Design of a Unique Protein Scaffold for Maquettes

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Heme proteins serve a wide variety of functions in the biological milieu.[1] Our approach to the study of protein structure function relationships is to use insight drawn from nature to design and synthesize minimal peptides which fold, assemble, and incorporate cofactors.[2−8] Molecular *maquettes*[2] are functional synthetic proteins which are simplified versions of their biological counterparts. This strategy is aimed to provide direct insight into protein design,[9−18] cofactor incorporation,[19−21] structure and function properties of redox proteins, and establishment of *in situ* catalytic properties. The four α-helix bundle[22,23] has been successfully used in this manner for construction of maquettes containing hemes,[2,7] porphyrin dimers,[3] flavins,[4] iron sulfur clusters,[5] and amino acid radicals.[6] Several of the heme-containing maquettes have been analyzed by NMR spectroscopy and show properties atypical of native-like structure. This Communication describes recent progress toward the design and structural characterization of a native-like maquette scaffold.

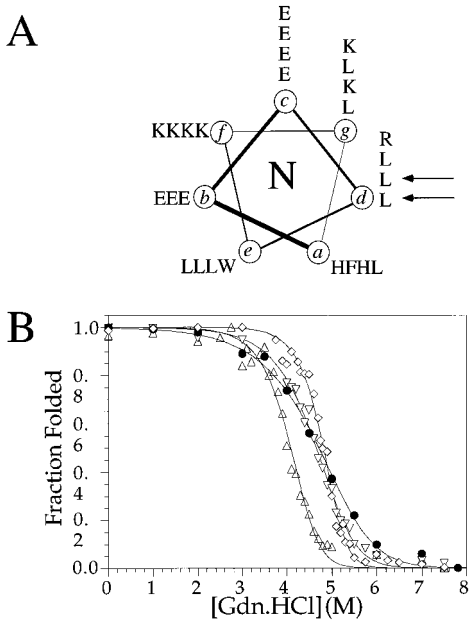

Figure 1. (A) Helical wheel diagram of a single helix of H10H24 with modified heptad *d* positions indicated with arrows and (B) the guanidine hydrochloride denaturation curves of (●) H10H24, (▽) H10H24-L13F, (Δ) H10H24-L6I, and (◇) H10H24-L6I,L13F followed by CD spectropolarimetry (15 μM peptide, 50 °C, 10 mM potassium phosphate, 100 mM KCl, pH 8.1 buffer).

Like the majority of designed proteins[24] the prototype heme protein maquette,[2] H10H24 (*Ac*-CGGGELWKL·HEELLKK·FEELLKL·HEERLKK·L-*CONH*$_2$), shows characteristics associated with non-native protein structure. We have explored computationally and experimentally the effect of substituting leucine residues with conformationally restricted β-branched and/or aromatic amino acids to increase hydrophobic core packing specificity. Since the three internal heptad *a* positions[25] (H10, F17, and H24) of H10H24 are engineered for heme binding and R27 is designed to modulate the heme redox potential, the best candidates for redesign were the leucine residues L6, L13, and L20 (see Figure 1A). Initial studies have focused on exchanging L6 for isoleucine and L13 for phenylalanine. Single and double variants at these two helix positions (H10H24-L6I, H10H24-L13F, and H10H24-L6I,L13F) were prepared,[26] and the effect of these substitutions on the coiled-coil thermodynamic stability and structural specificity determined. This series of maquettes displays a continuum of changes ranging from the poorly ordered and least stable prototype H10H24 to the uniquely structured and most stable double variant H10H24-L6I,L13F.

The secondary structure of all three H10H24 variants was investigated using circular dichroism, infrared and NMR spectroscopies, and the aggregation state probed with gel premeation chromatography. The CD spectrum of each peptide had minima at 222 and 208 nm in a ratio ($\Theta_{222}/\Theta_{208}$) of >1.0, the hallmark

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(26) All peptides were assembled on a continuous flow solid phase synthesizer employing the fluorenylmethoxycarbonyl (Fmoc)/^{t}Bu strategy. Following chain assembly the peptides were N-terminal acetylated, washed with DMF and CH_2Cl_2, and simultaneously cleaved/deprotected with trifluoroacetic acid/ethanedithiol/water (90:8:2 v/v/v). Each peptide was purified to homogeneity using reverse-phase C_{18} HPLC and the molecular mass confirmed by laser desorption mass spectrometry. The N-terminal cysteine sulfhydryl was then oxidized to the disulfide to yield α_2 that spontaneously self-assembles in solution to afford the dimeric $[\alpha_2]_2$.

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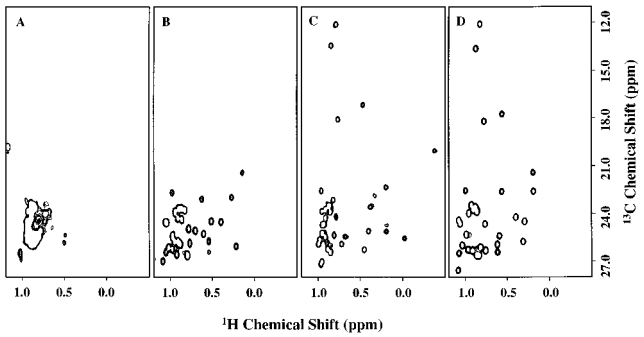

Figure 2. Methyl region expansions from the ^{13}C-HSQC spectra of (A) H10H24, (B) H10H24-L13F, (C) H10H24-L6I, and (D) H10H24-L6I,L13F. Conditions were ≈1.0 mM protein in 20 mM phosphate buffer [pH 7.20], 50 mM KCl, 8% D_2O, and 32 °C.

of coiled-coil α-helical structure[27] with >80% helical content. The location of the amide I′ band[28] between 1640 and 1655 cm^{-1} in the FTIR spectrum and numerous NH−NH NOEs in the ^{1}H−^{1}H NOESY spectrum of each peptide provide further evidence for predominately α-helical structure. Gel permeation chromatography demonstrated that the di-α-helical peptides spontaneously self-assemble in solution to afford the dimeric four helix bundles in the concentration range 0.1−100 μM.

The stability of the global protein structures was compared by following their guanidine hydrochloride induced denaturation by CD spectroscopy.[29] The peptides display cooperative transitions with stabilities similar to natural proteins (Figure 1B). H10H24-L6I and H10H24-L13F are more stable than the original H10H24 (ΔG^{H_2O} = 14.6 kcal/mol) with $\Delta\Delta G$ values of 2.7 and 4.7 kcal/mol, respectively. H10H24-L6I,L13F is the most stable (ΔG^{H_2O} = 23.0 kcal/mol) of the series, and its enhanced stability ($\Delta\Delta G$ = 8.4 kcal/mol) is slightly more than the sum of the $\Delta\Delta G$'s of the single modified versions suggesting that each heptad *d* position is independent of the other. In consonance with the high stability of H10H24-L6I,L13F, there is no observable loss in helicity between 5 and 95 °C in up to 3 M Gdn·HCl. These results indicate that single amino acid changes per helix can significantly change the cooperativity of the unfolding transition and presumably the uniqueness of the four helix bundle hydrophobic core.

These variants of the prototype H10H24 studied are well structured in solution as demonstrated by NMR spectroscopy. Each of the H10H24 variants demonstrates much better chemical shift dispersion and resolution than the prototype. Remarkably, the conservative substitution of only one residue per helix is sufficient to generate a unique four helix bundle structure. The ^{13}C-HSQC spectra for the peptides (Figure 2) provide direct evidence for existence of a non-native hydrophobic core for the prototype H10H24 and native-like cores of the variants.[30] The double variant possesses the most disperse and resolved ^{13}C-HSQC spectrum indicating a single, unique conformation in solution. These NMR data convincingly demonstrate the achievement of native-like structure in these proteins which is the direct result of an increase in side chain packing specificity.

These maquette scaffold structures are symmetrical. A C_2 symmetry axis is clearly demonstrated for H10H24-L6I,L13F (Figure 2; panel D) by observation of 32 methyl correlations in the ^{13}C-HSQC spectrum (64 expected). The considerable Hα resonance degeneracy from the largely α-helix secondary structure has impeded sequential proton resonance assignments. Moreover, the symmetry of the molecule makes interpretation of the NOEs ambiguous. To overcome these barriers, we are expressing H10H24-L6I,L13F in *E. coli* to economically provide uniformly ^{15}N and ^{13}C enriched protein that will enable assignment of all ^{1}H, ^{13}C, and ^{15}N resonances with a standard suite of triple resonance NMR experiments.

We have examined the heme binding properties of these novel peptides. Each of the peptides binds Fe(III)(protoporphyrin IX) (λ_{max} at 411 and 535 nm) with a K_D in the 10−20 nanomolar range for the first two hemes and a K_D in the 5−15 micromolar range for the second pair of hemes, values similar to those obtained for H10H24 and intermediate between myoglobin (80 pM)[31a] and cytochrome b_{562} (1.3 μM).[31b] The EPR spectrum of H10H24-L6I,L13F, is rhombic with *g*-values (2.91, 2.25, and 1.52) indicative of low spin ferric hemes.[32] Reduction by sodium dithionite results in a UV−vis spectra typical of bis-histidine ligated hemes with λ_{max} at 429, 529, and 560 nm. The heme binding constants and spectral properties throughout this maquette series suggests that the immediate heme environments are quite similar.

By replacing leucines in heptad *d* positions on each helix with either β-branched or aromatic amino acids we have demonstrated clear progress toward a maquette scaffold design having many features of native proteins. The well dispersed proton resonance frequencies, small line widths, and ^{1}H−^{13}C methyl carbon correlation information provide convincing evidence for native-like protein structure in the single and double variants of H10H24. The free energy and cooperativity values of protein unfolding are consistent with the generation of native-like proteins. Future efforts will focus on structural determination of the H10H24 variants presented here in order to establish the structural basis for their native-like properties and for further design to accommodate hemes and other biochemical cofactors.

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Supporting Information Available: Aromatic-amide proton region one-dimensional NMR spectra for H10H24, H10H24-L13F, H10H24-L6I, and H10H24-L6I,L13F; ^{1}H−^{1}H NOESY NMR spectra in the aromatic-amide proton region of the double variant H10H24-L6I,L13F; heme binding titration for H10H24-L6I,L13F; electron paramagnetic resonance spectrum of holo-H10H24-L6I,L13F (5 pages). See any current masthead page for ordering and Internet access instructions.

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