N-acyl-L-amino acid), we conclude that the α-hydroxyglycine moiety produced by PAM and reacted upon by PGL is of the *S* configuration.[17,19] It is therefore also evident that PAM-catalyzed oxygenation, in which the *pro-S* glycine hydrogen is stereospecifically abstracted,[10] proceeds with retention of configuration.

Elucidation of the stereochemical course of enzymatic amidation suggests new possibilities for the design of specific inhibitors and pseudosubstrates for PAM and PGL, a goal which is being actively pursued in this and other laboratories.

Acknowledgment. The financial support of the National Institutes of Health (GM 40540) is gratefully acknowledged.

(17) While Young and Tamburini[18] reported that only one diastereomer of a synthetic α-hydroxyglycine-peptide is converted to amide by thyroid carcinoma amidating enzyme, their work did not determine either the stereochemical correlation between the monooxygenase and lyase steps or the configuration of the α-hydroxyglycine species.

(18) Young, S. D.; Tamburini, P. P. *J. Am. Chem. Soc.* **1989**, *111*, 1933-1934.

(19) An analogous series of experiments was carried out with PGL and carboxypeptidase Y (CPY) using *N*-Ac-Phe-α-hydroxy-Gly, which is hydrolyzed slowly to *N*-Ac-Phe by CPY and readily N-dealkylated to *N*-Ac-Phe-NH_2 by PGL. We find that PGL reacts only with the same diastereomer of *N*-Ac-Phe-α-hydroxy-Gly which shows preferential reactivity toward CPY. Since CPY-catalyzed hydrolysis is highly *S*-enantioselective (see, for example: Bai, Y.; Hayashi, R.; Hata, T. *J. Biochem.* **1975**, *77*, 81-88), these results confirm those obtained with acylase.

Design of an Artificial Four-Helix Bundle Metalloprotein via a Novel Ruthenium(II)-Assisted Self-Assembly Process

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Design of a topologically predetermined macromolecular scaffold is the obligatory first step toward the de novo design of synthetic receptors and enzymes. Despite a few promising reports,[1] the biomimetic approach toward the design of artificial proteins continues to be a formidable task due to the incomplete understanding of the complex factors controlling peptide and protein folding.[2] Alternatively, an abiotic approach, which is not limited to the use of natural amino acids or long linear polypeptide chains, might permit more freedom in exploring new concepts in protein design and engineering.[3] Recently, the *metal ion-assisted self-organizing molecular process* has found considerable utility in the construction of supramolecular structures[4] and is rapidly emerging as a promising tool for the design of topologically predetermined peptides and proteins.[5] Here we describe one such process for the construction of an artificial metalloprotein. A 15-residue amphiphilic polypeptide, equipped at the N-terminus with a pyridyl functionality, was designed and shown to undergo intermolecular self-assembly, upon Ru(II) complexation, to form a remarkably stable 60-residue parallel four-helix bundle metalloprotein (Figure 1).

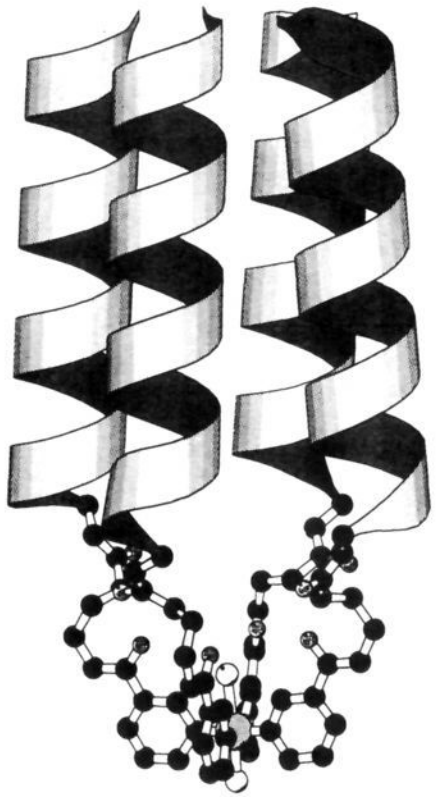

Figure 1. Computer-generated model of the parallel four-helix bundle metalloprotein. The polypeptide sequence is shown on the bottom.

The metal ion-assisted self-assembly process described in this study is a highly convergent approach in which a predefined number of small peptide subunits are forced, through metal ion complexation, to interact and form a large and topologically predetermined water-soluble protein-like structure. Intersubunit hydrophobic interactions supply the major driving force, while the chemoselective metal ion complexation to the pyridyl ligands protruding from each structural subunit provides the key element for controlling the overall topology and the number of subunits participating in the assembly process.[6,7] Intermolecular assembly of the peptide into the desired metalloprotein hinged on the ability to comply with the following criteria: the metal complex must be compatible with the four-stranded topology of the target structure, exclude or disfavor formation of undesirable ensembles,

† Searle Scholar, 1991-1994; Beckman Young Investigator, 1991-1993.

(1) (a) DeGrado, W. F.; Wasserman, Z. R.; Lear, J. D. *Science* **1989**, *243*, 622. (b) Hecht, M. H.; Richardson, J. S.; Richardson, D. C.; Ogden, R. C. *Science* **1990**, *249*, 884. (c) Kaumaya, P. T. P.; Berndt, K. D.; Heidorn, D. B.; Trewhella, J.; Kedzdy, F. J.; Goldberg, E. *Biochemistry* **1990**, *29*, 13. (d) Handel, T.; DeGrado, W. F. *J. Am. Chem. Soc.* **1990**, *112*, 6710. (e) Regan, L.; Clarke, N. D. *Biochemistry* **1990**, *29*, 10878. (f) Oas, T. G.; Kim, P. S. *Nature* **1988**, *336*, 42.

(2) (a) Richardson, J. S. *Adv. Protein Chem.* **1981**, *34*, 167. (b) *Prediction of Protein Structure and the Principles of Protein Conformation*; Fasman, G. D., Ed.; Plenum Press: New York, 1989. (c) Chothia, C. *Annu. Rev. Biochem.* **1984**, *53*, 537. (d) Baldwin, R. L. *TIBS* **1989**, *14*, 291. (e) Kim, P. S.; Baldwin, R. L. *Annu. Rev. Biochem.* **1982**, *51*, 459.

(3) (a) Bo, S. P.; DeGrado, W. F. *J. Am. Chem. Soc.* **1987**, *109*, 6751. (b) Mutter, M.; Vuilleumier, S. *Angew. Chem., Int. Ed. Engl.* **1989**, *5*, 535. (c) Hill, C. P.; Anderson, D. H.; Wesson, L.; DeGrado, W. F.; Eisenberg, D. *Science* **1990**, *249*, 543. (d) Karle, I. L.; Flippen-Anderson, J. L.; Uma, K.; Sukumar, M.; Balaram, P. *J. Am. Chem. Soc.* **1990**, *112*, 9350. (e) Sasaki, T.; Kaiser, E. T. *J. Am. Chem. Soc.* **1989**, *111*, 380. (f) Hahn, K. W.; Wieslaw, A. K.; Stewart, J. M. *Science* **1990**, *248*, 1544.

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(5) (a) Ghadiri, M. R.; Soares, C.; Choi, C. *J. Am. Chem. Soc.* **1992**, *114*, 825. (b) Ghadiri, M. R.; Choi, C. *J. Am. Chem. Soc.* **1990**, *112*, 1630. (c) Ghadiri, M. R.; Fernholz, K. A. *J. Am. Chem. Soc.* **1990**, *112*, 9633. (d) Ruan, F. Q.; Chen, Y. Q.; Hopkins, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 9403. (e) Liberman, M.; Sasaki, T. *J. Am. Chem. Soc.* **1991**, *113*, 1470.

(6) For a more detailed discussion of our design principles and the underlying rationale in constructing artificial metalloproteins by a metal ion-assisted self-assembly process, see ref 5a.

(7) The peptide was synthesized by standard Merrifield solid-phase methods and characterized by amino acid analysis and mass spectrometry. The details of the molecular modeling used in designing the polypeptide sequence will be described elsewhere.

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and exhibit high kinetic and thermodynamic stabilities. In addition, since the peptide is endowed with a number of reactive side-chain functionalities, the exchange-inert metal complex[8] forming reaction must be exquisitely chemoselective. The well-known affinity of ruthenium(II) for the nitrogen-containing aromatic heterocycles,[9] as well as our recent success in using the $Ru_5Cl_{12}^{2-}$ cluster[10] for the chemoselective functionalization of bipyridyl peptides,[5a] prompted us to examine the feasibility of forming the hitherto unknown exchange-inert *trans*-[Ru(pyridyl peptide)$_4$Cl$_2$] complex. We have found that the desired complex can be formed readily in high yield simply by incubating an appropriate amount of the peptide with a freshly prepared solution of the $Ru_5Cl_{12}^{2-}$ cluster.[11]

Chemoselective functionalization of the pyridyl moieties is supported by absorption spectroscopy. The ruthenium–peptide complex displays an absorption spectrum similar to that of the *trans*-[Ru(py)$_4$Cl$_2$] complex with characteristic absorption bands at 256 and 377 nm. Molecular weight estimation using size exclusion chromatography, atomic absorption spectroscopy, and electrospray mass spectrometry was also used to unequivocally establish the formation of a tetrameric peptide–ruthenium complex.[12,13] Furthermore, the CD spectrum of the complex (6.2×10^{-6} M in 10 mM sodium borate, pH 7.6 at 21 °C) is indicative of a highly α-helical structure[14] (>90% α-helicity, $[\theta]_{222} = -29\,000$ deg·cm^2·dmol^{-1}). In an amphiphilic peptide, α-helical secondary structure is induced mainly through interhelical hydrophobic interactions.[15] Therefore, considering that the formation of a tetrapyridyl metal complex necessarily draws the N-termini of four peptides close in space, there are only two thermodynamically allowed modes of parallel interstrand helix–helix interactions. Either the desired parallel four-helix bundle topology is formed in which the hydrophobic surface of each helix is engaged with the neighboring helices, or only three of the peptide subunits interact to form a three-stranded helical coiled-coil structure with the fourth subunit adopting a random orientation in solution.[16] The four-helix bundle structure lacking a solvent-exposed hydrophobic surface should show CD spectra independent of the metallopeptide concentration, while the triple-stranded structure having an unmatched amphiphilic peptide available for further intermolecular interaction should display concentration-dependent CD spectra.[3a,5a] The metalloprotein complex displays concentration-independent CD spectra in the measured range 2–500 μM. Additional support for the formation of a four-helix bundle topology is provided by the guanidine hydrochloride denaturation studies[17] (Figure 2). The metalloprotein displays a highly cooperative and concentration-independent denaturation curve with a transition at 5.5 M GdnHCl and the free energy of stabilization $\Delta G(H_2O) = 5.6 \pm 0.8$ kcal·mol^{-1}, $m = 1025 \pm 145$ cal·mol^{-1}·M^{-1}. In contrast, denaturation of the free peptide strongly depends on the polypeptide concentration, indicating, as expected, uncontrolled formation of various intermolecular aggregates in the absence of the metal ion complex.

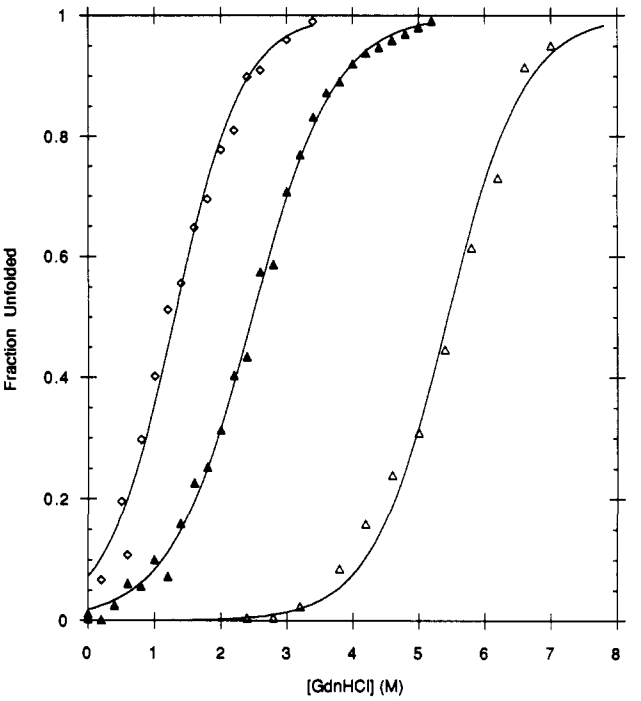

Figure 2. Guanidine hydrochloride denaturation curves were determined by measuring the mean residue ellipticity at 222 nm for the free peptide at (◇) 1.02×10^{-4} M and (▲) 4.35×10^{-4} M concentrations and (△) the Ru(II) four-helix bundle metalloprotein at 1.1×10^{-4} or 5.2×10^{-6} M concentrations in 100 mM HEPES, pH 7.0 at 25 °C. (△) $[D]_{1/2} = 5.5$ M, $\Delta G(H_2O) = 5.6 \pm 0.8$ kcal·mol^{-1}, $m = 1025 \pm 145$ cal·mol^{-1}·M^{-1}. (◇) $[D]_{1/2} = 1.3$ M, $\Delta G(H_2O) = 1.5 \pm 0.1$ kcal·mol^{-1}, $m = 1160 \pm 73$ cal·mol^{-1}·M^{-1}. (▲) $[D]_{1/2} = 2.5$ M, $\Delta G(H_2O) = 2.4 \pm 0.06$ kcal·mol^{-1}, $m = 956 \pm 22$ cal·mol^{-1}·M^{-1}. Solid lines are drawn on the basis of the parameters obtained by nonlinear least-squares fits of the data (see ref 5a for more detail).

In summary, we have presented a novel metal ion-assisted self-assembly process for the design and construction of a 60-

(8) For the earliest definition, see: Taube, H. *Chem. Rev.* **1952**, *50*, 69. Also see: Van Wart, H. E. *Methods Enzymol.* **1988**, *158*, 95.

(9) (a) Sundberg, R. J.; Bryan, R. F.; Taylor, I. F., Jr.; Taube, H. *J. Am. Chem. Soc.* **1974**, *96*, 381. (b) Matthews, C. R.; Erickson, P. M.; Van Vliet, D. L.; Perersheim, M. *J. Am. Chem. Soc.* **1978**, *100*, 2260. (c) Bowler, B. E.; Meade, T. J.; Mayo, S. L.; Richards, J. H.; Gray, H. B. *J. Am. Chem. Soc.* **1989**, *111*, 8757 and references cited therein. (d) Chang, I.-J.; Gray, H. B.; Winkler, J. R. *J. Am. Chem. Soc.* **1991**, *113*, 7056. (e) Isied, S. S.; Taube, H. *Inorg. Chem.* **1976**, *15*, 3070.

(10) For the characterization and the use of blue solutions of ruthenium(II) ($[Ru_5Cl_{12}]^{2-}$ cluster anion) in the formation of various ruthenium complexes, see: (a) Rose, D.; Wilkinson, G. *J. Chem. Soc. A* **1970**, 1791. (b) Gilbert, J. D.; Rose, D.; Wilkinson, G. *J. Chem. Soc. A* **1970**, 2765.

(11) A degassed solution of ruthenium trichloride hydrate (0.3 mg, 1.4 μmol) in 120 μL of 50% ethanol–water was placed under argon in a 1.0-mL Schlenk tube. The mixture was heated at 90 °C for 2 h to afford the ruthenium(II) blue solution. The peptide (10 mg, 5.7 μmol) was then added and heating continued for 30 min. The reaction mixture was allowed to cool to room temperature, directly applied to a Sephadex G-25 gel filtration column, and eluted with water. The peptidic fractions (monitored by absorption spectroscopy at 230 nm) were collected and purified by cation-exchange chromatography (CM Sephadex C-25, 0–2 M NaCl salt gradient in 50 mM TRIS pH 7.6), followed by reversed-phase HPLC (Vydac C$_8$, 5–95% aqueous acetonitrile, 0.1% TFA gradient) to afford 7.0 mg of the desired metalloprotein.

(12) Size exclusion chromatography was performed on a calibrated Superose 12HR 10/30 column (Pharmacia) in 6 M guanidine hydrochloride, 100 mM HEPES, pH 7.0, with a flow rate of 0.4 mL·min^{-1}; $MW_{found} = 7350$, $MW_{calcd} = 7192$. Atomic absorption spectroscopy was performed in duplicate on a Perkin-Elmer 5000 and was found to be consistent with 4:1 peptide:Ru stoichiometry (±10%). Concentration of the metalloprotein stock solution was determined by amino acid analysis. Electrospray mass spectrometry was performed on a SCIEX API-III biomolecular mass analyzer to give the following characteristic fragmentation products. [Ru(peptide)$_4$Cl$_2$]·(TFA)$_2$·(H$_2$O)$_2$: average mass = 7451.6 ± 3.8. [Ru(peptide)$_4$Cl$_2$]·CH$_3$CN·H$_2$O: average mass = 7247.3 ± 4.4. [Ru(pyridyl peptide)$_2$Cl$_2$]: average mass = 3681.5 ± 1.3. Ru(pyridyl peptide): average mass = 1856.0 ± 1.2.

(13) Formation of related metal complexes such as the cis-[Ru(peptide)$_4$Cl$_2$], [Ru(peptide)$_5$Cl]$^+$, or [Ru(peptide)$_6$]$^{2+}$ was predicted to be strongly disfavored due to the unfavorable steric and electronic effects.

(14) For 100% helix the value of $[\theta]_{222} = 32\,000$ deg·cm^2·dmol^{-1} was used. See: Chen, Y.; Yang, J.; Chau, K. *Biochemistry* **1974**, *13*, 3350.

(15) (a) Kaiser, E. T.; Kezdy, F. J. *Science* **1984**, *223*, 249. (b) Taylor, J. W.; Kaiser, E. T. *Pharmacol. Rev.* **1986**, *38*, 291. (c) DeGrado, W. F.; Lear, J. D. *J. Am. Chem. Soc.* **1985**, *107*, 7684.

(16) Double-stranded parallel coiled-coil structures are unstable with helical subunits of fewer than 29 residues and cannot give rise to the observed 5.6 kcal·mol^{-1} free energy of stabilization. See: (a) Lau, S. Y. M.; Taneja, A. K.; Hodges, R. S. *J. Biol. Chem.* **1984**, *259*, 13253. (b) Talbot, J. A.; Hodges, R. S. *Acc. Chem. Res.* **1982**, *15*, 224. Furthermore, the possibility of an alternating up–down configuration of four peptides can be easily dismissed. In such a configuration, the protein would be predisposed to form various intermolecular polymeric aggregates. Size exclusion chromatography (calibrated G-25, 2.0 × 90 cm, 100 mM HEPES, pH 7.5, flow rate 1.0 mL·min^{-1}) gave a sharp peak at the expected retention time, indicating the formation of a nonpolymeric ensemble with the expected apparent molecular weight of the four-helix bundle metalloprotein.

(17) (a) Pace, N. C. *Trends Biotechnol.* **1990**, *8*, 93 and references cited therein. (b) Santoro, M. M.; Bolen, D. W. *Biochemistry* **1988**, *27*, 8063. (c) Pace, N. C.; Shirley, B. A.; Thomson, J. A. In *Protein Structure*; Creighton, T. E., Ed.; IRL Press: Oxford, 1989; p 311.

residue four-helix bundle metalloprotein. The availability of such a highly convergent and synthetically simple approach for the construction of protein-like structures should benefit future designs of functional macromolecules.

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A Racemic Protein

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Most natural products exist as a single stereoisomer. In contrast, many synthetic chiral compounds exist as 1:1 mixtures of two enantiomeric forms. Such racemic mixtures may have desirable properties over the optically pure materials. For example, many racemic mixtures crystallize such that the two enantiomers are related by an inversion center.[1] This greatly simplifies the crystallographic phase problem.[2] In addition, the availability of the enantiomer of a natural product can provide a substance that is identical to that product except in a chiral environment.[3,4] We report herein the synthesis and characterization of a racemic protein via the synthesis of the two enantiomers separately.

We chose to study the rubredoxin protein native to the anaerobic bacterium *Desulfovibrio desulfuricans* 27774, hereafter RbDD.[5-8] RbDD consists of 45 amino acids including four cysteine residues that act to bind iron or other metal ions. This protein was chosen for study for several reasons, including its small size, its metal binding ability, and the relatively high hydrogenase-like activity of its Ni^{2+} complex.[7] The sequence in the proteins we have synthesized differs from the natural sequence[6] in that the amino terminus lacks a formyl group, and a nonliganding cysteine was changed to alanine. Synthesis was performed using a Milligen/Biosearch 9050 PepSynthesizer and *N*-fluorenylmethoxycarbonyl amino acid pentafluorophenyl esters as described previously.[9] RbDD was synthesized using either all L-amino acids or all D-amino acids. After reduction with β-mercaptoethanol (10 mM) for 2 h at 55 °C, the peptide isomers coelute on the reverse-phase HPLC column in a 27% to 32% gradient of water (0.1% TFA)/acetonitrile (0.1% TFA). Anaerobic metal titrations[10] clearly show that both L- and D-rubredoxin bind metal ions such as Co^{2+} and Fe^{2+} with high affinities with dissociation constants $<10^{-7}$ M. A representative titration is shown in Figure 1. The Fe^{2+} complexes could be oxidized to the characteristically red Fe^{3+} complexes upon exposure to air. As expected, no measurable differences between the L and D proteins were observed.

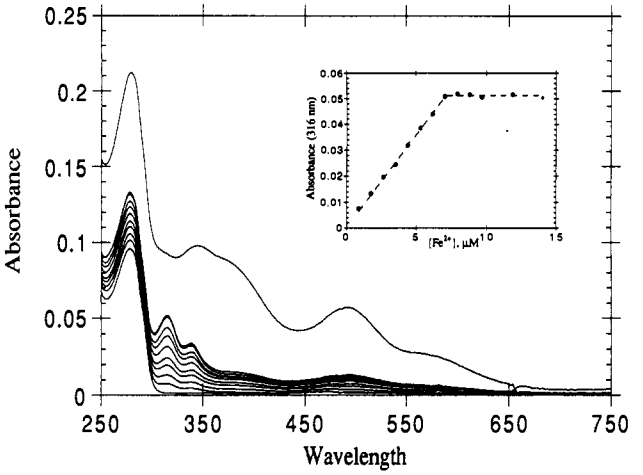

Figure 1. Fe^{2+} titration, Fe^{3+} saturation absorption spectra of D-RbDD. Similar spectra were obtained with $FeCl_2$ titrations of L-RbDD. L- and D-RbDD bind Fe^{2+} ($K_d \leq 5.8 \times 10^{-8}$) with subsequent oxidation to Fe^{3+} upon exposure to oxygen. Extinction coefficients determined from these titrations are for Fe^{2+}, $\epsilon_{316} = 6900\ M^{-1}\ cm^{-1}$, and for Fe^{3+}, $\epsilon_{490} = 7100\ M^{-1}\ cm^{-1}$. The titration curve is shown in the inset and depicts the tight-binding of D-rubredoxin for Fe^{2+}.

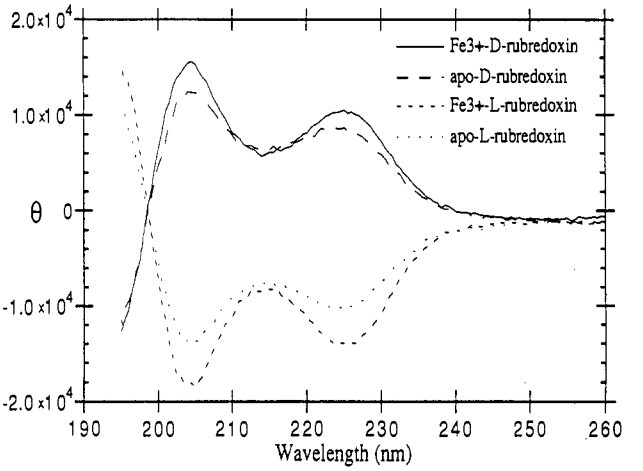

Figure 2. Circular dichroism spectra of Fe^{3+}-substituted RbDD and apo-RbDD isomers. Purified and reduced RbDD was dissolved in 5 mM Tris·HCl buffer to 9 μM, and 1.2 equiv of $FeCl_2$ was added anaerobically, with a final pH of 7.0. After oxidation of the metalated protein, 0.5-mL samples were transferred to a CD cuvette (Hellma No. 282 QS, 0.200-cm path length). Scans were taken from 260 to 195 nm, with five repeats to obtain an average spectrum. Units of θ are in deg·cm^2·(dmol of amino acids)$^{-1}$.

Circular dichroism (CD) spectra were taken using an AVIV Model 60DS circular dichroism spectropolarimeter. CD spectra of the reduced apoproteins as well as the Fe^{3+} complexes are shown in Figure 2. The spectra of both the apo and metalated forms are mirror images of each other. As further evidence for the different behavior of the enantiomers with regard to chiral probes, chymotrypsin digests were performed on both the apo and Fe^{3+} forms. RbDD has six potential chymotrypsin digestion sites within 45 residues. The metal-bound peptides were treated with chy-

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