Communications to the Editor

De Novo Design of a Zn^{2+}-Binding Protein

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We recently described the design, expression, and preliminary characterization of a protein intended to adopt an antiparallel four-helix bundle consisting of four identical, 16-residue helices connected by three identical loops.[1] This work proceeded in three stages involving the characterization of (1) a tetrameric peptide (α_1),[2] (2) a dimeric helix–loop–helix peptide (α_2), and (3) a protein (α_4) with four helical sequences connected by three identical loops.[1] Recently, we have begun introducing metal-binding sites into α_4 to probe its conformational properties and to set the stage for the design of catalytically active proteins. In the present work we modified α_2 and α_4 to introduce a Zn^{2+}-binding site intended to approximate that of carbonic anhydrase, which has His residues at three corners of a tetrahedral binding site.

The known structure of hemocyanin[3] provided a clue as to how to design such a protein. This binuclear copper protein contains two antiparallel α-helical pairs with three His residues converging on a single Cu atom. Two of these His residues are separated by a single turn of a helix, while the third His originates from the neighboring helix. It appeared that an analogous arrangement of His residues might be capable of binding Zn^{2+} if the site were placed near the surface of α_2 or α_4. We substituted His for Leu_7 and Lys_{11} on one helix, and for Leu_7 on a second helix (Figure 1A), into a computer model[4] of α_2. The ϵ-N of these His residues could be positioned at three corners of a tetrahedron or octahedron by introducing energetically reasonable torsional angles into the side chain; solvent molecules serve as the remaining ligand or ligands.

This computer model led to the design of two Zn^{2+}-binding peptides, H3α_2 and H3α_4, which contain three His residues introduced into α_2 and α_4, respectively. H3α_2 was expected to homodimerize through a hydrophobic interaction to form a four-helix bundle with two Zn^{2+}-binding sites on opposite faces of the bundle, while H3α_4 was expected to form a monomolecular four-helix bundle with a single Zn^{2+}-binding site. As a control, we also synthesized H2α_2, which consists of α_2 with two His residues substituted at positions 7 and 11 within a single helix (Figure 1B). If the peptides containing three His residues coordinate Zn^{2+} by using all three His residues, the control peptide should bind Zn^{2+} with reduced affinity.

The three proteins assemble into compact, α-helical proteins similar to α_2 and α_4. H2α_2 and H3α_2 eluted from a size-exclusion column (Sephadex G50) calibrated with globular proteins[1,2] with apparent aggregation numbers of 2.4 and 2.0, respectively, in agreement with those expected for the dimers. H3α_4 eluted with an apparent aggregation number of 1.2, consistent with a monomer. Only minor changes in elution volume were observed when the eluting buffer contained 0.5 mM zinc acetate. Further, at 10.0 μM peptide concentration, the CD spectra of H3α_2 and H2α_2 were similar to the spectra of α_2, and the spectra of H3α_4 and α_4 were also similar. No significant spectral changes were observed upon addition of 1.0 mM $ZnCl_2$.

B
Helix 1 : G-E-L-E-E-L-L-K-K-L-K-E-L-L-K-G
Helix 2 : G-E-L-E-E-L-H-K-K-L-H-E-L-L-K-G
Helix 3 : G-E-L-E-E-L-H-K-K-L-L-E-L-L-K-G
Loop : P-R-R

H2α_2: Ac-Helix 1 - Loop - Helix 2 - $CONH_2$
H3α_2: Ac-Helix 3 - Loop - Helix 2 - $CONH_2$
H3α_4: Ac-Helix 1 - Loop - Helix 3 - Loop - Helix 2 - Loop - Helix 1- $CONH_2$

Figure 1. (a) Computer model of one subunit of the H3α_2 dimer, showing only the backbone atoms, His side chains, and the bound Zn^{2+}. (b) Sequences of H2α_2, H3α_2, and H3α_4. Peptides were synthesized by the solid-phase method with a Milligen 9050 peptide synthesizer, purified by reverse-phase HPLC, and judged to be better than 95% pure by analytical HPLC. The correct composition of the peptides was confirmed by amino acid analysis; fast atom bombardment mass spectrometry gave the appropriate $(M + H)^+$.

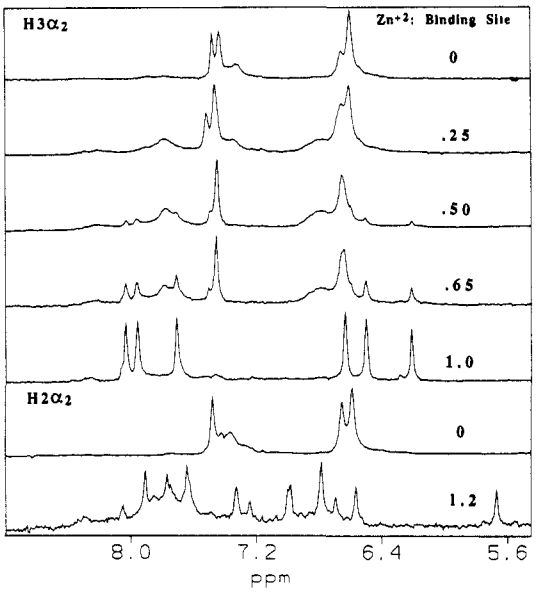

Figure 2. Downfield region of the ^1H NMR spectra of H3α_2 and H2α_2 as a function of Zn^{+2} concentration. Peptide (2.5 mM) in ^2H_2O (50 mM NaOAc-d_3, pH 7.5) was titrated with zinc acetate. Spectra were recorded on a 600-MHz spectrometer (Bruker AM-600) at 20 °C.

NMR spectroscopy indicates that H3α_2 binds Zn^{2+} in a 1:1 complex. The resonances associated with the δ and ϵ C–H protons of the three His residues of H3α_2 are broad and overlapped in the absence of Zn^{2+} (Figure 2), but separate into sharp, well-resolved peaks after addition of a single equivalent of Zn^{2+}. Titration of H3α_2 with Zn^{2+} shows that the binding is tight and stoichiometric at 2.5 mM protein concentration, as no further changes in the spectrum were observed when more than 1 equiv of Zn^{2+} was added. Also, the Zn^{2+}-saturated form of H3α_2 is in slow exchange with the free protein. Thus, H3α_2 appears to form a well-defined complex with Zn^{2+}, and the large change in chemical shifts suggests that all three His residues ligate the metal. The H3α_4 protein also formed a (1:1) complex with Zn^{2+}, with spectral properties similar to that formed by H3α_2 (data not shown). By contrast, the His resonances of H2α_2 showed large changes in chemical shift upon addition of Zn^{2+}, but far more resonances than imidazole ring protons were observed at saturating Zn^{2+} concentrations (Figure 2). This finding indicates that

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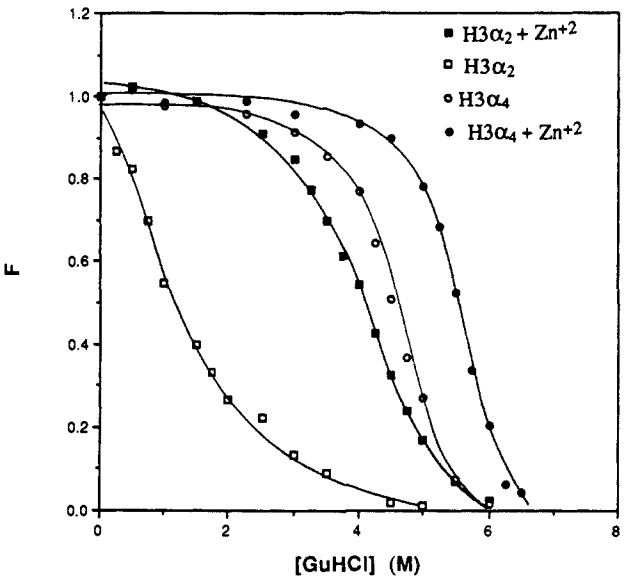

Figure 3. Fraction of initial ellipticity at 222 nm, (F), as a function guanidine hydrochloride concentration for 5.0 μM H3α_2 and H3α_4 in the presence and absence of 1.0 mM $ZnCl_2$.

Table I. Free Energy of Association of the Proteins in 3.0 M Guanidine Hydrochloride (5.0 μM in the Presence and Absence of 1.0 mM $ZnCl_2$)[a]

	midpoint (M)		ΔG_0 (kcal/mol)		
peptide	$-Zn^{+2}$	$+Zn^{+2}$	$-Zn^{+2}$	$+Zn^{+2}$	$\Delta\Delta G$
α_2	4.5	4.5	9.9	9.9	0.0
H2α_2	2.9	4.0	7.0	9.0	1.0
H3α_2	1.3	4.1	4.9	8.7	1.9
H3α_4	4.4	5.6	10.5	13.3	2.8

[a] ΔG_0 was calculated at various concentrations of guanidine hydrochloride as described previously[2] and the data for each peptide were extrapolated or interpolated to 3.0 M guanidine hydrochloride. This guanidine concentration was chosen to minimize errors associated with lengthy extrapolations. However, similar results were obtained when the data were extrapolated to 0 M guanidine hydrochloride. Midpoint refers to the concentration of guanidine·HCl at which the ellipticity at 222 nm was half that observed in its absence.

multiple, slowly exchanging complexes with similar stabilities are formed.

The conformational stability of all three peptides increased upon addition of Zn^{2+}. Figure 3 illustrates the variation in θ_{222} (a measure of helical content) of H3α_2 and H3α_4 as a function of guanidine hydrochloride concentration. In the absence of Zn^{2+}, the H3α_2 dimer is less stable than α_2 (Table I). This is consistent with our expectation that substitution of a polar His residue for Leu at position 7 of the helices (largely buried in models) would destabilize the folding of H3α_2. In the presence of 1.0 mM Zn^{2+} H3α_2 shows a large increase in stability, with a corresponding decrease in the free energy of dimerization of -1.9 kcal/(mol of bound Zn^{2+}) (Table I). In contrast, α_2 showed no change in stability in the presence of 1.0 mM Zn^{2+} (Table I), providing further evidence that the substituted His residues were essential for binding. In the absence of Zn^{2+}, the stability of H2α_2 was intermediate between H3α_2 and α_2, consistent with the fact that only one Leu was changed to His in H2α_2 (Table I). In the presence of Zn^{2+}, the protein showed increased stability, but the enhancement was about half that for H3α_2. Finally, H3α_4 unfolded at considerably higher guanidine concentrations (Figure 3) and showed an increase in stability of -2.8 kcal/mol in the presence of 1.0 mM Zn^{2+} (Table I).

These data establish the feasibility of designing metalloproteins and represent a significant step toward the de novo design of catalytically active proteins. As expected from previous work on monomeric helices bearing His residues separated by a single turn of α-helix,[6] H2α_2 bound Zn^{2+}, although the complex was not unique nor was the increase in stability as great as for H3α_2. Thus, it appears that all three His residues complex Zn^{2+} in H3α_2 and H3α_4. We are currently determining their specificity and affinity for other ions.

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Direct Evidence for an Equilibrium between Early Photolysis Intermediates of Rhodopsin

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Recently we showed that bathorhodopsin (Batho) produced by photolysis of rhodopsin does not decay directly to lumirhodopsin (Lumi), but rather forms a blue-shifted intermediate (BSI), which then decays to Lumi.[1] BSI was first observed in artificial visual pigments,[2,3] and time-resolved spectral studies later confirmed the existence of this photolysis intermediate in native rhodopsin as well.[1] The temperature dependence[1] and linear dichroism[4] of the time-resolved spectra were shown to be best fit by a mechanism involving an equilibrium between Batho and BSI (mechanism b in Figure 1[5]). In this communication we present more direct evidence of this equilibrium mechanism that excludes the simple sequential path (mechanism a in Figure 1).

Figure 1 shows the time evolution of the Batho, BSI, and Lumi species implied by a sequential decay mechanism, with equilibrium, following rhodopsin photolysis. While the kinetics of the Batho and BSI concentrations will depend somewhat on the equilibrium constant in mechanism b, spectral overlap of different intermediates makes detecting such subtle kinetic differences after excitation with a single laser pulse difficult. A more practical way to discriminate between mechanisms a and b is to remove part of the Batho, formed by an initial laser excitation of rhodopsin, with a second laser pulse that is absorbed only by Batho and to then monitor the subsequent kinetics at a wavelength only absorbed by Batho. Thus, also shown in Figure 1 are the Batho time evolutions, assuming either mechanism a or b, that would occur following photolysis of this intermeiate by a second light pulse coming 93 ns after the initial photolysis pulse. For mechanism a, photolysis of Batho would result in a reduction of Batho concentration followed by continued exponential decay. However, if an equilibrium exists between Batho and BSI, the kinetics of Batho decay would differ. Waiting for 93 ns after the initial rhodopsin excitation allows a significant amount of BSI to form. If an equilibrium mechanism is valid, this BSI will back-react to form more Batho after the second photolysis pulse. Thus, after Batho photolysis there will be an initial rise in Batho concentration followed by an exponential decay. The difference in kinetics following double excitation provides a means to discriminate between these two mechanisms.

In the experiment reported here excitation of rhodopsin by a 0.5-mJ, 7-ns pulse of 532-nm light formed Batho, which was detected by using absorbance measured at 620 nm.[6] After 93

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(5) An alternative mechanism

$$\text{Batho} \rightarrow \text{Lumi}$$
$$\downarrow\uparrow$$
$$\text{BSI}$$

had been shown to be inconsistent with the time-dependent spectral data.[1]

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