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Metal-Assisted Stabilization and Probing of Collagenous Triple Helices

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Collagen, the most abundant protein in mammalian tissues, has a unique tertiary structure. Collagen stalks consist of right-handed supercoils of three left-handed polyproline II-type helices with sequences of $(Gly\text{-}X\text{-}Y)_n$.[1] In studies on the structure, folding, and biological activities of collagen, synthetic peptides that mimic the native triple-helical structure have been utilized.[2] To stabilize the triple-helical conformation of relatively short collagenous peptides, several strategies to knot three strands by covalent linkage have been developed.[3] Although these approaches were successful in gaining thermal stability of the helix, the synthetic procedures are generally cumbersome, and final purification of large branched molecules is required. We describe here an alternative strategy to assemble three collagenous peptides in a postsynthetic manner by the coordination of the Fe(II) ion to built-in bipyridine (bpy) ligands (Figure 1a). Bpy is a well-characterized metal acceptor which is utilized for various supramolecular assemblies including α-helical coiled coil peptides.[4]

Bpy-containing collagen model peptides listed in Figure 1b were synthesized by the conventional solid-phase technique. The bpy groups were successfully introduced to N-termini of the peptides by usual carbodiimide coupling.

Trimer formation of **bpy-P23** by the coordination of the Fe(II) to the bpy ligands was initially tested. Adding the Fe(II) to the peptide solution,[5] the color turned magenta and showed new absorption maxima at 378 and 545 nm. The stoichiometry of the metal−peptide complex was determined by UV−vis titration. The absorbance at 545 nm reached a plateau at the molar ratio of Fe(II):**bpy-P23** = 1:3. Other bpy-containing peptides, **bpy-G24** and **bpy-P5** also showed similar results. These results indicated that the $Fe^{II}(bpy\text{-}peptide)_3$ complex formed by simply adding the Fe(II) solution to the bpy-peptides.

The conformational status of the peptides was next analyzed using circular dichroism (CD) spectrometry. The solutions of **bpy-P23** and **Ac-P23** were allowed to fold at 4 °C for 2 days. These peptides showed positive bands around 225 nm, a characteristic feature of the collagen triple helix in the presence and absence of Fe(II) (Figure 2a). To investigate the effect of the $Fe^{II}(bpy)_3$ complex formation on the stability of the triple helix, the melting temperature (T_m) of the triple helix in Fe^{II}(**bpy-P23**)$_3$ was subsequently estimated and compared with those of metal-free **bpy-P23** and **Ac-P23** (Figure 2b). The melting curves of metal-free **bpy-P23** and **Ac-P23** were very similar with an identical T_m value of 33 °C. In the presence of Fe(II), the T_m value was estimated to be 46 °C. Thus, knotting three collagenous peptide strands by the metal chelation was shown to increase the thermal stability of the triple-helical conformation.

In this experiment, four stereoisomers of $Fe^{II}(bpy)_3$ complex, *fac*-Λ, *fac*-Δ, *mer*-Λ, and *mer*-Δ could be formed. In Figure 2a, the Fe(II)-complexed form of **bpy-P23** showed positive and negative bands around 295 and 315 nm, respectively, which were negligible in the absence of the Fe(II) ion, indicating predominant formation of the Δ-isomers at the metal center.[6] To clarify the origin of this diastereoselectivity, we examined the relationship between the chirality of the metal center and the conformation of the peptide moiety. As expected, a shorter counterpart, Fe^{II}(**bpy-P5**)$_3$ did not form a triple helix, showing no significant positive band around 225 nm (Figure 3a). In the same measurement, the metal center was racemic, and there was almost no signal observed around 315 nm (Figure 3b). In comparison with CD profiles of Fe^{II}(**bpy-P23**)$_3$, we concluded that the diastereoselective formation of the Δ-isomers was not due to the chirality of the N-terminal Pro residue but due to the supercoiling of the triple helix.

We also examined the effect of the flexibility of the linkage site between the bpy group and the peptide moiety on the diastereoselectivity by comparing CD profiles of **bpy-G24** to that of **bpy-P23** in the presence of Fe(II). Both peptides showed similar positive bands around 225 nm of the triple helices (Figure 3a). However, the molar ellipticity of the negative band (315 nm) at the metal

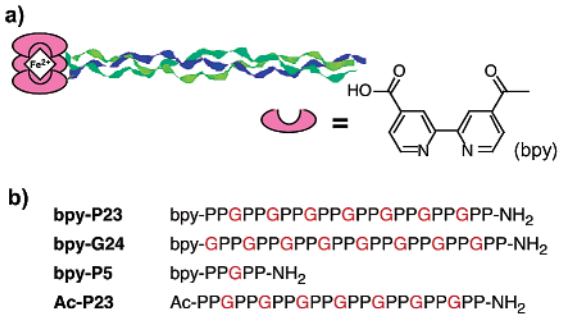

Figure 1. (a) Schematic representation of the triple-helical collagenous peptide assembled by N-terminal $Fe^{II}(bpy)_3$ complex. (b) Structures of synthetic peptides.

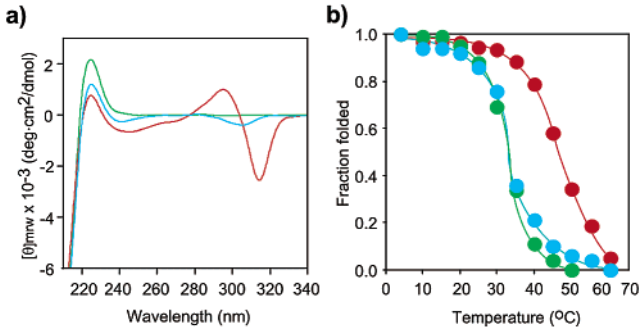

Figure 2. (a) CD profile of **Ac-P23** (green) and **bpy-P23** in the presence (red) and absence of Fe(II) (blue) (b) Triple helix → random coil transition curves of **Ac-P23** (green) and **bpy-P23** in the presence (red) and absence of Fe(II) (blue) determined by CD at 225 nm.

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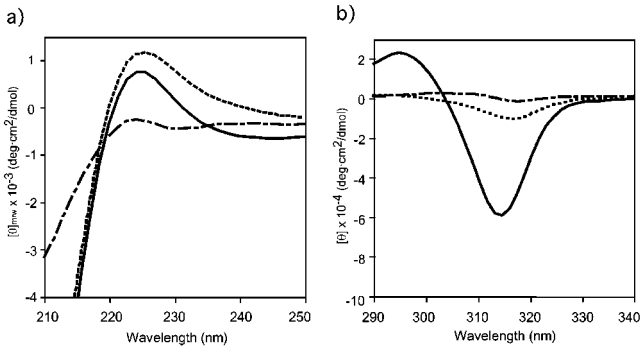

Figure 3. Supercoil-induced diastereoselective metal center formation. (a) CD spectra of peptide moieties and (b) $Fe^{II}(bpy)_3$-moieties of **bpy-P23** (solid), **bpy-P5** (dotted-broken) and **bpy-G24** (dotted) in the presence of Fe(II). The ordinates are plotted by mean residue ellipticity $[\Theta]_{mrw}$ of the peptides and molar ellipticity $[\Theta]$ of the $Fe^{II}(bpy)_3$ complex, respectively.

center of Fe^{II}(**bpy-G24**)$_3$ was approximately 7 times smaller than that of Fe^{II}(**bpy-P23**)$_3$ (Figure 3b). This result suggests that introduction of flexible Gly residue at the linkage site decreased the efficiency of the supercoil-induced diastereoselective metal center formation. Similar conformation-dependent induction of the $M(bpy)_3$ center was previously demonstrated using a bpy-containing α-helical trimer system.[4d,6b] In those cases, left-handed Λ-isomers predominantly formed, probably induced by the left-handed-coiled coils. This is in good agreement with our finding that the right-handed collagen supercoil induces an opposite twist at the metal center. The folding status of collagenous peptides is generally investigated by estimating the CD signal around 225 nm. Hence, it is difficult to know the conformation of collagen model peptides in the presence of other proteins such as collagen-specific molecular chaperones.[7] Our system described here leads to the potential use of CD signal of $M(bpy)_3$ at the far UV region as a probe to detect the conformational status of collagenous peptides.

We further examined the effect of metal-complex formation of the refolding kinetics of **bpy-P23**. The refolding kinetics was monitored at time intervals using ellipticity gain at 225 nm after rapid temperature jump from 80 to 4 °C. The refolding rate of metal-free **bpy-P23** is as slow as that of **Ac-P23** (see Figure 4). The rate was enhanced in the presence of Fe(II), especially in the initial fast phase. We suggest that the rapidly formed $Fe^{II}(bpy)_3$ complex act as a nucleation site, similarly to the covalent linkages in other trimeric collagen model systems[8] and in C-propeptides of native procollagen.[9]

In conclusion, the metal-assisted facile assembly of collagenous peptides described here is useful for stabilizing and probing the collagenous triple helix as well as to enhance triple helix formation. This system opens new opportunities in the use of collagenous peptides for studies on collagen folding, structure, and biochemistry. The metal-assisted assembly of such peptides may also be useful for a pivot for supramolecular architecture based on collagenous triple helix.

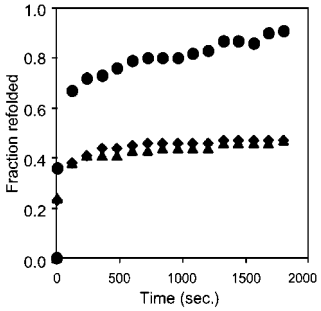

Figure 4. Refolding of triple helix of **Ac-P23** (▲) and **bpy-P23** in the presence (●) and absence (◆) of Fe(II) was monitored by CD at 225 nm.

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Supporting Information Available: Details for the synthesis of peptides, UV−vis spectra, CD measurements, thermal stability of the $Fe^{II}(bpy)_3$ complex, and a modeled structure of $Fe^{II}(bpy\text{-}P23)_3$ (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(5) For all of the spectrometric analysis, we used peptide solutions in 25 mM sodium acetate buffer (pH 5.2) and freshly prepared $Fe(NH_4)_2(SO_4)_2$ solution in the same buffer.

(6) The preferred chirality of $Fe^{II}(bpy)_3$ complex was assigned to be Δ-configration based on the CD profile of each isomer of the similar Fe^{II}-(bpy-peptide)$_3$ complex reported in refs 4d and 6b. (b) Case, M. A.; Ghadiri, M. R.; Mutz, M. W.; McLendon, G. L. *Chirality* **1998**, *10*, 35−40.

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