media under identical conditions (0.1M supporting electrolyte). The latter more closely approaches the value of 61 mV[13] expected for this system.[14] The new family of electrolytes raises expectations that faster heterogeneous electron transfer rates may be measured in low-polarity media,[15] in addition to aiding mechanistic studies through increased accuracy of diagnostic parameters such as peak potentials and voltammetic wave shapes.[16]

There is no reason to think that $[NBu_4][B(C_6F_5)_4]$ is the optimum supporting electrolyte for all electrochemistry in low-polarity media, and analogues specifically designed to meet the needs of other redox systems are easily envisioned. When combined with ultramicroelectrode technology,[17] which already minimizes problems of ohmic loss, and advances in the use of very small concentrations of supporting electrolytes,[18] this family of salts holds significant promise in aiding further advances in applications of electrochemistry.[19]

Experimental Section

Experiments were carried out under a nitrogen atmosphere using Schlenk or dry-box conditions, and solvents were dried and distilled, except for *tert*-butyl methyl ether, which was used as received (Aldrich, anhydrous). $[NBu_4][B(C_6F_5)_4]$ was prepared by metathesis of an aqueous solution of $Li[B(C_6F_5)_4]\cdot n\,Et_2O$ ($n = 2-3$) (Boulder Scientific Co.) with a solution of $[NBu_4]Br$ in methanol. The precipitate was washed with water, dried under vacuum, and recrystallized several times from CH_2Cl_2/Et_2O (purified yield ca. 70%). Conductance values were obtained with a YSI Model 3200 conductance meter and a conductivity cell with a cell constant of 0.1 cm^{-1}. Voltammetry was performed using a Princeton Applied Research Model 273 potentiostat without ohmic compensation. A standard three-electrode cell configuration was employed using working electrodes composed of polished disks (Pt or glassy C), a Pt wire auxiliary electrode, and a Ag/AgCl reference electrode.

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Combinatorial Synthesis of Four-Helix Bundle Hemoproteins for Tuning of Cofactor Properties**

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The aim of protein design is to create novel proteins with tailored structural and functional properties. Besides a rational approach by site-directed mutations of known proteins, a general strategy is to generate a large pool of different molecules and to screen this library for single substances with the desired properties. This goal is realized by phage display

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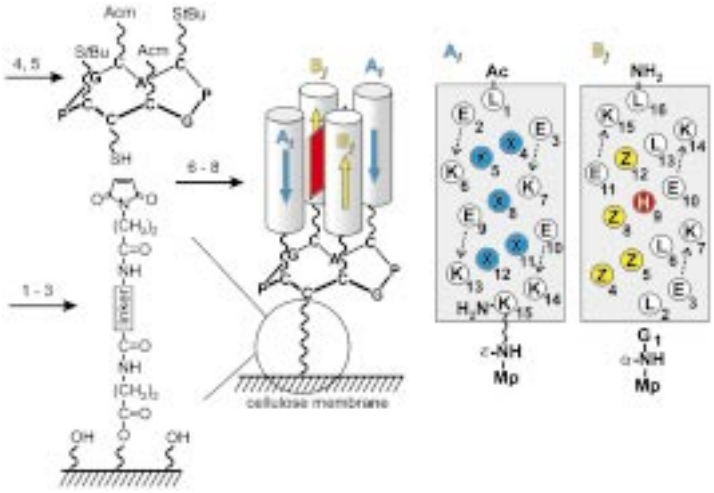
of proteins produced by DNA-based variations and then selection, for example, by their affinity to immobilized ligands[1] as well as by chemical solid-phase synthesis of libraries with short linear or cyclic peptides.[2]

Extensive research efforts are being directed towards a de novo design of proteins that provide a wide variability in biomimetic chemistry.[3] In particular the interaction of synthetic proteins with the cofactor Fe^{III}–protoporphyrin IX (heme) has been studied.[4] The difficulty in screening physical parameters has limited the studies of variants to rather small numbers.[5] The assembly of synthetic proteins from antiparallel α-helices on a cyclic peptide is an efficient strategy for the synthesis of predetermined structures.[6] This method was successfully applied to tailor a synthetic cytochrome b analogue, which was also active in bioelectronic applications when bound to a gold surface[7] and to metalloproteins capable of light-induced electron transfer.[8] These studies have focused on the need for an optimized protein environment to control the physical properties of a cofactor. Herein we report the synthesis of a library of hemoproteins from small libraries of peptide building blocks attached to a template that is bound to a cellulose membrane. The strategy is based on a combination of spot synthesis,[9] the concept of template-assembled synthetic proteins (TASP),[6] and solid-phase characterization of physical parameters.

A schematic presentation of the protein design[7, 8] and the synthesis is shown in Scheme 1. The cellulose membrane was



Scheme 1. Stepwise assembly of the cellulose-bound library of synthetic Fe^{III}–protoporphyrin IX modular proteins. The positions of the varied amino acids labeled X_k and Z_l of helices A_i and B_j, respectively, and the heme-ligating histidine are colored. The numbers above the arrows refer to the synthetic steps described in the Experimental Section.

modified with β-alanine, to which a linker and 3-maleimidopropionic acid were coupled. The maleimidopropionyl (Mp) group reacts selectively with the unprotected cysteine residues of the cyclic template. The successive removal of the S*t*Bu and acetamidomethyl (Acm) protecting groups of the cysteine residues and the coupling of the maleimidopropionyl moiety of the helical peptides A_i and B_j ensured the antiparallel orientation of the helix pairs. Small libraries have been synthesized using a parallel peptide synthesizer by variation of residues X_k ($k = 4, 5, 8, 11, 12$) and Z_l ($l = 4, 5, 8, 12$) in the amphiphilic helices A_i and B_j, respectively; these residues were found by molecular modeling to form the hydrophobic binding pocket of the heme. Since a variation of all the natural amino acids at these nine positions would need 5×10^{11} proteins we have limited the variation to hydrophobic amino acids of different size and a few polar ones, including Gly, Ala, Val, Leu, Ile, Phe, Tyr, and Gln. The 22 different combinations of these amino acids for the shielding helices A_i and the 21 for the binding helices B_j resulted in 462 modular proteins. Finally Fe^{III}–protoporphyrin IX was incorporated into the proteins by incubating the membranes with a solution of the cofactor.

The peptides could be collected at any stage of the synthesis by acid cleavage of the linker from cutout spots. Figure 1 shows the analytical reversed-phase HPLC and mass spectrometry of the final products formed from five different sets

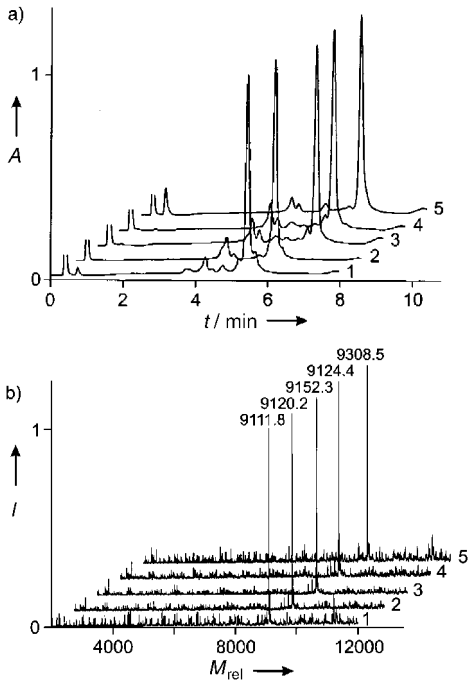


Figure 1. a) Analytical reversed-phase HPLC monitored at 215 nm of four-helix bundle proteins after cleavage of the linker from cutout spots and precipitation with diethyl ether. b) Deconvoluted electrospray mass spectra of the samples used for (a). The traces 1–5 have been recorded with the proteins assembled from helices A_8B_{12}, $A_{13}B_{11}$, $A_{18}B_9$, A_9B_{11}, and A_3B_{12}, respectively. For the varied amino acids see Figure 3. The masses reveal the correct primary structure of the template assembled four-helix bundle proteins.

of helices. Electrospray mass spectrometry was also used to characterize all the helices, the template, several cellulose-assembled test peptides, and the intermediate products. Importantly, no impurities of template-assembled helix bundles with less than four helices were detected (Figure 1b) and hence the four-helix bundles were pure enough for direct screening. For comparison of the cellulose-bound hemoproteins with their soluble variants two four-helix bundle proteins with helices $A_{10}B_9$ and $A_{10}B_{11}$ (see Figure 3) were assembled on a previously described template[7b] in milligram quantities and characterized in solution.

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Figure 2 shows a picture of a sheet with 42 cellulose-bound proteins with a heme group in the oxidized and the reduced state. The spectra were recorded from the spots of the hemoprotein $A_{10}B_9$. The protein complexes show typical

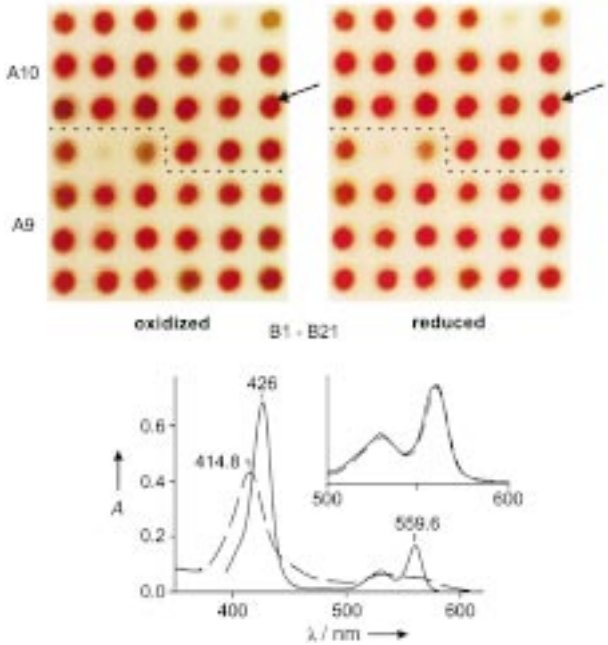

Figure 2. Top: Cellulose membrane with 42 different covalently bound proteins with ligated heme in the oxidized (left) and reduced (right) state. The dashed line indicates the frontier between the proteins with shielding helix A_9 (bottom) and A_{10} (top). A total of 21 different binding helices B_j were combined with the shielding helices. The arrows indicate the protein used to record the spectra shown below. Bottom: UV/Vis spectra monitored from the cellulose-bound Fe^{III}-protoporphyrin IX protein assembled from helices A_{10} and B_9 (oxidized state, dashed line) and after addition of dithionite in the reduced state (solid line). Inset: expanded region of the spectrum in the reduced state superimposed with that of a reference protein with the same helices in solution (- · -).

spectra for bis-histidine-ligated b-type cytochromes with a Soret band at about 414 nm in the oxidized state and at about 427 nm in the reduced state. The complexes display distinct α- and β-bands at about 560 and 530 nm, respectively, in the reduced state. To check the applicability of this solid-phase characterization the spectra of the soluble proteins were compared with those of the corresponding cellulose-bound peptides and found to be superimposable (see inset in Figure 2).

Two of the shielding and two of the binding helices (B_{20} and B_{21}) were designed to be either nonamphiphilic or non-α-helical. Proteins containing these helices did not bind heme (see the spots in Figure 2) or showed an undefined spectrum of the heme. For the remaining 399 proteins the width at half the maximum intensity of the α-band in the reduced state varied between 14.4 and 20.3 nm with a maximal deviation of 6% in repeated recordings. These complexes were screened for the midpoint potential of the heme group. The midpoint potential was estimated by determining the fraction of total complexes reduced at an ambient potential of -95 mV versus the standard hydrogen electrode (SHE). Figure 3 shows the spectra of two proteins at this potential fitted to the isosbestic

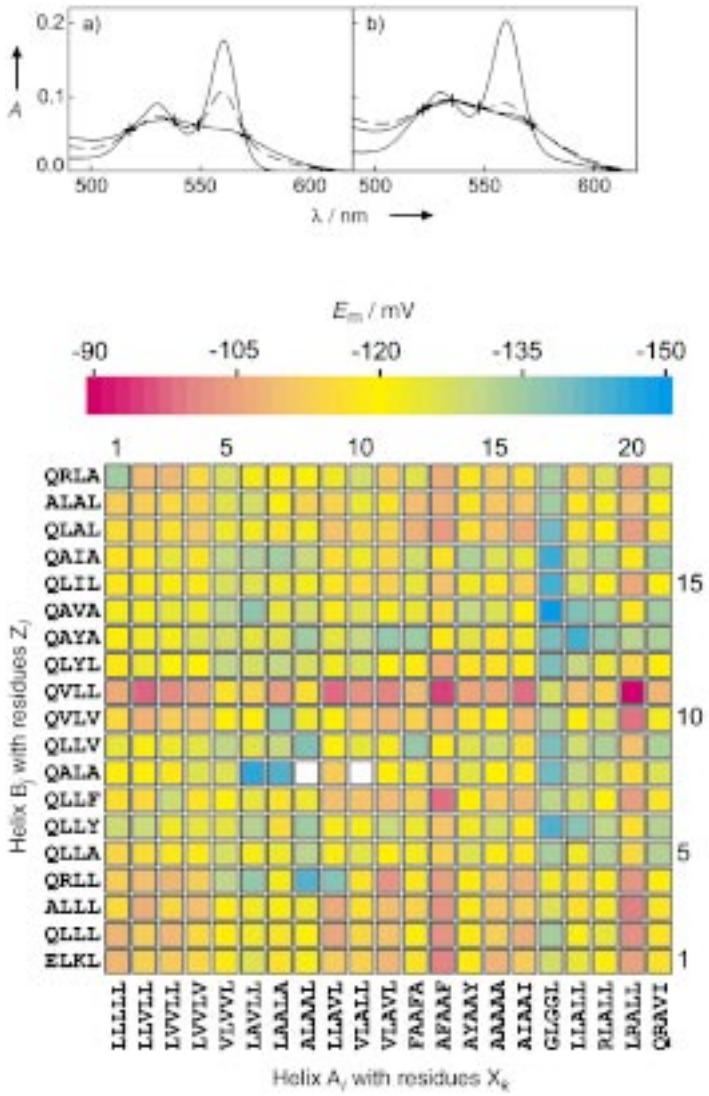

Figure 3. Redox midpoint potentials of cellulose-bound four-helix bundle proteins with inserted Fe^{III}–protoporphyrin IX. Top: Spectra of the proteins with helices $A_{13}B_{17}$ (left) and helices $A_{17}B_{14}$ (right) in the oxidized and reduced state (solid lines). The isosbestic points marked by crosses were taken from these spectra and used to normalize the spectrum monitored at -95 mV in the presence of the redox buffer 2,5-dihydroxy-*p*-benzoquinone (4 mM) under argon (- · -). The respective midpoint potentials of -103 and -148 mV were estimated using the Nernst equation and the reduced fraction at -95 mV by comparing the absorbance of the three spectra at the maximum of the α-band. Bottom: The estimated midpoint potentials of the proteins assembled from helices A_i and B_j are color-coded according to the scale at the top. The amino acids at the positions X_k ($k = 4$, 5, 8, 11, 12) and Z_l ($l = 4$, 5, 8, 12) are given along the axis.

points of the spectra of the oxidized and the reduced protein. The estimated midpoint potentials varied in the range of -90 to -150 mV as summarized in the color-coded diagram in Figure 3. The combinatorial approach was successful in finding proteins that tune the heme to a midpoint potential that is 80 mV more positive than the redox potential of our previously designed heme proteins of about -170 mV[8] and even more positive than those of other synthetic heme proteins[4a] that have redox potentials close to the value of -210 mV for histidine/imidazole-coordinated heme in water.[10] The redox potentials of the two reference proteins

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synthesized in solution were both 10 mV more negative than their immobilized counterparts. The heme group could be removed by washing the membrane with DMF. When the heme group was incorporated a second time, a maximal difference of 5% between the two independent redox measurements of 57 modular hemoproteins with shielding helices A_5, A_6, and A_{19} was found. This does not only show the excellent reproducibility of the repeated use of the cellulose-bound protein library, but also the possibility of successive screening with different cofactors such as other metalloporphyrins or chlorophyll derivatives.

A relationship between the midpoint redox potentials and the varied amino acids is not readily apparent. For example, the change of 20 mV in the midpoint potential by a switch of Val and Leu at the positions Z_5 and Z_{12} in helices B_9 and B_{11} is not easy to interpret, but in general the effects of the binding and the shielding helix seem to be additive. Figure 3 indicates the trend that a combination of helices that form complexes with more positive midpoint potentials display the highest values, as for example the combinations of helix B_{11} with A_{13} or A_{20}. This observation is consistent with a tight packing of all four helices being needed to exclude water from the heme-binding pocket and favor a high redox potential.[10] Further analysis of the data using neural networks[11] is currently in progress and may help to design new variants with even higher midpoint potentials.

A method for the efficient synthesis of large numbers of four-helix bundle proteins from peptide libraries has been described. The parallel solid-phase synthesis has led to spatially separated spots of homogenous protein–heme complexes that could be directly screened for physical properties. Automation with pipetting and screening robots and the extension to a large number of proteins is possible. The reversible binding of cofactors allows the successive screening of the protein library. The range of the detected redox potentials demonstrates the applicability of the described method to tune physical parameters of immobilized de novo proteins. The method is not limited to two different helices and can also be of general use for the synthesis of large numbers of receptorlike molecules, new metalloproteins, or the search for new catalysts.

Experimental Section

The helical peptides were synthesized using a multiple batch solid-phase peptide synthesizer (Advanced ChemTech) with standard 9*H*-fluoren-9-ylmethoxy)carbonyl (Fmoc)/benzotriazol-1-yl-*N*-tetramethyluronium tetrafluoroborate (TBTU) protection/activation. 3-Maleimidopropionic acid was coupled to the *N*-terminus of helices B_j or the ε-amino group of the C-terminal lysine of helices A_j.[8a] All deprotected peptides were purified by preparative reversed-phase HPLC. Cellulose membranes were derivatized with β-alanine (1; Scheme 1).[9] The 1-hydroxy-1*H*-benzotriazole (HOBt) ester of the modified Rink linker, *p*-[(*R*,*S*)-α-[1-(9*H*-flouren-9-yl)-methoxy-formamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid, was coupled by adding 2 μL (×2) of a 0.3 M solution, which resulted in spots of 8 mm diameter (2). Any residual free amino groups were acetylated[9] and 3-maleimidopropionic acid was coupled to the linker as a symmetrical anhydride (3). Synthesis of the cyclic decapeptide template (4) was as described[7b] except for an additional trityl-protected cysteine (trityl = triphenylmethyl). The trityl-deprotected (5) template was coupled to each of the spots in 0.3 M sodium-phosphate (pH 7.1)/acetonitrile (2/1, (6)). The deprotection of the S*t*Bu groups with dithiothreitol and of the Acm groups with mercuric acetate under Ar and coupling of the helices (7) were adapted from described procedures.[8] The synthesis was monitored by cutting out spots of the membrane and cleaving the modified Rink linker by incubation with TFA/CH_2Cl_2/water/triisopropyl silane (50/45/4/1) for 60 min. Fe^{III}–protoporphyrin IX (heme) was incorporated into the cellulose-bound proteins by incubation with 50 μM heme in 100 mM Tris-HCl (pH 7.5)/DMF (4/1) followed by washing with the solvent (×3, (8)). The spectra were recorded with a diode-array spectrophotometer (J&M, Germany) by placing light guides on opposite sides of the protein spots with the membrane submerged in buffer in a microtiter plate without wells. The spectra were analyzed automatically by software of local design.

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