Design, Synthesis, and Properties of a Novel Cytochrome *b* Model

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Abstract: The modular strategy of a template-assembled synthetic protein (TASP) was used for the de novo synthesis of a 122-residue, antiparallel four-helix bundle protein which accommodates two bis-histidine ligated heme groups. The cyclic decapeptide template contains four cysteine residues with different protecting groups which allow coupling of the unprotected helices carrying bromoacetyl units either at the N-terminus or the ϵ-amino group of a C-terminal lysine residue. The amphiphilic helices realize a water-soluble model of the cytochrome *b* core with two parallel heme-binding helices alternating with two antiparallel helices shielding the two hydrophobic heme binding sites. The spectral properties resemble those of the natural protein. Characterization by mass spectrometry and circular dichroism support the anticipated structure. The free energy of folding shows a stabilizing effect by the two heme groups which have respective redox midpoint potentials of −106 and −170 mV. This modular protein combines the advantage of the structural organization of a TASP with the incorporation of functional groups.

Introduction

The complex assembly of most enzymes from subunits and structural domains serves for recognition, regulation, and other functions in addition to catalysis. The design of a protein harboring only the catalytic center or the cofactor binding site is a promising approach toward the use of essential or new protein functions. A de novo design of such proteins can be based on the domain of a known protein by adaptation of structural elements accessible to today's techniques. The principles stabilizing α-helices[1] and four-helix bundles[2] have been extensively investigated. The assembly of a water-soluble four-helix bundle is driven to a great extent by the formation of a hydrophobic core. But the association of helical or helix−turn−helix peptides includes several possibilities of the interfacial packing[3] which are difficult to predict. However, the covalent binding of secondary structure forming peptides to predetermined positions of a cyclic template,[4,5] termed template-assembled synthetic protein (TASP),[6] offers the possibility of overcoming this protein folding problem. In addition, the deficit in designing an optimal van der Waals packing of a cofactor like the tetrapyrrole group may be compensated by the increased stability and invariant orientation of the branched template with amphiphilic helices. Therefore, we have chosen this approach to realize an antiparallel four-helix bundle binding two heme groups as in cytochrome *b*, which constitutes the catalytic hearts of the cytochrome bc_1 and the cytochrome *bf* complexes.[7] These complexes function in the respiratory and the photosynthetic electron transport, respectively. In a previous design of a tetraheme synthetic protein by Robertson et al.,[8] a single helix has been dimerized by a disulfide bridge between N-terminal cysteins which assembled to a bundle of four identical helices oriented in parallel. Cytochrome *b* binds only two heme groups between two parallel helices. The previous predictions of the folding[7] are consistent with the recently published structure of the mitochondrial cytochrome bc_1 complex[9] which shows details of how the heme groups are shielded by two helices in an antiparallel orientation. Glycine residues are conserved in these protecting helices at the height of the heme-ligating histidine residues, presumably to provide the space for a tight packing of the heme edges. The compact binding pocket of the hemes buried in the interior of the four-helix bundle made it an attractive feature to conserve the hydrophobic interior and to design a hydrophilic outside. This construct reduces the exposure of the heme edges to the solvent and should improve the possibility of tuning the redox potential.

Experimental Section

Materials. Fmoc-protected amino acids, TBTU, DIEA, piperidine, and PAL-PEG-PS resin, were purchased from Perseptive Biosystems. Preloaded Fmoc-Gly-NovaSyn TGT resin was obtained from Nova Biochem. HPLC grade acetonitrile was purchased from SDS. All other

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(6) Abbrevations: Acm, acetamidomethyl; Aloc, allyloxycarbonyl; Bu_3-SnH, tributyltin hydride; CD, circular dichroism; DCM, dichloromethane; DIEA, *N*,*N*′-diisopropylethylamine; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; ESI, electrospray ionization; Fmoc, 9-fluorenylmethoxycarbonyl; FPLC, fast-performance liquid chromatography; GuHCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; PAL, 5-(4-(aminomethyl)-3,5-bis(methyloxy)phenoxy)valeric acid; $PdCl_2(PPh_3)_2$, dichlorobis(triphenylphosphine)palladium(II); SPPS, solid-phase peptide synthesis; TASP, template assembled synthetic protein; TBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; Trt, trityl; UV, ultraviolet.
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chemicals were obtained from Aldrich/Sigma or Merck and were of highest available grade.

Synthesis of the Helical Peptides 3 and 7. The synthesis of the helical peptides Br-H1 (**3**) and Br-H2 (**7**) (see Results) began by solid-phase peptide synthesis using Fmoc-protected amino acids on PAL-PEG-PS resin in a Milligen Model 9050 peptide synthesizer. A 4-fold excess of Fmoc L-amino acid was activated for 7 min with TBTU/DIEA and recirculated through the resin for 30 min. The Fmoc group was removed by washing the resin with 20% (v/v) piperidine in DMF for 7 min. After removal of the last Fmoc protecting group, **1** was bromoacetylated by adding an 8-fold excess of bromoacetic anhydride in DMF (c = 0.25 M) over the molar loading of the resin and stirring the suspension at room temperature for 1 h. The other peptide was acetylated by stirring the resin in a solution of 0.3 M 1-acetylimidazole in DMF for 30 min to yield **4**. The completeness of the reactions was determined by the ninhydrin test.[10]

The N^{ϵ}-Aloc group of Lys27 of **4** was removed through Pd-catalyzed hydrostannolysis with tributyltin hydride.[11] The resin (750 mg, 0.06 mmol of peptide) was suspended in a solution of 1.68 mg (2.4 μmol = 0.04 equiv) of $PdCl_2(PPh_3)_2$ and 12 μL (0.21 mmol = 3.5 equiv) of acetic acid in 7.5 mL of DCM. The suspension was bubbled for 5 min with argon, 130 μL (0.48 mmol = 8 equiv) of Bu_3SnH was added, and the reaction mixture was stirred for 15 min at room temperature. To make sure that the removal of the Aloc group was complete, the procedure was repeated. The resin was then washed with DCM (3×), 0.5% (v/v) DIEA in DMF (1×), 0.5% (w/v) sodium diethyl dithiocarbamate in DMF (1×), and DMF (4×). The N^{ϵ}-amino group of Lys27 in **5** was bromoacetylated as described above for the N-terminus of peptide **1**. After cleavage from the resin with 23/1/1 (v/v/w) TFA/anisole/DTT and precipitation with diethyl ether, peptides **3** and **7** were purified by preparative HPLC using a gradient of 30−55% and 5−50% acetonitrile over 30 min, respectively, and then lyophilized.

Synthesis of the Cyclic Template 11. The linear decapeptide **8** was synthesized manually using the same chemistry as described above but using N^{α}-Fmoc-Gly-NovaSyn-TGT resin (1 g, 0.22 mmol/g). To prevent racemization[12] a 6-fold excess of Fmoc-Cys(Trt)-OH and Fmoc-Cys(Acm)-OH was preformed to a neutral symmetrical anhydride as described by Atherton et al.[13] except for the use of diisopropylcarbodiimide. After deprotection of the last Fmoc group to yield **8**, the side chain protected peptide **9** was cleaved from the resin with 20 mL of 5/1/4 (v/v/v) AcOH/methanol/DCM for 2 h at room temperature. The resin was washed with 3 × 7 mL of the cleavage mixture, and to the combined eluents was added 620 mL of *n*-hexane. The solvent was removed under reduced pressure, and the precipitate was purified by reversed phase HPLC using a gradient of 40−80% acetonitrile over 25 min and then lyophilized. **9** (100 mg, 0.066 mmol) was dissolved in a solution of 46 μL (34 mg, 0.26 mmol) of DIEA in 120 mL of DMF, and 42 mg (0.13 mmol) of TBTU was added. The solution was stirred for 8 h at room temperature, and the completion of the cyclization to **10** was determined by analytical reversed phase HPLC (Poros R1 column, 5−60% acetonitrile in 7 min) and mass spectrometry. The solvent was removed under reduced pressure, and to remove the trityl protecting groups, **10** was dissolved in 5 mL of 19/1 TFA/DTT and stirred at room temperature for 30 min. **11** (56 mg, 84% yield) was obtained by precipitation with cold diethyl ether. The cyclic template **11** was purified by preparative HPLC using a gradient of 5−30% acetonitrile over 25 min.

Synthesis of 13, $T(H1)_2$. The coupling of the unprotected bromoacetylated helix Br-H1 (**3**) with the cyclic template **11** was carried out by combining 70 mg (i.e. 15.2 μmol with nine TFA counteranions per peptide molecule) of **3** with 7 mg (7 μmol) of **11** in 1 mL of deoxygenated (by bubbling with Ar) 3/2 (v/v) 0.15 M sodium phosphate buffer (pH 7.5)/acetonitrile. The solution was stirred under Ar at room temperature, and the progress of coupling to form **12** was monitored by analytical HPLC. After the termination of the ligation reaction, after 3 h, approximately 10 μL of mercaptoethanol was added to deactivate the surplus bromoacetyl groups. The product was desalted two times on a Pharmacia HiTrap desalting column eluted with 10% acetic acid and finally lyophilized.

12 (50 mg, i.e. 5 μmol with 18 TFA counteranions) was dissolved in 4 mL of 20% acetic acid, and the pH of the solution was adjusted with aqueous ammonia to pH 4. Mercury(II) acetate (40 mg, 126 μmol) was added, and the pH was readjusted with aquous ammonia to pH 4. At room temperature the solution was stirred under Ar for 1 h, then 500 mg (3.2 mmol) of DTT was added and the resulting suspension stirred for 4 h. The precipitate was removed by centrifugation, and the solution was lyophilized. The product **13** was purified by preparative HPLC using a gradient of 25−45% acetonitrile over 30 min and then lyophilized.

Synthesis of 14, MOP1. **7** (27.3 mg, i.e. 6.67 μmol with seven TFA counteranions) was combined with 30 mg (3.03 μmol with 18 TFA conteranions) of **13** in 500 μL of 3/2 (v/v) deoxygenated 0.15 M sodium phosphate buffer (pH 7.5)/acetonitrile and stirred under Ar at room temperature. The coupling reaction was monitored by analytical HPLC and observed to be complete after 4 h. The product was purified by preparative HPLC using a gradient of 25−55% acetonitrile over 30 min and then lyophilized. For simplicity, the *mo*dular *p*rotein $T(H1)_2$-$(H2)_2$ is termed in the following MOP1.

Reversed Phase HPLC. Analytical HPLC was carried out on a Waters model 600E system equipped with a Waters model 996 photodiode array detector. A 4.8 × 100 mm stainless steel column packed with PerSeptive Poros R1 material was run at a flow rate of 5 mL/min. Preparative HPLC was performed on a Waters model 4000 system equipped with a Waters model 486 tunable absorbance detector and a Waters DeltaPak C18 PrepPak column (40 × 300 mm, 15 μm) at a flow rate of 50 mL/min. The runs used different gradients of 0.1% TFA (buffer A) versus 4/1 acetonitrile/water plus 0.1% TFA (buffer B). Crude peptides containing unprotected cysteine residues were dissolved in 50 mM DTT in water plus acetonitrile. The other peptides were dissolved in mixtures of buffers A and B. All purified peptides were analyzed by ESI mass spectrometry.

Mass Spectrometry. ESI mass spectrometry of crude and purified peptides was performed on a Finnigan model TSQ 700 tandem quadrupole mass spectrometer with an electrospray interface. Apomyoglobin of horse skeletal muscle with an average mass of 16 950.5 Da was used for calibration. The peptides were dissolved in 1/1 (v/v) water/methanol containing 1% HAc and directly injected into the electrospray interface. The mass of the peptides was calculated with the program pepmatch (Finnigan). The mass spectra were analyzed by the programs Biomass Calculation and Biomass Deconvolution (Finnigan).

Size-Exclusion Chromatography. Size-exclusion chromatography was performed on a Hewlett-Packard Model HP 1050 Ti HPLC system using a Pharmacia Superdex 75 column (10 × 300 mm) equilibrated with 50 mM potassium phosphate buffer (pH 7) and 100 mM NaCl. To estimate the molecular mass of MOP1 and its bis-heme complex in solution, the column was calibrated with bovine serum albumin (67 kDa), ovalbumin (43 kDa), α-chymotrypsinogen (25 kDa), myoglobin (17.6 kDa), RNase A (13.7 kDa), aprotinin (6.5 kDa), and vitamin B_{12} (1.36 kDa). Peptide and protein samples were eluted at a flow rate of 0.5 mL/min, and the absorbance was monitored at 215 nm. To examine a possible concentration-dependent aggregation, samples of 200 μL of MOP1 and its heme complex in a concentration range 20−150 μM were chromatographed.

Incorporation of Heme. The incorporation of Fe(III)−protoporphyrin IX (heme) was performed as described by Choma et al.[14] except that a 2-fold excess of heme over the heme-binding sites was added in DMSO at once to give a maximal final concentration of 0.1 mM in 50 mM Tris/HCl (pH 8). The solution was stirred at room temperature for 30 min before most of the unbound heme was removed by passing 1.5 mL aliquotes through a Pharmacia HiTrap desalting column

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equilibrated with 50 mM potassium phosphate (pH 7). The eluents containing the heme peptide were collected, desalted by a passage through a Pharmacia PD10 column, and lyophilized. Further purification of the heme peptide was accomplished by size-exclusion chromatography using a Pharmacia Superdex 75 FPLC column (10 × 300 mm) equilibrated with 50 mM potassium phosphate (pH 7) and 100 mM NaCl at a flow rate of 0.5 mL/min.

Extinction Coefficients. The extinction coefficient of the MOP1 bis-heme complex at pH 7 and of free heme at pH 7.5 were determined by comparing the spectra in 50 mM potassium phosphate buffer and 100 mM NaCl with that of the pyridine−hemochrome[15] using the dual-wavelength difference extinction coefficient of reduced *minus* oxidized bis(pyridyl)−Fe(II,III)−protoporphyrin IX at 557−540 nm of $\epsilon_{557,r-540,o} = 23.98\ mM^{-1}\ cm^{-1}$. Peptide concentrations were estimated from the absorbance of tryptophane at 280 nm using an extinction coefficient of 5700 $cm^{-1}\ M^{-1}$.

Redox Potentiometry. The midpoint potential of the heme groups in the bis-heme complex of MOP1 at 8 μM in 50 mM potassium phosphate buffer (pH 7) and 100 mM NaCl at 20 °C was determined by redox potentiometry essentially as described by Choma et al.[14] The redox mediators[16] used were 10 μM pyocyanine; 15 μM each of 2-hydroxy-1,4-naphthoquinone, anthraquinone-2-sulfonate, and anthraquinone-2,6-disulfonate; 20 μM 2,5-dihydroxy-*p*-benzoquinone; 25 μM 1,4-naphthoquinone; and 40 μM duroquinone. The potential between 50 and −300 mV was measured with a platinum electrode and an Ag/AgCl reference electrode.

Stability Studies. The stability of the modular protein MOP1 and its bis-heme complex was studied by monitoring the molar ellipticity at 222 nm, the wavelength of maximal fluorescence emission of tryptophane, and the absorbance at the Soret band of the complex at 412 nm as a function of the guanidine hydrochloride (GuHCl) concentration. Aliquots of the stock solution of the bis-heme complex in 50 mM potassium phosphate buffer (pH 7) and 100 mM NaCl were mixed with appropriate volumes of the buffer and GuHCl stock solution (6 M in buffer) to give a final peptide concentration of 1.7 μM. The concentration of the GuHCl stock solution was determined by measuring the refractive index.[17] The resulting solutions were incubated for 30 min at 20 °C prior to the measurements of the spectra in the range 250−700 nm. The data were fitted to the equation $\Delta G_{obs} = \Delta G_{H_2O} - m[\mathrm{GuHCl}]$, where ΔG_{obs} is $-RT \ln[f/(1-f)]$, ΔG_{H_2O} is the free energy of folding in the absence of GuHCl and m depends on the cooperativity of the transition.[18] f is the fraction of folded peptide and can be determined from $f = (\Phi_{obs} - \Phi_u)/(\Phi_f - \Phi_u)$. Φ_{obs} is the observed signal of the method applied, and Φ_f and Φ_u are the signals in the folded and unfolded state, respectively. For UV−vis spectroscopy Φ represents the heme absorption difference $A_{412} - A_{700}$.

Fluorescence emission spectra of the two tryptophanes in MOP1 were measured with a Spex Fluorolog II fluorometer. Samples of approximately 1.5 μM MOP1 were prepared as described above and excited at 280 nm, and the emission spectra were measured at a scan rate of 1 nm/s between 295 and 400 nm. The fraction of folded peptide f was plotted against the GuHCl concentration, where Φ represents the wavelength of maximum fluorescence emission.

Circular Dichroism Spectroscopy. CD spectra were recorded on a Mark V dichrograph from ISA Jobin Yvon. The quartz cuvette with an optical path length of 1 mm was thermostated at 20 °C and contained 20 mM potassium phosphate buffer (Merck Suprapur), pH 7, and the peptide or the peptide heme complex at approximately 12 μM. From each sample three spectra were recorded in the range of 185−250 nm and averaged. For studies with GuHCl the spectra were recorded between 200 and 250 nm in 50 mM buffer *plus* 100 mM NaCl. The mean residue molar ellipticity was calculated by dividing the measured ellipticity by the number of 122 residues. In addition, the absorbance spectrum of the complex between 360 and 450 nm was recorded. The fraction of folded peptide f (Φ represents the mean residue molar ellipticity at 222 nm) was plotted as a function of the GuHCl concentration. The denaturation curves were fitted as described above.

Results

Protein Design. Our goal is to synthesize a water-soluble protein with a cofactor harbored by a four-helix bundle in a controlled binding pocket. A promising approach to such a de novo protein is a combination of the TASP concept of Mutter with that of salt-bridge-stabilized amphiphilic helices.[1] A binding pocket of a redox protein can be designed by following the structural proposal of cytochrome *b*, which is a membrane-spanning subunit of the mitochondrial cytochrome bc_1 and the photosynthetic cytochrome *bf* complexes.[7,19,20] The structure indicates indeed a bis-heme binding core formed by a bundle of four transmembrane α-helices A−D. The two heme groups are bound by the His residues of the parallel helices B and D which alternate in the bundle with the antiparallel helices A and C. Our strategy was to conserve the anticipated interior packing of the hemes by hydrophobic residues and to change the other residues to hydrophilic and helix-stabilizing ones. The amphiphilic antiparallel helices should also shield the heme-binding pockets against the hydrophilic exterior. The amino acids of the two heme-binding helices B and D and that of the two shielding helices A and C have been adapted to design the hydrophobic face of helix H1 and H2, respectively, in our design:

Br-H1 : BrAc-GGEL**R**REL**H**EKLAKQ**F**EQLVKL**H**EE**R**AKKL-NH_2

Helix B[19,20]: AM**R**YI**H**ANGASL**F**FLAVYI**H**IF**R**G

Helix D[19,20]: RFFSL**H**YLLPFVIAALVAI**H**IWAF

Maquette[8]: CGGGELWKL**H**EELLKK**F**EELLKL**H**EERLKKL

Br-H2: Ac-LEEL**W**KK**G**EELAKKLQEALEK**G**KKLAK(AcBr)-NH_2

Helix A[20]: WWIW**G**IVLAFTLVLQIVT**G**IVL

Helix C[20]: **TW**IV**G**MVIYLLMMGTAFM**G**YVL

For comparison, the sequences of the helices from *Rhodobacter capsulatus* with the conserved residues marked in bold are shown in addition to the sequence of the Maquette designed by Robertson et al.[8] Helix H1 was synthesized with the two heme-ligating His separated by 13 amino acids with a central Phe and two Arg at a distance of three amino acids to His. The Arg residues provide positive charges near the position of the propionate groups of the hemes. In the shielding helix H2 two Gly were positioned at the same height as the His residues in H1 and a Trp residue near the N-terminal end by adopting the highly conserved residues of the natural helices A and C. The hydrophilic outer surface of the amphiphilic helices was designed to stabilize the helix by salt bridges between Glu and Lys.[1,21] The C-termini of the helices were amidated, and the N-terminal amino group of the shielding helix was acetylated for increased stability of the helices. This arrangement of the synthetic four-helix bundle protein was fixed by a template with four Cys via bromoacetyl groups attached to two N-terminal

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Figure 1. Model of the de novo protein MOP1 bis-heme complex. The backbone is shown as ribbon. Amino acid side chains are shown to illustrate the thioether linkage of the four Cys of the template with the N-terminal two Gly of helix H1 and the C-terminal Lys of helix H2. The His residues which bind the two heme groups and the Phe residue in the center of helix H1 are presented as stick structures. N and C indicate the N- and C-terminus of helices H2 and H1, respectively. The program Insight II (Biosym) was used.

Gly and the side chain of a C-terminal Lys of the heme-binding and shielding helix, respectively. A model of this design is shown in Figure 1.

Synthesis. The synthesis scheme of the helices for the cytochrome *b* model is shown in Figure 2. The helical peptides were synthesized by automated solid-phase peptide synthesis. H1 (**1**) was modified with a bromoacetyl group at the α-amino group of the N-terminal Gly (**2**). After removal of all protecting groups and cleavage from the resin, Br-H1 (**3**) was purified by HPLC. The synthesis of the helical peptide H2 was followed by modification of the N-terminus with an acetyl group (**4**). The antiparallel orientation of helix H2 was achieved by using Aloc as a protecting group for the C-terminal Lys. After selective removal of the Aloc group (**5**), the free ϵ-amino group was modified by a bromoacetyl group (**6**). Deprotection and cleavage from the resin yielded the helical peptide Br-H2 (**7**), which was purified by HPLC.

As template for the chemoselective ligation of the unprotected, purified, and bromoacetylated helical peptides via thioether bridges,[22] a cyclic decapeptide with four Cys was used. A secondary structure of a cyclic β-sheet with two turns formed

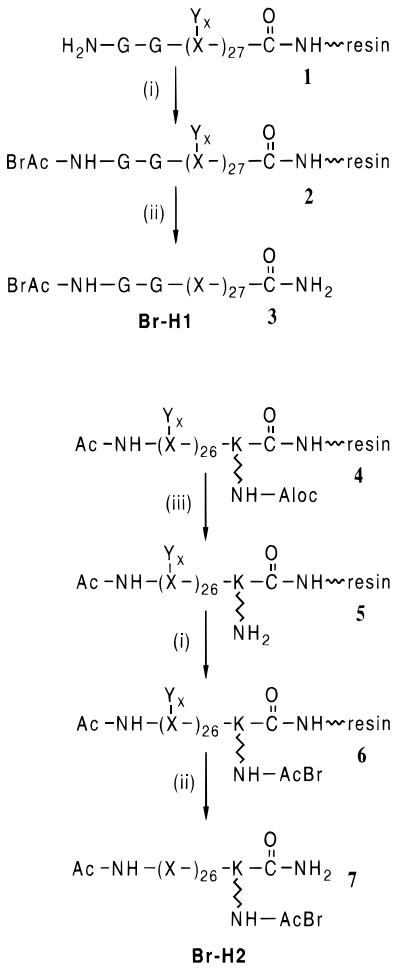

Figure 2. Synthesis scheme of the bromoacetylated peptides Br-H1 (**3**) and Br-H2 (**7**). The helical peptides were synthesized on a resin with a peptide amide linker. Synthesis steps: (i) 8 equiv of bromoacetic anhydride in DMF, 1 h; (ii) 23/1/1 TFA/DTT/anisole, 2 h; (iii) Pd-$(PPh_3)_2Cl_2$/Bu_3SnH/AcOH in DCM; all steps were performed at room temperature. Further details in the Experimental Section. The sequences of amino acids are given in the text. Y_X symbolizes the protecting group of amino acid X.

by Pro-Gly has been proposed for a similar sequence[23] as shown in Figure 3. The linear decapeptide **8** was synthesized manually on an extremely acid-labile resin with special care to avoid racemization of Cys. The Cys residues with alternating protection by the Acm and Trt group serve as the selectively addressable functional groups. The decapeptide was cleaved from the resin without deprotection of the side chains (**9**) and cyclized in solution (**10**). After selective cleavage of the Trt protecting groups of the cyclic template (**11**), Br-H1 (**3**) was ligated to the two deprotected Cys residues (**12**). The remaining two Acm protecting groups were cleaved with mercuric acetate. The fully deprotected template **13** was coupled with the helical peptide Br-H2 (**7**) to the four-helix bundle protein $T(H1)_2(H2)_2$. For simplicity this *mo*dular *p*rotein is designated in the following as MOP1.

Every synthesis step was followed by analytical HPLC and ESI mass spectrometry, and the products **9**, **11**, **13**, and **14** were

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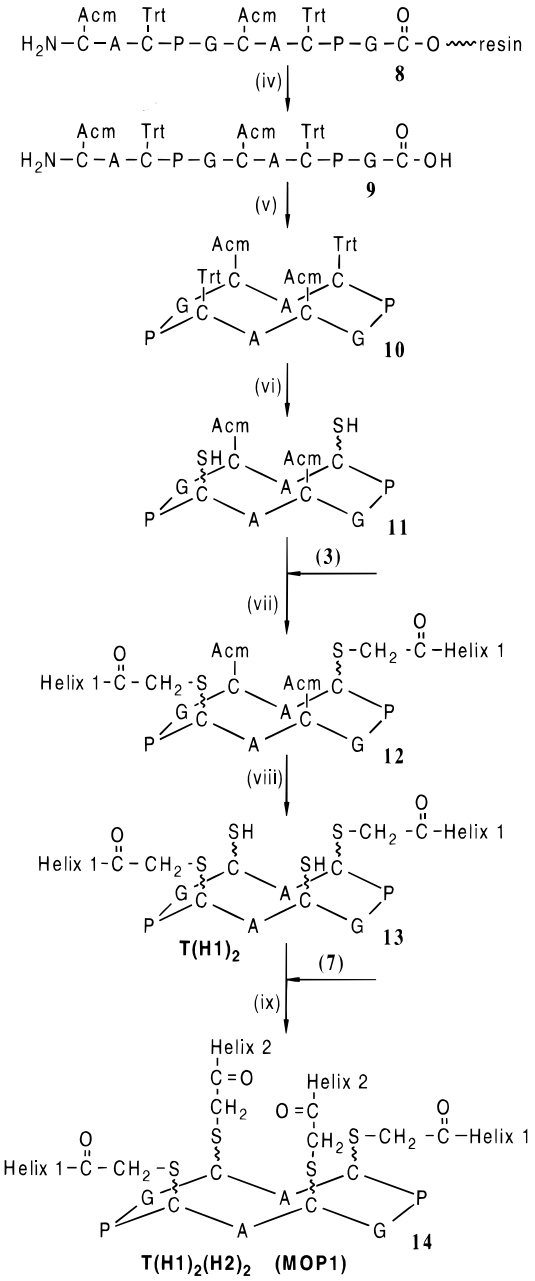

Figure 3. Synthesis scheme of the cyclic decapeptide template and the protein MOP1. The linear template **8** was prepared on a resin with the extremely acid-labile 4-carboxytrityl linker. After cleavage from the resin the protected peptide **9** was purified by reversed phase HPLC. Further HPLC purification was performed after steps vi, viii, and ix. Synthesis steps: (iv) 5/1/4 acetic acid/methanol/DCM, 2 h; (v) TBTU/DIEA in DMF, 8 h; (vi) 19/1 TFA/DTT, 30 min; (vii) 2.2 equiv of Br-H1, pH 7.5, 3 h; (viii) (1) $Hg(OAc)_2$, pH 4, 1 h; (2) DTT; (ix) 2.2 equiv of Br-H2, pH 7.5, 4 h. All steps were performed at room temperature.

purified by preparative HPLC. Analytical chromatograms of the critical coupling of Br-H1 and Br-H2 to the template are shown in Figure 4, top and bottom, respectively. They show an almost quantitative coupling of the bromoacetylated peptides with the thiol group of the unprotected Cys. The calculated and determined masses of the intermediate products are shown in Table 1. The mass spectrum of MOP1 is shown in Figure 5. The detected mass of 14 294.5 ± 1.6 Da verifies the correct primary structure, and the spectrum shows the high purity of the protein.

Cofactor Binding. Heme in DMSO added to an aqueous

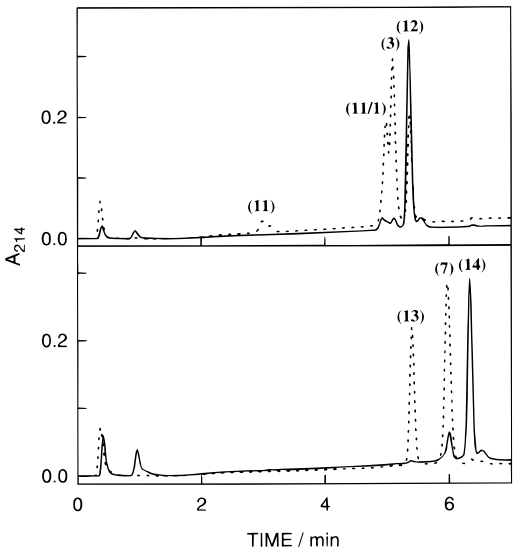

Figure 4. Analytical reversed-phase high-performance liquid chromatography of the chemoselective coupling steps. (top) Reaction of the helical peptide Br-H1 (**3**) with the cyclic template **11** to the bis-helix template $T(H1)_2Acm_2$ (**12**). The chromatograms of the reaction mixture after 45 min and 3 h are represented by the dotted and solid lines, respectively. The component (**11**/**1**) was identified by ESI-MS as the monohelix template $T(H1)Acm_2$. (bottom) Ligation of Br-H2 (**7**) with the Acm-deprotected bis-helix template **13** to $T(H1)_2(H2)_2$, MOP1 (**14**). The dotted and solid lines are the chromatograms of the reaction mixture after 2 min and 4 h, respectively. For all chromatograms a linear gradient of 0−56% acetonitrile in 7 min was used.

Table 1. Masses of Intermediates and the Final Protein Determined by ESI Mass Spectrometry during the Synthesis

peptide	calcd av mass (Da)	found mass (Da)
template **9**	1507.9	1506.9
template **10**	1489.9	1489.1
template **11**	1005.3	1004.5
Br-H1 **(3)**	3578	3577.7 ± 0.2
Br-H2 **(7)**	3298.8	3299.5 ± 0.3
$T(H1)_2Acm_2$ **(12)**	7999.4	7998.0 ± 0.5
$T(H1)_2$ **(13)**	7857.2	7857.3 ± 0.5
$T(H1)_2(H2)_2$ **(14)**	14293.1	14294.5 ± 1.6

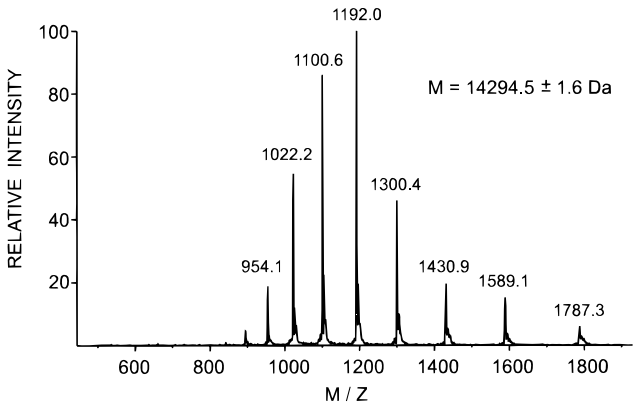

Figure 5. Electrospray ionization mass spectrum of the purified modular protein MOP1. The spectrum reveals the high purity and that the protein with a calculated mass of 14 293.1 Da possesses the correct primary structure.

solution of MOP1 is spontaneously incorporated. Figure 6 shows the UV−vis spectrum of the oxidized free heme group in buffer for comparison and the spectra of the oxidized and reduced heme groups bound to MOP1 in solution. The spectrum of the oxidized complex displays a characteristic Soret band at

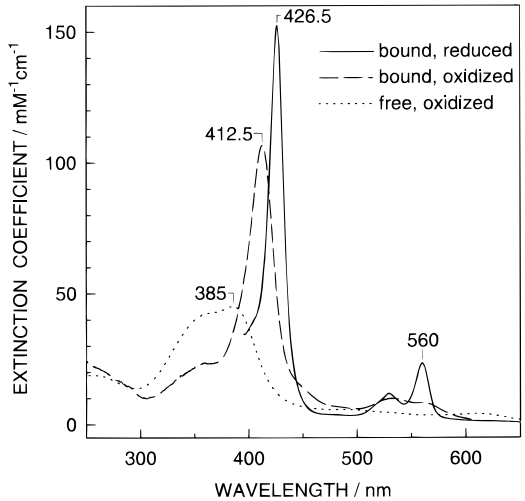

Figure 6. UV−vis spectra of oxidized heme in aqueous solution (dotted line) and of MOP1 with oxidized (broken line) and with reduced (solid line) bound heme groups. The MOP1-bound heme groups were reduced with a minimum amount of solid sodium dithionite. The extinction coefficient is given per heme.

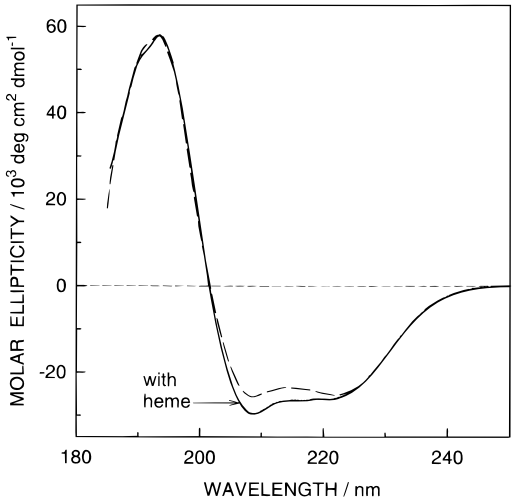

Figure 7. Circular dichroism spectra of the protein MOP1 (12 μM) (broken line) and its bis-heme complex (12 μM) (solid line) with the hemes in the oxidized state in 20 mM potassium phosphate (pH 7) at 20 °C.

412.5 nm and a broad band around 532 nm. Reduction of the complex with sodium dithionite leads to a characteristic red-shift of the Soret or γ-band to 426.5 nm and to distinct α- and β-bands at 530 and 560 nm, respectively, which are typical for bis-histidine ligated *b*-type cytochromes. The ratio of the absorbance of 6.5 at the γ- and α-bands of the reduced complex is also very close to that of 6 found for natural *b*-type cytochromes and indicates that the two heme groups are in a well-defined orientation between two histidines. The width of the α-band at half-maximum height is 16 nm. The stoichiometry of heme groups per MOP1 has been determined by addition of an exact amount of a 2.2−2.8-fold molar excess of heme over the protein with a concentration determined from the Trp absorbance at 280 nm. The resulting spectrum was analyzed by superimposing an appropriate sum of the spectra of the free and bound oxidized heme. These spectra have been normalized by their extinction coefficients. This reveals two heme groups bound per MOP1 with an error of ±4%. The absorbance of the two bound heme groups could not be distinguished neither at 293 K from the spectra measured for the redox titration nor at 78 K, where the spectrum at a reduction of 25% of total heme was compared with an appropriate sum of those in the fully reduced and oxidized state (not shown).

Circular Dichroism. Figure 7 shows the CD spectra of MOP1 and its bis-heme complex. The mean residue molar ellipticity at 222 nm θ_{222} was −25 400 and −26 200 deg cm^2 dmol^{-1} for the empty MOP1 and its bis-heme complex, respectively. From these values helicities of about 71 and 81% can be estimated if 100% helicity is referred to a value of −35 700 deg cm^2 dmol^{-1} as calculated from the formula of Chen et al.[24] or of −32 200 deg cm^2 dmol^{-1} as determined for tropomyosin,[25] respectively. If all residues fold as modeled in Figure 1 we would expect a helicity of 89%. The high positive value at 190 nm indicates also a high helix and a low random coil content of the protein with respect to the negative peak of the ellipticity of random coil structures in this range. Upon heme binding the ratio of $\theta_{222}/\theta_{209}$ changes from 0.98 to 0.89.

Size-Exclusion Chromatography. A possible association of MOP1 and its bis-heme complex was analyzed by size-exclusion chromatography. Figure 8 shows the calibration curve of a Sephadex 75 column with a set of globular proteins. The elution time of MOP1 and its bis-heme complex indicates an apparent molecular mass of 18.6 and 18.8 kDa, respectively. The retention times did not depend on the protein concentration between 20 and 150 μM, indicating no formation of dimers. The highly symmetric size-exclusion chromatogram of the bis-heme MOP1 in the inset indicates a homogeneous sample. It should be mentioned that no loss of heme was observed during the slow passage through the column although free heme is tightly adsorbed to Sephadex. This indicates an extremely high binding constant.

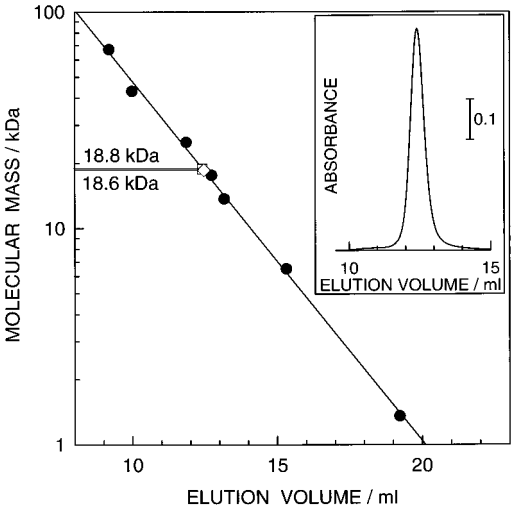

Figure 8. Size-exclusion chromatography of the de novo protein MOP1 and its bis-heme complex. The elution volumes of a set of globular proteins (●) (low molecular weight kit) was determined at a flow rate of 0.5 mL/min with a Pharmacia Superdex 75 (1 × 30 cm) size-exclusion FPLC column equilibrated with 50 mM potassium phosphate (pH 7) and 100 mM NaCl. Retention volumes of the proteins were determined by following the absorbance at 215 nm. The straight line is the linear regression of the calibration values. The elution volumes of MOP1 (◇) and its bis-heme (oxidized) complex (□) indicate molecular weights of 18.6 and 18.8 kDa, respectively. The insert shows the size-exclusion chromatogram of the MOP1 bis-heme complex.

Stability. To characterize the stability of MOP1 and its bis-heme complex, we have measured three different properties of the molecule as a function of guanidine hydrochloride (GuHCl)

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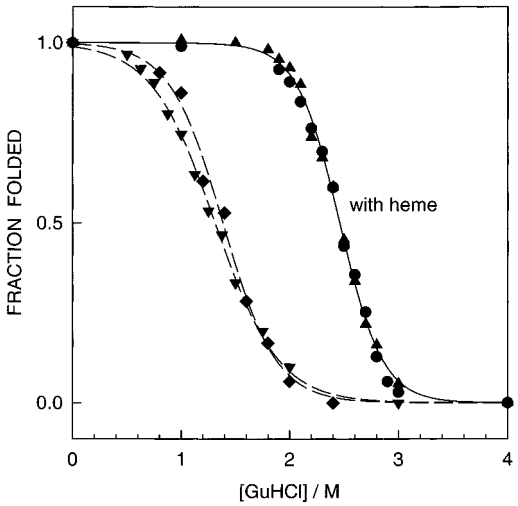

Figure 9. Guanidine hydrochloride (GuHCl)-induced unfolding of the empty MOP1 protein monitored by the circular dichroism at 222 nm (▼) and the wavelength of the maximum fluorescence emission of the two tryptophane residues (◆) and of the MOP1 bis-heme complex in the oxidized state monitored by the circular dichroism at 222 nm (▲) and the binding of the oxidized hemes to the modular protein monitored by the absorbance at 412 nm at 20 °C (●). The folded fraction *f* (cf. text) is plotted against the GuHCl concentration. The lines are based on the parameters obtained by a nonlinear least-squares fit of the data points to the equation described in the Experimental Section. The respective values for a folded fraction of 1 and 0 were for circular dichroism −26 200 and +240 deg cm^2 dmol^{-1} for the bis-heme MOP1, −25 400 and +160 deg cm^2 dmol^{-1} for the empty MOP1, for the fluorescence maximum 342 and 354 nm, and for the absorbance at 412 nm 0.35 and 0.145.

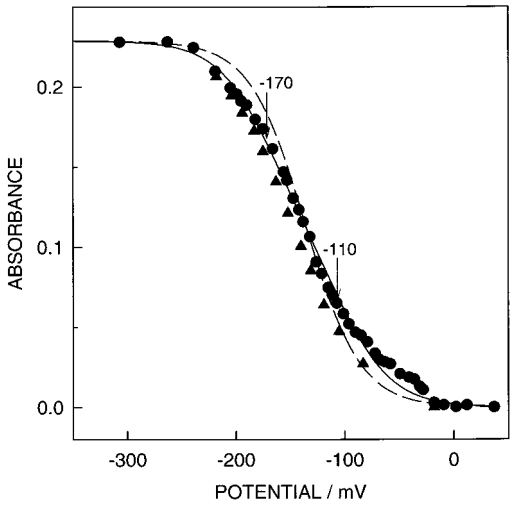

Figure 10. Redox titration of the MOP1 bis-heme complex. The difference of the absorbance at the α-band (560 nm) of the reduced *minus* that of the fully oxidized species is plotted against the ambient redox potential. The solid line is based on the midpoint potentials of −110 and −170 mV obtained by a nonlinear least-squares fit of the data points of this reductive titration to two Nernst equations with $n = 1$ and equal contributions of each component. For comparison a fit with a single Nernst equation with $n = 1$ is given by the broken line. The data for the titration in the reductive and the subsequent oxidative direction are represented by full circles and triangles, respectively, indicating midpoint potentials which differ by less than 10 mV.

concentration (Figure 9). The molar ellipticity at 222 nm gives an integral over the helical conformation of the entire protein, the wavelength of the maximum fluorescence emission of the two Trp residues[26] indicates the degree of isolation of the indole rings from solvent water at the open end of MOP1, and the difference of the absorbance between the bound and the free heme at 412 nm monitors the incorporation of the oxidized hemes. Using the two-state transition model[18,27] to analyze the data in Figure 9, the free energy of folding in the absence of denaturant (ΔG_{H_2O}) at 20 °C is −13.7 ± 2 and −30.4 ± 3 kJ/mol (standard deviation, four titrations) for the empty MOP1 and its bis-heme complex, respectively. Thus, binding of the two hemes stabilizes the structure of MOP1 by 16.7 kJ/mol. For the respective values of *m* we found −9.9 ± 1 and −12.4 ± 1.5 kJ mol^{-1} M^{-1}. No significant difference between the three parameters has been observed, which indicates the absence of stable intermediates and a high cooperativity during the folding process.

Redox Potential. The electrochemical midpoint potential was determined by monitoring the absorbance in the α-band at 560 nm as a function of the ambient redox potential. Figure 10 shows a redox titration in the reducing followed by one in the oxidizing direction. The analysis of four titrations uncovered two distinct midpoint potentials of −106 and −170 mV vs standard hydrogen electrode (standard deviation of both values is ±8 mV) for the two heme groups. The minor deviation in the redox titration curve with a midpoint near −40 mV is possibly due to poor equilibration by the redox mediators. The spectra indicated no difference of the absorbance between the heme groups (not shown).

Discussion

Design. The de novo design of the modular protein MOP1 represents an attempt toward the synthesis of a structurally defined protein with a binding pocket for a cofactor. On the basis of the TASP concept, we were able to synthesize an antiparallel four-helix bundle protein by ligating different helices with the desired topology to the template. The structure should contain an optimized cavity for a heme even if helix-destabilizing residues have to provide the necessary space. In this respect it is an advantage that the branched chain architecture of a TASP increases the stability in comparison to a linear sequence.[5] The problem of protein folding and association to a defined topology of a four-helix bundle is difficult to handle with linear sequences even without bound cofactors[3,28] but is overcome by the topological restraints introduced by a template connecting amphiphilic helices.[29]

To realize the target structure of an α-helix bundle most of the known principles to stabilize this secondary structure have been used as detailed above. A stable folding and the relative orientation of amphiphilic helices are driven by hydrophobic forces. This provides the rationale to adopt the hydrophobic core of an integral membrane protein with a bound cofactor to a soluble protein of similar function. Following a natural structure should provide an advantage to construct a pocket for the incorporation of voluminous cofactors which will heavily disturb the association of a four-helix bundle.

We constructed a bis-heme binding protein strongly related to the heme-binding core of the natural protein with two heme binding helices and two shielding helices. The recent crystal structure of cytochrome *b*[9] proved that the predictions which were the basis of our design are essentially consistent with the

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structure. Indeed the carboxyl groups of both hemes point to the end of the four-helix bundle as intended to orient by the two arginine residues of helix H1 in our design. In the structure the helices terminate close to the edge of the heme groups and the binding pockets are shielded against the hydrophilic phases by loops connecting the helices on both sides of the membrane. In our design the helices are longer and shielding of the hydrophobic interior may be provided by the tryptophane residues of helix H2 at one end and the template at the other end of the molecule. We do not know if the helices in our molecule are tilted as in the natural structure, but a coiled structure seems to be necessary for a ligation of the two heme iron by the histidine residues.

Synthesis. With respect to a chemical synthesis of new proteins this design offers the possibility of assembling building blocks of unprotected peptides. The development of chemoselective ligation methods for unprotected peptide fragments made it possible to synthesize such a structural complex and homogeneous TASP. Dawson and Kent[30] first synthesized a simple TASP by using a chemoselective ligation method. Mutter and co-workers have demonstrated the use of the oxime and thioether approach for the synthesis of an antiparallel four-helix bundle TASP[31] and of a TASP with up to three different loop sequences coupled at their N- and C-termini onto a cyclic peptide of 14 amino acids.[32] But this approach needs extensive preparative chemistry to synthesize the cyclic peptide with differently protected aminoxyacetic acid side chains. We have incorporated commercially available orthogonally protected cysteine derivatives into the template which simplified the synthesis considerably and can be extended. The use of the chemoselective bromoacetyl−thiol chemistry yields a homogeneous product as can be seen in the mass spectrum shown in Figure 5.

Size-Exclusion Chromatography. The size-exclusion chromatography shows that MOP1 and its bis-heme complex are monomeric species in solution in a large concentration range. Their respective apparent molecular masses of 18.6 and 18.8 kDa are somewhat larger than their calculated masses of 14.3 and 15.5 kDa. This coincides with the apparent molecular mass of 18.1 kDa found for cytochrome b_{562},[33] a natural four-helix bundle protein,[34] which is almost 6 kDa higher than the actual mass. This effect has been attributed to the assymmetric shape of the four-helix bundle protein which can be described as a cylinder 25 Å in diameter and 50 Å in length[33] and is very similar to the anticipated shape of our protein in Figure 1. The small difference of 0.2 kDa between the apparent mass of MOP1 and its bis-heme complex reveals that there is no significant change in the hydrodynamic radius and that the two heme groups are well integrated into the heme binding pockets.

Redox Potentials. The midpoint redox potentials of the two MOP1 bound hemes of −106 and −170 mV are significantly higher than the midpoint of −220 mV of a synthetic four-helix bundle which accommodates one bis-histidine ligated heme group[14] and the midpoint of −215 mV of an imidazole−histidine ligated single heme octapeptide.[35] In line with these values are also the potentials of −205 and −215 mV of the noninteracting heme groups of the two synthetic two heme four-helix bundle cytochromes of Robertson et al. These values around −220 mV seem to be typical for bis-histidine ligated heme groups in a hydrophilic environment. A close packing of the four-helix bundle MOP1 around the two heme groups together with the electrostatic interactions of the hemes with the arginine residues may raise the potential to −170 mV. If compared to natural complexes, closest to our values are those of −45 and −150 mV found for the two hemes in the cytochrome *bf* complex.[36] In the natural protein cytochrome, b_H and b_L refer to the hemes with high and low redox potentials, which would correspond to the positions of the hemes in MOP1 near the open end and the template, respectively, when the orientation of the helices is used as reference. However, to what extent a distinct heme environment or/and the electrostatic interaction between two almost indentical heme groups cause the difference in the midpoint potentials has still to be settled.

Stability. The free energy of folding of the empty MOP1 of $\Delta G_{H_2O} = -13.7$ kJ/mol is rather small if compared to the synthetic four-helix bundle Ac-α4 of Handel et al.[37] ($\Delta G_{H_2O} = -64$ kJ/mol) but almost the same as that of apocytochrome b_{562} ($\Delta G_{H_2O} = -13.4$ kJ/mol), the apoprotein of a natural heme binding four-helix bundle with a molecular mass of 12.3 kDa.[33] Our design is not in favor of a protein with maximum stability without a bound heme. The insertion of four glycine and two valine residues causes a considerable destabilization of the helices. These amino acids were used to optimize the heme binding sites by following the natural protein. A criterion for the design of an optimized binding pocket may be the increase of stability upon binding of the cofactors. It is 8.4 kJ/mol per heme group in our design, a slightly higher value than that of 7.9 kJ/mol found for the single heme bound by the synthetic four helix bundle of Choma et al.[14] Remarkable is the high cooperativity of the unfolding of the MOP1 bis-heme complex if compared with other de novo designed peptides. The *m* value ($m = -12.4$ kJ mol^{-1} M^{-1}), which is a measure of the cooperativity of the transition, is furthermore approximately proportional to the difference in solvent-accessible surface area between the folded and unfolded states. This suggests a reasonably well packed molecule with a hydrophobic interior shielded from solvent exposure.

Conclusion. The synthesis of a topologically defined four-helix bundle protein with two heme groups bound in a well-defined hydrophobic environment in accordance with the initial design has been successful. The spectroscopic properties of the bound heme groups are very similar to those of natural *b*-type cytochromes. Although we adopted our design very close to the heme binding core of the *b*-subunit of the cytochome bc_1 complex, there are significant differences between the design and the natural protein as the wavelength of maximum absorbance or the more negative redox potentials of the hemes in the synthetic protein. However, with our approach, we do not intend to mimic nature or draw conclusions from our model protein to the structure and function of the natural protein but we consider this heme-binding MOP1 as a starting compound

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for the investigation of the influence of the protein hull on the properties of the bound cofactor.

The advantages of the modular TASP design for the chemical synthesis of cofactor binding proteins are that its topology is predetermined and that the design can easily be modified. We are able to bind at a specific position several variants of a helix or to change the relative position of the helices by different templates. Another advantage is the possibility to place a labeled or nonproteinogenic amino acid at any position. Future development will be to find the strategy for an optimized binding pocket of the cofactor. The concept offers the possibility for combinatorial binding of single helices for optimization of the needed properties.

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