170 mV vs SHE (see Figure 7), which were also lower than those of the natural globins with $E_m$ values around −70 mV, with no reversible $O_2$ binding ability. Denaturation experiments with the designed globins in the apo forms revealed that the thermodynamic stabilities of these secondary structures were much higher than those of the natural globins, but the folding cooperativity of the designed globins was lower and not native-like. These observations posed an open question: what is the relationship between protein function and folding

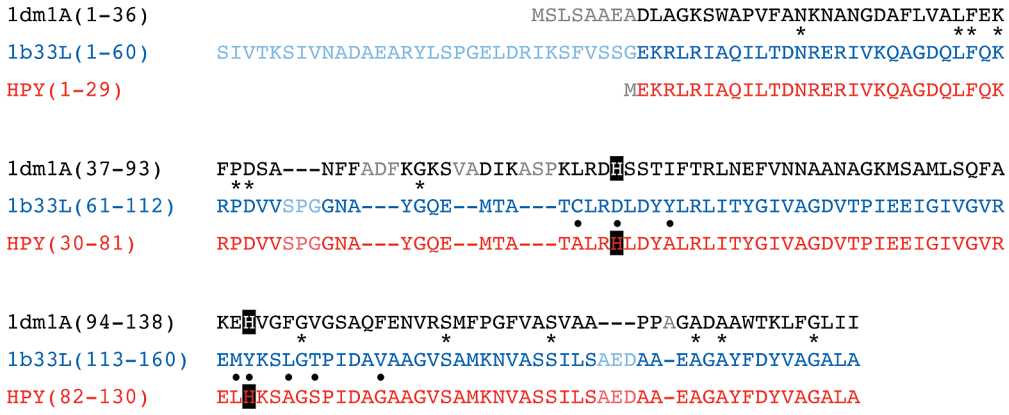


FIGURE 2: Sequence alignment of cyanobacterial phycocyanin, aplysia myoglobin, and HPY. The sequence alignment between myoglobin (1dm1A) and phycocyanin (1b33L) was performed on the basis of the structure alignment shown in Figure 1. The identical amino acid residues between myoglobin and phycocyanin are indicated by asterisks (*). The different residues between phycocyanin and HPY, which were mutated in the phycocyanin sequence to bind heme, are indicated by dots (•). The distal and proximal histidine residues in myoglobin and the corresponding His residues in HPY are highlighted by white letters in black backgrounds.



properties; is it possible to improve the function by realizing the native-like folding? In the present study, we designed and synthesized a heme-binding protein with native-like folding properties by multiple mutagenesis of a natural phycocyanin to address this question and to clarify the evolutional relationship between globins and phycocyanins.

## MATERIALS AND METHODS

*Protein Design*. The amino acid sequence for the heme-binding site in a cyanobacterial phycocyanin was designed on the bases of the structural alignment with natural myoglobins by the protein 3D structure comparison software MATRAS (*18*) and of molecular model building and simulation by the computer graphics software Discovery Studio 2.0 (Accelrys Software Inc.) (see Figure 2 for the sequences). The detailed design procedures are described in the Results and Discussion section.

*Synthesis*. The apo form of the designed heme-binding phycocyanin (HPY) was synthesized from an artificial gene encoding the whole protein sequence with the optimal codons of *Escherichia coli* (*19*). The gene was synthesized by polymerase chain reactions (PCR) with several oligonucleotides containing either the template or complementary sequences of the gene and was cloned into the pRSET-C vector (Invitrogen), as described previously (*20*). The coding sequence of the gene was confirmed by a dye terminator method, using an ABI PRISM 377XL DNA sequencer (Applied Biosystems). The protein was synthesized by expression of the gene in an *E. coli* strain BL21(DE3) under the control of the T7 promoter, which was induced by the addition of isopropyl $\beta$-D-thiogalactopyranoside (IPTG).

*Purification*. The synthesized protein was extracted from the harvested cells by centrifugation after intense sonication with a urea lysis solution containing 6 M urea, 0.5 M NaCl, 1 mM EDTA, and 0.1% octyl $\beta$-D-glucopyranoside at 4 °C. The extract was dialyzed overnight against deionized water containing 0.05% trifluoroacetic acid (TFA) at 4 °C. After the removal of the insoluble materials in the dialyzed extract by centrifugation, the protein was purified by reverse-phase HPLC[1] with a mobile phase containing 0.05% TFA (*20*). The fractions containing the purified protein were collected, evaporated, and dialyzed against 2 mM Tris-HCl (pH 8) and subsequently against deionized water at 4 °C. The resultant protein solution was lyophilized and yielded a white powder. The identity and purity of the protein were verified by analytical reverse-phase HPLC and laser desorption mass spectrometry. The protein concentrations in an aqueous solution were determined spectrophotometrically, using $\varepsilon_{280} = 7000\ M^{-1}\ cm^{-1}$ for apo-HPY.

*Heme-Binding Titration*. The thermodynamic affinity of designed proteins for protoheme was estimated by binding titration experiments at 20 °C. A concentrated hemin solution (1−2 mM) in dimethyl sulfoxide (DMSO) was gradually added to an aqueous solution containing the apoprotein at approximately 10 $\mu$M in TN buffer containing 10 mM Tris-HCl (pH 8.0) and 200 mM NaCl. After each addition of a small amount of the hemin solution, the resulting protein solution was incubated for 5−10 min with stirring. The UV−visible absorption spectrum of the solution was measured in the wavelength region from 200 to 750 nm. The addition of heme and the spectral measurements were repeated 15−20 times. The absorbance at 412 nm, which is the Soret absorption peak of the heme-binding protein, was plotted against the added amounts of hemin. The titration profiles were computationally analyzed to determine the dissociation constants ($K_d$) according to an equation for ligand binding to macromolecules (*21*). For other experiments with heme-binding HPY (holo-HPY), the heme was associated with the apo-HPY by titration of the protein solubilized in TN buffer at 0.1−0.2 mM with concentrated hemin (10−15 mM) dissolved in DMSO. The solvent was replaced with a buffer solution by dilution and concentration with a Centriprep YM-10 (Amicon).

*Redox Titration*. To estimate the redox midpoint potentials, potentiometric titration was performed under anaerobic conditions with a continuous flow of gaseous nitrogen in TN buffer at 20 °C (*13*, *22*). The ambient redox potential was adjusted by the addition of sodium dithionite and potassium ferricyanide in the reductive and oxidative directions, respectively. The redox potential was measured with a combination redox electrode MI-800-410 (Microelectrodes, Inc.), and the optical absorption spectra were recorded throughout the titration on a Hitachi U-3000 spectrometer. The potentials measured against the Ag/AgCl reference electrode are reported against the standard hydrogen electrode (SHE) in the text. Equilibration between the electrode and the reaction mixture was facilitated with the

[1]Abbreviations: CD, circular dichroism; Gd-HCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; rmsd, root-mean-square deviation; UV, ultraviolet.

following redox mediators at 10−30 μM: 2,3,5,6-tetramethyl-*p*-phenylenediamine, 1,4-naphthoquinone, 1,2-naphthoquinone, phenazine ethosulfate, phenazine methosulfate, duroquinone, and 2-hydroxy-1,4-naphthoquinone. Heme reduction was monitored by the increase in the α absorption bands. The titration data were analyzed by fitting with the Nernst equation (*21*).

*Spectrometry*. UV−visible absorption spectra were recorded using a Hitachi U-3000 spectrometer and quartz cuvettes with a 1.0 cm path length. Circular dichroism (CD) spectra were recorded at 20 °C using a JASCO J700 spectropolarimeter equipped with a JASCO electronic thermal controller PTC and a rectangular quartz cuvette with a 0.2 cm path length. Thermal denaturation of proteins was measured by monitoring the CD signal intensity at 222 nm with the same CD instrument. MALDI-TOF mass analyses were conducted with either a Reflex mass spectrometer (Bruker Daltonics) using sinapinic acid as the matrix in the linear positive mode or an Ultraflex mass spectrometer (Bruker Daltonics) in the positive MS/MS mode (*23*).

## RESULTS AND DISCUSSION

In total, 98 structures of 21 different phycocyanin molecules and 209 structures of 10 different myoglobin molecules had been deposited in the Protein Data Bank (PDB) by the beginning of 2005, when we started this study. The pair with the most similar structures between phycocyanin and myoglobin was identified by a comparison of the scores from the structural alignment by MATRAS (*18*). A cyanobacterium, *Mastigocladus laminosus*, phycocyanin (PDB ID 1b33L) and a sea hare, *Aplysia limacine*, myoglobin (1dm1A) were yielded the structurally closest pair, with a MATRAS score of 557. The structure and sequence alignments between the phycocyanin and myoglobin molecules are shown in Figures 1 and 2, respectively. The pairwise 3D alignment (Figure 1A) shows that the main-chain structures coincided well with each other, except for the N-terminal regions from residue 1 to residue 32 for phycocyanin and residue 1 to residue 7 for myoglobin. The aligned $C_\alpha$ positions gave an rmsd of 3.7 Å. The sequence alignment based on the 3D alignment (Figure 2), however, revealed that the sequence identity was only 8.1% for the phycocyanin sequences against the myoglobin sequence, and thus no substantial similarity between the two sequences was detected. Based on the structure and sequence alignments, residue replacements were performd on the cyanobacterial phycocyanin to transform the phycobilin-binding protein to a novel heme-binding protein. Both residues Asp-83 and Tyr-115 of phycocyanin, which correspond to His-64 and His-93 of myoglobin, respectively (Figure 2), which are the distal and proximal His residues that are crucial for the heme-related functions of myoglobin, were replaced with histidine in expectation that the mutant protein would acquire the myoglobin function. Furthermore, 3D models of phycocyanin mutants were computationally constructed to bind heme in the protein core, in a similar manner as myoglobin binds the cofactor in the heme-binding site (Figure 3). Many atomic bumps between the inserted heme and the protein core residues were observed in the 3D models of phycocyanin. Thus, the phycocyanin sequence was additionally changed to remove the bumps by replacing some of the bumping residues with smaller ones, as shown in Figure 2. Moreover, the phycocyanin N-terminal chain that could not be aligned with the myoglobin backbone (see Figures 1 and 2) was deleted, because the N-terminus forms the site for intermolecular contacts between natural phycocyanin molecules, and it did not

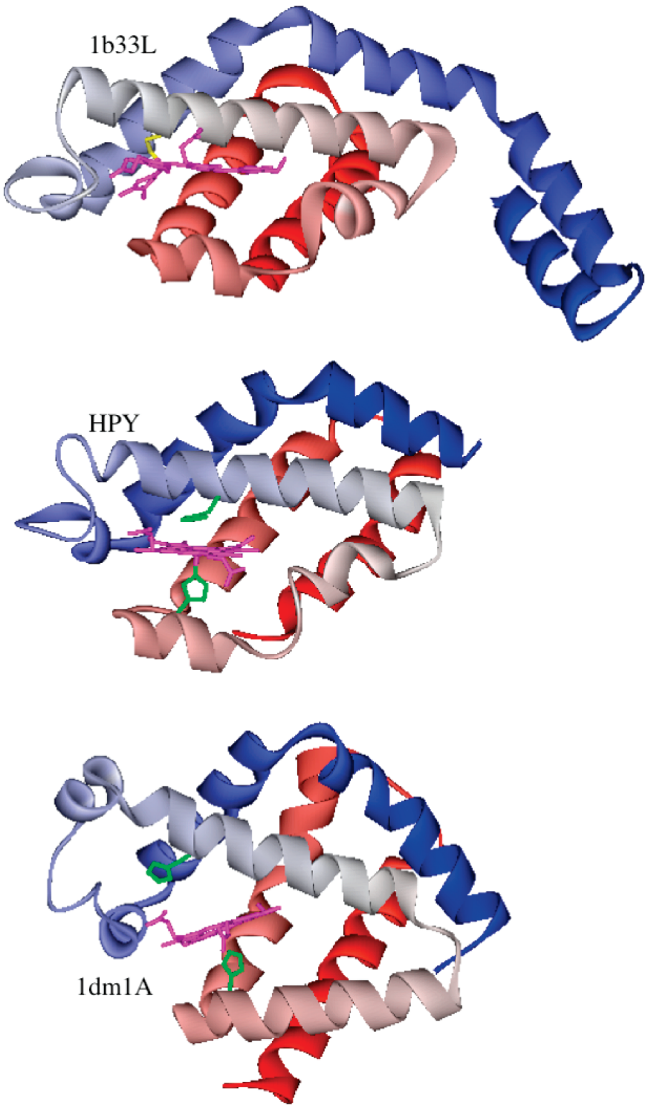


FIGURE 3: Computational model of the designed heme-binding phycocyanin, HPY (middle), compared with the X-ray crystallographic structures of cyanobacterial phycocyanin (1b33L) (top) and aplysia myoglobin (1dm1A) (bottom). The polypeptide main chains are depicted with solid ribbons from blue N termini to red C termini in the color gradient. The prosthetic groups, i.e., heme in myoglobin and HPY and phycobilin in phycocyanin, are represented with magenta sticks. The distal and proximal histidine residues in myoglobin and the corresponding His residues in HPY are colored green. The yellow sticks indicate the Cys-81 in phycocyanin, which is covalently attached to the phycobilin molecule. The HPY model was constructed using the molecular graphics software, Discovery Studio, equipped with the Protein Modeling and Simulation modules, based on the phycocyanin structure.

seem to be needed for the functional transformation of phycocyanin to myoglobin in the present study. The finally obtained model of the phycocyanin variant appears to have a well-formed pocket for the ligation and accommodation of heme (Figure 3).

The phycocyanin variant designed for binding heme was successfully synthesized by expressing the synthetic gene coding the protein sequence with the *E. coli* optimum codons (*19*) in *E. coli* (see Materials and Methods). The variant protein in the apo form was purified from the cell extracts and was solubilized in an aqueous solution. The circular dichroism (CD) spectrum of the solubilized HPY is displayed in Figure 4 and indicates that the protein main chain is well folded to form an α-helix-rich conformation, as in the original phycocyanin molecule and also

in the apo form of myoglobin. The mean residue ellipticity of HPY at 222 nm was 19500 deg cm$^2$ dmol$^{-1}$ and gave an α-helix content of 60.8%, and these values are similar to those of the natural apomyoglobins and our computationally designed globins (*14*, *15*). No significant changes in the CD spectrum were observed upon heme binding (see below).

The synthesized HPY bound one heme per protein molecule, without significant changes in the secondary structure (Figure 4). The $K_d$ value of HPY for heme was estimated at approximately 0.3 μM, based on binding titration experiments under equilibrium conditions (see Materials and Methods). The UV−visible absorption spectra of holo-HPY are shown in Figure 5. The spectrum of the oxidized form exhibited absorption peaks at 408 and 532 nm and clearly differed from that of the free ferric heme (Figure 5A). The reduced form exhibited absorption peaks at 424.5, 529, and 558 nm (Figure 5B). Upon the addition of gaseous carbon monoxide (CO), the ferrous heme HPY quickly formed a stable CO complex (Figure 5B), exhibiting absorption peaks at 420, 539, and 567 nm. The absorption peaks of the ferrous form are characteristic of those of natural *b*-type cytochromes with a low-spin bis-His-coordinated protoheme. This is in contrast to the natural globins, which have a high-spin five-coordinated heme iron under the experimental conditions. On the other hand, the peaks of the ferric form are 4−5 nm shorter than the typical values for the six-coordinated protoheme iron and are indicative of a high-spin five-coordinated protoheme iron. The formation of the CO complex of ferrous heme−HPY and its absorption spectra also agree with the reaction of natural globins with CO. These spectral data of HPY, as well as those of the natural and designed globins, are summarized in Table 1.

The ferrous heme HPY, however, easily reoxidized upon exposure to the air, and the $O_2$-bound form was not detected under the experimental conditions. Furthermore, recursive reduction by the addition of sodium dithionite ($Na_2S_2O_4$) and oxidation by air irreversibly damaged the heme, as judged from the absorption spectra. These properties of the heme HPY are similar to those of the designed globins (*14*, *15*) but differ from the properties of natural globins, which form stable complexes with $O_2$. These results indicate that the natural globins are specifically designed to be protected against autoxidation and to form a stable complex with $O_2$, whereas the formation of the CO complex is due to the chemical properties of the prosthetic group, rather than to the natural design of globins.

Thermal denaturation of the apo and holo forms of HPY was observed by increasing the temperature from 5 to 95 °C while monitoring the CD signal at 222 nm (Figure 6). The apoprotein clearly exhibited a two-phase denaturation profile with a folding intermediate. The first transition, from the folded state to the intermediate state, had a midpoint temperature ($T_{1/2}$) at approximately 30 °C. The second transition, from the intermediate to the fully unfolded state, had a $T_{1/2}$ at 55 °C. The holoprotein denatured via a two-state transition with a $T_{1/2}$ at 60 °C, without

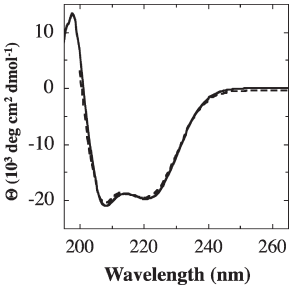


FIGURE 4: CD spectra of designed phycocyanins. The spectra of the apo and holo forms are shown by the solid and dotted lines, respectively. The spectra were recorded in 10 mM sodium phosphate (pH 7.0) and 200 mM NaCl at 20 °C. The protein concentrations were 10 μM, as determined spectrophotometrically.

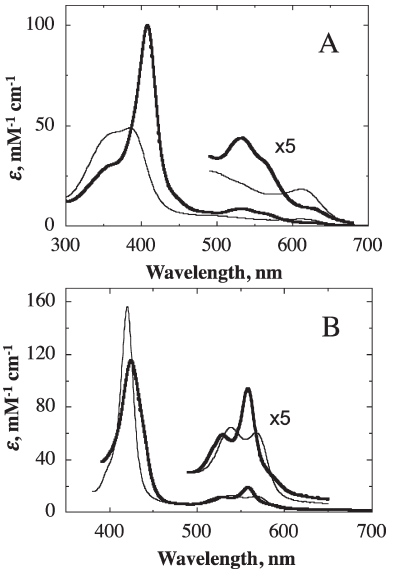


FIGURE 5: UV−visible absorption spectra of holo-HPY. (A) The spectra of holo-HPY as compared with free hemin in the ferric forms. (B) The spectra of the heme protein in the ferrous and ferrous CO-bound forms are displayed by the bold and thin lines, respectively. These spectra were recorded in 10 mM Tris-HCl (pH 8.0) and 200 mM NaCl, with heme concentrations of 5 μM, at 20 °C.

Table 1: Spectral and Electrochemical Properties of Natural and Designed Heme Proteins with the Globin Fold

| | absorption maxima[b] | | | |
|---|---|---|---|---|
| protein | ferric (nm) | ferrous, deoxy (nm) | ferrous, CO (nm) | $E_m$[c] (mV vs SHE) |
| horse myoglobin | 409, 503, 630 | 434, 556.5 | 423, 540.5, 578 | +70 |
| designed globins[a] | | | | |
| DG1 | 413, 535 | 425, 529, 558 | 421, 539, 568 | −171 |
| DG2 | | | | −189 |
| DG3 | 413.5, 534 | 426, 530, 559.5 | 420.5, 540, 568 | −196 |
| DG4 | | | | −186 |
| HPY | 408, 532 | 424.5, 529, 558 | 420, 539, 567 | −120 |

[a] Design procedures and detailed biochemical properties were described in refs *14* and *15*. The spectral data of DG1 were cited from ref *14*, whereas the other data of designed globins were obtained in the present study. [b] The absorption spectra and redox potentials were measured in a buffer solution containing 10 mM Tris-HCl (pH 8.0) and 200 mM NaCl at 20 °C. [c] $E_m$ values were determined within a standard deviation of ±10 mV by curve fitting with the single Nernst equation with $n = 1$ for the redox titration data, as shown in Figure 7.

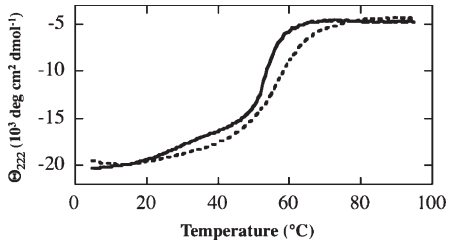


FIGURE 6: Thermal denaturation of apo- and holo-HPY. The CD signals at 222 nm are plotted against the temperature for the apo (solid line) and holo (dashed line) forms of HPY. The experimental conditions, except for the temperature, were the same as those in Figure 4.

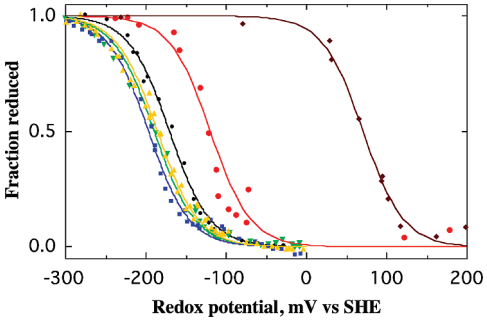


FIGURE 7: Redox titrations of holo-HPY and designed and natural globins. The fractions of the reduced forms of HPY (red circles), the designed globins DG1−4 (black circles, green triangles, blue squares, and yellow triangles), and horse myoglobins (brown diamonds) are plotted against the solution redox potentials under equilibrium conditions. The data were obtained from the spectral changes of the heme proteins in the α- and β-band regions with the redox potential, monitored by a combination redox electrode at 20 °C. The theoretical curves were drawn by fitting to the data using the Nernst equation ($n = 1$).

an apparent folding intermediate. These protein-folding results, i.e., destabilization of the folding intermediate and reinforcement of the stability of the whole protein by heme binding, coincide with the differences in the properties between the native apo- and holo globins (*24*, *25*) and suggest that HPY maintained a well-folded, native-like heme protein structure.

To investigate the functional differences between myoglobin and HPY, we performed potentiometric (equilibrium) redox titration experiments. Figure 7 shows a titration profile of the holo-HPY, along with the results of natural and computationally designed globins (*14*, *15*). All of these heme proteins with the globin fold underwent one-electron reduction/oxidation processes with the redox midpoint potentials ($E_m$) listed in Table 1. The heme bound to HPY underwent one-electron reduction/oxidation with $E_m = -120 \pm 10$ mV. As shown in Figure 7 and Table 1, this $E_m$ value of HPY is 190 mV lower than the value of natural myoglobin but 50−80 mV higher than the values of the designed globins. The higher $E_m$ value of HPY, as compared with those of the designed globins, seems to be due to the more hydrophobic environment inside the heme-binding pocket of HPY, since the designed globins have a rather fluctuated, molten globule-like protein structure (*14*, *15*) and water molecules may easily access the protein core. A water molecule will preferentially stabilize the oxidized state ($Fe^{3+}$) of the heme, since it has a net positive charge (+1) whereas the reduced state ($Fe^{2+}$) has no net charge, and the electrostatic interaction between water and heme would thus lower the redox potential. The putatively more hydrophilic environments in the heme-binding sites of the designed globins are also expected from the fact that the $E_m$ value of the designed globins is close to those of the designed heme-binding four-helix bundle proteins (*8*−*13*), in which the bound heme is apparently exposed to the aqueous environment based on the 3D models. On the other hand, natural globins are designed to stabilize the ferrous state relative to the ferric state to give positive values of $E_m$, possibly for protection against autoxidation.

Neuroglobin and cytoglobin, recently discovered members of the vertebrate globin family, display ferric and ferrous heme visible spectra indicative of six coordination, which is quite distinct from the five-coordinate myoglobin and hemoglobin. However, they form an oxy-ferrous state, though unstable in comparison with myoglobin and hemoglobin, by replacing the ligated distal histidine with $O_2$ (*26*, *27*). The similar $O_2$-binding function was observed in a designed four-helix bundle heme protein (*28*). According to these results on the natural and artificial six-coordinate heme proteins, the $O_2$ binding abilities should be acquired through the loose ligation of the distal histidine to promote the accessibility of $O_2$ to the heme iron and exclusion of water from the protein core around heme to prevent the heme oxidation. In the present study, the heme-binding site was designed simply by positioning the two histidine residues and making the space in the protein core. Thus, there may be room for redesign of the well-packed core in the presence of heme, by which water molecules are excluded to prevent the heme oxidation, for yielding an $O_2$ carrier protein.

In contrast with natural globins, natural phycocyanins have evolved to be functionalized for efficient transfer of absorbed light energy to a photosynthetic reaction center. Thus, they are not designed to preferentially stabilize any specific redox state of the bound heme. This seems to explain the functional difference between HPY and natural globins. The present study indicated that, in the ferrous state, the heme-bound proteins that are not specifically designed to be protected from autoxidation are easily oxidized and irreversibly damaged by $O_2$, even though the proteins preserve the globin-like 3D structure and native-like folding properties. The amino acid sequences of natural globins and other heme proteins should be highly selected to adapt to the current aerobic environments on earth and to prevent autoxidation. It remains unclear how the natural redox proteins have evolved to obtain the robustness against $O_2$, and this should be an important subject for protein engineering and antioxidation biology.

## ACKNOWLEDGMENT

We thank Ms. Yukiko Takai (Tokyo Institute of Technology) and Prof. Motonori Ota (Nagoya University) for helpful suggestions for the structural alignment between myoglobins and phycocyanin.

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