Metal-Dependent Intramolecular Chiral Induction: The Zn^{2+} Complex of an Ethidium−Peptide Conjugate

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Introduction

Ethidium bromide is an aromatic organic dye that for many years has been known to bind via intercalation to the minor groove of DNA.[1] When the ethidium cation binds to DNA, the chirality of the right-handed double helix is imposed upon ethidium (Et), producing induced circular dichroism (ICD).[2] Other examples of ICD have been observed in the heme group of hemoglobin and myoglobin[3] as well as synthetic porphyrin assemblies,[4] and in host−guest assemblies.[5] In every case, an ICD spectrum is produced as a result of the close association of an achiral chromophore with a chiral moiety (e.g., a biopolymer). During the course of our ongoing research into artificial hydrolytic nucleases,[6] we prepared an ethidium−peptide conjugate that displays metal-dependent ICD. Here we report the synthesis and spectroscopic characterization of a metal−peptide assembly which may represent a first example of metal-dependent intramolecular chiral induction.

Experimental Section

Materials. Ethidium′ (*N*-8-glycyl ethidium; Et′) was prepared as reported previously.[7] The peptide was synthesized using standard solid phase methods employing Fmoc chemistry, as previously reported.[6] Buffer solutions were prepared with distilled, deionized water and passed through Chelex (BioRad) to remove any metal ions. Solutions of $ZnCl_2$ were prepared by dissolving zinc metal in HCl and diluting to volume with water.

Instrumentation. HPLC purification was performed on a Hewlett-Packard 1050 HPLC system equipped with a Vydac semipreparative C_{18} column. UV/visible spectra were recorded at 20 °C unless otherwise noted on a Hewlett-Packard 8452A diode array spectrophotometer using a 1 cm path length cell. CD spectra were recorded at 20 °C unless otherwise noted on either a Jasco J-500 or an AVIV CD spectropolarimeter using either a 0.2 or 1 cm path length cell. Fluorescence polarization measurements were made at 20 °C with excitation at 480 nm on an ISS K2 multifrequency phase fluorimeter. Amino acid analysis of Et′-Pep was carried out using a Beckman System 6200 with a 12 cm sodium column for the analysis of hydrolyzed peptide amino acids. Electrospray ionization mass spectrometry (ESI MS) was performed on a Finnegan LCQ mass spectrometer. The concentrations of solutions of ethidium−peptide conjugate were determined by UV−visible spectroscopy using $\epsilon_{292} = 50\,000\ M^{-1}\ cm^{-1}$.

Et′-Pep: [Ethidium′-DPDELEHAAKHEAAAK-$CONH_2$]$^+$. Et′-Pep was synthesized by a solid phase coupling procedure previously reported for the synthesis of metallointercalator−peptide conjugates.[6,8] The ethidium−peptide conjugate was simultaneously deprotected and cleaved from the resin by treatment with TFA and isolated as previously described.[6] The crude Et′-Pep was purified by reversed-phase HPLC using a semipreparative C_{18} column (VYDAC) affording Et′-Pep as an orange powder in 3−5% overall yield. Amino acid analysis observed (calculated): Asp 1.969 (2), Glu 3.097 (3), His 1.936 (2), Ala 4.899 (5), Pro 1.051 (1), Leu 1.036 (1), Lys 1.983 (2). ESI MS (I): $(M + H)^{2+}$ *m/z* 1071.3 (calculated 1071.0); $(M + 2H)^{3+}$ *m/z* 714.6 (calculated 714.3); $(M + 3H)^{4+}$ *m/z* 536.2 (calculated 536.0). UV/visible (H_2O) [λ_{max}, nm (ϵ, $M^{-1}\ cm^{-1}$)]: 292 (50 000), 460 (5600). HPLC $t_r = 18.0$ min.

Et′-Pep·Zn.[9] Stoichiometric amounts of aqueous solutions of $ZnCl_2$ (pH 7, typically <50 μL of 1 mM $ZnCl_2$) were added to aqueous solutions of Et′-Pep (typically 0.4−0.6 mL of 0.050 mM Et′-Pep). Both solutions were carefully buffered to pH 7.2 with either 10 mM PIPES [*N*,*N*′-bis(ethanesulfonate)piperazine] or 25 mM tris·HCl. Titrations at 10 °C indicate that Et′-Pep·Zn is formed with the addition of 1 equiv of Zn^{2+} ions. UV/visible (H_2O) [λ_{max}, nm (ϵ, $M^{-1}\ cm^{-1}$)]: 292 (48 000), 490 (5600).

Results and Discussion

The synthesis of the ethidium−peptide conjugate, Et′-Pep, was carried out using procedures established previously in our laboratory for the conjugation of peptides to rhodium complexes.[6,8] The peptide is 16 amino acids long, with two histidines at positions *i* and *i* + 4 along the peptide in order to place them on the same side of an α-helix and allow coordination of both side chain imidazole groups to a metal ion.[6,10] While still attached to the resin on which the peptide was synthesized, the side-chain-protected peptide was coupled to *N*-8-glycyl ethidium (Et′) via peptide coupling of the glycyl carboxylate of Et′ and the amine terminus of the peptide (Scheme 1).

Aqueous solutions of Et′-Pep display UV/visible spectra that are nearly identical to the sum of the unconjugated Et′ and the peptide. The high-energy UV transition at around 200 nm is associated with the peptide, whereas the UV peak and shoulder at 292 and 310 nm, respectively, and the visible transition at 462 nm are from the Et′ moiety. The spectra of Et′ and Et′-Pep differ from that of unmodified Et, with the Et′ $\pi-\pi^*$ transition red-shifted slightly and the $n-\pi^*$ transition blue-shifted ∼20 nm upon functionalization of the exocyclic amine.[11] The CD spectrum of Et′-Pep is dominated by a negative signal in the UV centered around 222 nm that is associated with the peptide amides. The percent α-helicity for Et′-Pep in the absence of zinc ions is 22%, which is typical for our family of oligopeptides.[6,12] The α-helicity of the peptide without ethidium is 15%, and for the peptide in the presence of Zn^{2+} there is 28% helicity.

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(9) ^1H NMR experiments were not possible owing to precipitation of the Et′-Pep·Zn complex at the concentrations required for NMR.
(10) Proline cis/trans isomerization either does not occur or does not appear to influence the behavior of the peptide. (a) Grathwohl, C.; Wüthrich, K. *Biopolymers* **1976**, *15*, 2025−2041. (b) Grathwohl, C.; Wüthrich, K. *Biopolymers* **1981**, *20*, 2625−2633.
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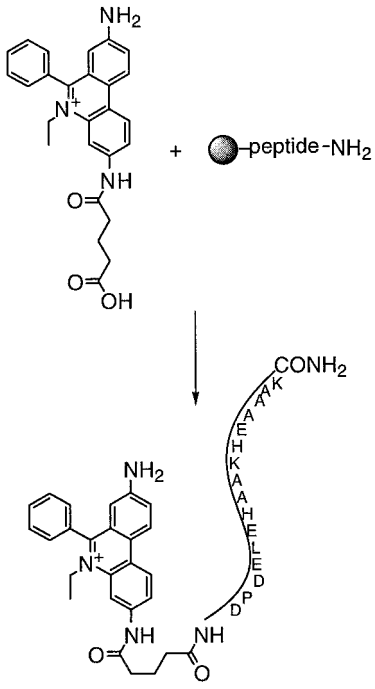
Scheme 1



Upon addition of zinc ions to aqueous solutions of Et′-Pep, a color change from orange to pink was observed. In contrast, no spectroscopic changes are evident for Et′ (lacking the peptide) with high concentrations of Zn^{2+}. Thus in the case of Et′-Pep, Zn^{2+} coordination directly perturbs the ethidium chromophore; this observation is consistent with the notion that, in the Et′-Pep·Zn complex, Zn^{2+} may now associate also with the Et′ moiety. The spectroscopic changes for Et′-Pep with Zn^{2+} were most marked at low temperature. At 10 °C and pH 7, a red shift in the low-energy absorbance is evident from 460 to 490 nm, accompanied by a change in the intensity of the 292 nm absorbance (Figure 1). Two isosbestic points are evident at 340 and 480 nm, supporting a clean equilibrium between Et′-Pep and Et′-Pep·Zn. Buffered solutions of Et′-Pep were titrated with buffered solutions of $ZnCl_2$ or $ZnSO_4$ at various temperatures in the range 10−40 °C. The change in absorbance at 510 nm was measured as a function of Zn^{2+} ions added. The binding isotherm for the data at 10 °C reaches a maximum with 1 equiv of zinc ions added, pointing toward a 1:1 stoichiometry (Figure S1, Supporting Information). Scatchard plots of the data at 10, 20, 30, and 40 °C reveal association constants for the equilibrium in eq 1. The association constant, K, at 10 °C and pH 7 is 1.2 ×

$$[\text{Et}'\text{-Pep}]^{n+} + Zn^{2+} \overset{K}{\rightleftharpoons} [\text{Et}'\text{-Pep}\cdot Zn]^{(n+2)+} \qquad (1)$$

10^5 M^{-1}, while for comparison association constants for zinc ions and histidine are typically ∼10^7 M^{-1} for Zn^{2+} + His and 10^{12} M^{-1} for Zn^{2+} + 2His.[13] This lowered affinity is understandable on the basis of values for $\Delta H°$ and $\Delta S°$ extracted by measuring K as a function of temperature (see Figure 1 inset). We find $\Delta H° = -52$ kJ mol^{-1} and $\Delta S° = -84$ J K^{-1} mol^{-1}. Note the significant negative entropy associated with this reaction.

Addition of zinc ions to Et′-Pep significantly increases the amount of α-helicity in the peptide, increasing to 54% at 20 °C (Figure 2).[14] This effect is clearly synergistic, since the peptide

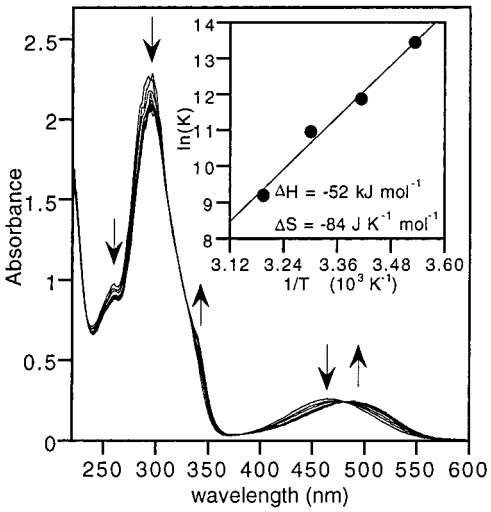


Figure 1. Titration of an aqueous solution of Et′-Pep with an aqueous solution of $ZnCl_2$, monitored by UV/visible spectroscopy. Both Et′-Pep and $ZnCl_2$ solutions were buffered with 10 mM PIPES, pH 7.0, [Et′-Pep] = 34 μM, [Zn^{2+}] = 1.0 mM, added in 5−10 μL aliquots. Path length = 1 cm; T = 10 °C. The absorbance was corrected for change in volume.

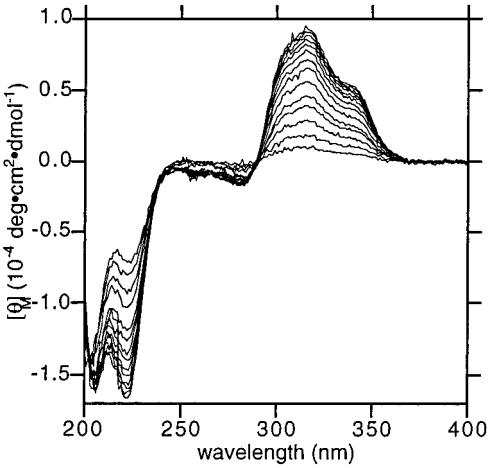


Figure 2. Titration of an aqueous solution of Et′-Pep with an aqueous solution of $ZnCl_2$ monitored by CD spectropolarimetry. Both Et′-Pep and $ZnCl_2$ solutions were buffered with 25 mM tris·HCl, pH 7.1 [Et′-Pep] = 67 μM, [Zn^{2+}] = 0.5 mM, added in 6 μL aliquots. Path length = 0.2 cm; T = 20 °C. The molar ellipticity was corrected for change in volume.

alone achieves α-helicity levels of only 25−30% in the presence of zinc. The sharp temperature dependence observed in the UV/visible titration was likewise seen in the CD experiment, with 77% α-helicity at 5 °C.[15] More dramatically, a large positive ICD signal centered at 315 nm with a shoulder at ∼340 nm is associated with the binding of zinc ions. Surprisingly, the n−π* visible transition at 490 nm is nearly absent in the CD spectrum (data not shown). The ICD signal is zinc ion dependent; it is also not observed under conditions of significant α-helicity in the absence of Zn^{2+}.[16]

(12) For 100% helix, $[\theta]_{222} = -31\,500$ deg cm^2 dmol^{-1}. Lehrman, S. R.; Tuls, J. L.; Lund, M. *Biochemistry* **1990**, *29*, 5590−5596.

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(15) The percent α-helicity of peptides has been shown to be highly temperature dependent, with the most α-helicity occurring at low temperature. See ref 12.

(16) The CD spectrum of a zinc-free solution of Et′-Pep containing 20% v/v TFE at 1 °C has no ICD signal at 315 nm, while displaying a CD signal at 222 nm corresponding to 51% α-helicity.

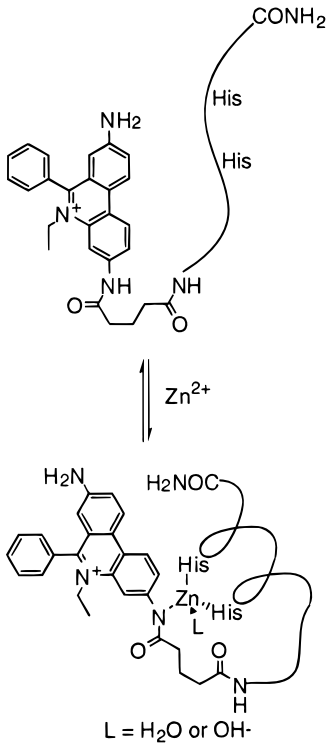
The formation of the Et′-Pep·Zn complex is not only temperature dependent but also highly pH dependent. The pH dependence of the equilibrium in eq 1 was probed by CD, UV/visible spectroscopy, and potentiometric titration. There is a steep pH dependence of the visible chromophore at 490 nm, as well as the ICD signal in the CD. A clear maximum is evident at around pH 7.2, with a drop-off of signal at both low and high pH (Figure S2, Supporting Information). The disappearance of both CD signals at low pH corresponds nicely to the pK_a of histidine (ca. 6.1), suggesting that the protonation of histidine side chains results in the dissociation of the Et′-Pep·Zn complex with the concomitant loss of α-helicity and ICD.[17] Further evidence in support of this hypothesis was provided by the potentiomentric titration of Et′-Pep both in the presence and in the absence of zinc ions. The pK_a for the two histidines in Et′-Pep under all conditions is approximately 6.0, and in the presence of zinc ions, a total of three protons have pK_a values in the pH range of 6.0−7.5.

The binding of Zn^{2+} by Et′-Pep can further be examined by observing the fluorescence polarization. Aqueous solutions of Et′-Pep were excited at 480 nm, which corresponds to the isosbestic point in the UV/visible titration. A decrease in fluorescence polarization was observed when zinc ions were added to a solution of Et′-Pep. This behavior is consistent with a faster rate of tumbling and a smaller average spherical radius in solution upon binding of the zinc ions.

The experimental evidence here indicates that Et′-Pep undergoes a significant structural change upon the binding of zinc ions. The large negative entropy associated with the folding of the bulky Et′-Pep molecule to coordinate a zinc ion reflects this change and is responsible for the low apparent binding constant, as well as the significant temperature dependence. Because of the perturbation in the visible transition of the ethidium chromophore upon the binding of Zn^{2+}, it follows that the zinc ion is coordinated via one of the exocyclic nitrogen atoms. Amide nitrogen atoms are well-known to coordinate zinc ions, and in fact Zn^{2+} is known to induce deprotonation of coordinating amides.[18] On the basis of the changes in the visible and CD spectra we believe that Et′-Pep forms a tridentate chelate which coordinates to zinc through two side-chain histidine nitrogens of the peptide, and the exocyclic amide nitrogen of ethidium (Scheme 2). We indicate an additional coordination site as water or hydroxyl, but the data for the complex at pH 7.0 do not allow us to establish the number of coordinated solvent molecules or ions.

The chiral α-helical peptide now closely associated as a result of Zn^{2+} coordination imposes the ICD on the achiral Et′ moiety. The fact that under conditions where α-helicity is >50% in a zinc-free solution there is no ICD signal rules out a simple helix-dependent mechanism of chiral induction. Under these conditions, the chiral helix is not in proximity of the ethidium, and

Scheme 2



hence there is no ICD signal. It is also unlikely at the concentrations studied (30−60 μM) that intermolecular interactions are occurring. Instead, when zinc ions are present, Et′-Pep folds into the tertiary conformation necessary to allow coordination of the His_2N(amide) ligand set to the metal ion. The Zn^{2+} ion serves a structural role, defining the folding and three-dimensional structure of the Et′-Pep molecule in much the same manner as for Zn^{2+} ions in the zinc-finger regulatory proteins.[19] Indeed, given the synergy in α-helix formation with Zn^{2+} coordination and Et conjugation, perhaps the hydrophobic ethidium moiety presents a close surface against the α-helix not unlike the β-sheet structure of the zinc finger. Certainly zinc coordination promotes the synergistic α-helix formation and conjugate folding. The chelate once formed forces the helical peptide into close proximity of Et′ and produces the ICD signal.[20]

The coordination of zinc ions to Et′-Pep therefore induces significant changes in the secondary and tertiary structure of the peptide, while simultaneously inducing chirality onto the ethidium chromophore. The extreme sensitivity of this system to changes in temperature and pH evinces the subtle effects of the equilibria involved in this metal-promoted assembly.

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Supporting Information Available: The binding isotherm for Et′-Pep + Zn^{2+} and the plot of CD signal vs pH for Et′-Pep·Zn. This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) The behavior at high pH is more complex, since the chromophore associated with the helix does not track with the disappearance of the ICD signal (see Figure S2, Supporting Information). One possible explanation of this phenomenon is that at high pH the equilibrium in eq 1 is in competition with a hydroxylated form of the complex, Et′-Pep·Zn$(OH)_n$, where some α-helicity is retained through peptide coordination to the zinc ion, but the chelate is opened, thus losing the ICD.

(18) Examples of non-peptide amides with Zn^{2+}-induced deprotonation: (a) Kimura, E.; Koike, T.; Shiota, T.; Iitaka, Y. *Inorg. Chem.* **1990**, *29*, 4621−4629. (b) Battistuzzi-Gavioli, G.; Borsari, M.; Saladini, M.; Sola, M. *Inorg. Chem.* **1991**, *30*, 498−502. Examples of peptide amides with Zn^{2+}-induced deprotonation: (c) Daignault, S. A.; Arnold, A. P.; Isab, A. A.; Rabenstein, D. L. *Inorg. Chem.* **1985**, *24*, 3984−3988. (d) Rabenstein, D. L.; Daignault, S. A.; Isab, A. A.; Arnold, A. P.; Shoukry, M. M. *J. Am. Chem. Soc.* **1985**, *107*, 6435−6439. (e) Arnold, A. P.; Stanley, D. M.; Collins, J. G. *FEBS Lett.* **1991**, *289*, 96−98.

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(20) The Et′-Pep·Zn complex does not appear to cleave plasmid DNA under conditions optimized to test for hydrolysis.[6] We believe that the closed chelate structure of Et′-Pep·Zn prevents it from binding to DNA due to steric hindrance.