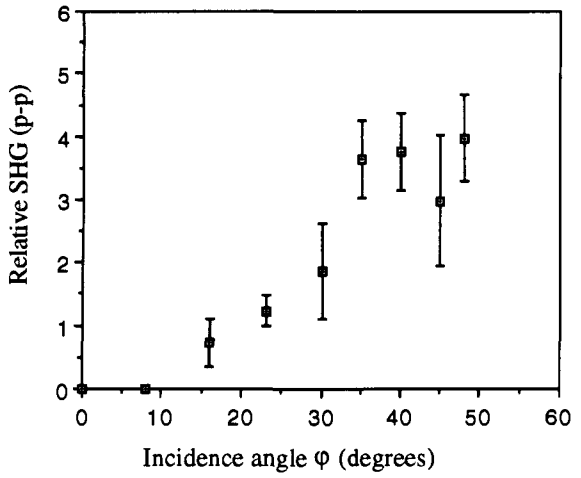

Figure 2. Relative SHG intensity (p–p) as a function of incidence angle φ (from the normal; up to limit imposed by sample holder) for PE*co*MA–**2**–PS sample (A_{470} = 0.12); several measurements.

duration, 5-mJ pulse energy). After the light passed through the sample at incidence angle φ (from normal to the sample surface), three filters and a polarizer were used to isolate either s- or p-polarized components of product 532 nm light, which were then quantitated with a photomultiplier (Hamamatsu R928); subsequent division by squared input intensities (measured with a photodiode) gave normalized average relative output intensities $I^{2\omega}_{p-p}$ and $I^{2\omega}_{s-p}$, each varying from pulse to pulse. With this apparatus, significant SHG could not be detected from samples prepared without dye (nonhyperpolarizable), or with **1** as the dye (nonoriented); however, SHG was clearly observed (signal/noise > 50) from PE*co*MA–**2**–PS and PE*co*MA–**3**–PS samples, varying with incidence angle φ as for monolayers prepared by Langmuir–Blodgett techniques (Figure 2);[4] SHG was also observed in samples prepared without PS overcoat, though these tended to cloud after several weeks in air. Surprisingly, SHG efficiency continued to increase with the amount of dye applied beyond the A_{470} = 0.02 for known[5] monolayers of similar compounds, up to 4-fold at A_{470} = 0.10–0.20 (beyond which the samples became visibly cloudy): evidently further dye molecules do not form an SHG-inactive centrosymmetric bilayer as expected, but maintain a common orientation as they associate with carboxylic groups a short way deeper in the PE*co*MA film.

Comparison, for φ = 45°, of average $I^{2\omega}_{p-p}$ (2.90 ± 0.75) and $I^{2\omega}_{s-p}$ (0.120 ± 0.066) for PE*co*MA–**2**–PS (A_{470} = 0.12) enabled calculation[4] of a tilt angle (deviation from an average direction) of 21° (±3°), which compares very favorably with materials prepared by LB and other techniques.[2,4,6]

Besides providing one part of a permanent hydrophilic/hydrophobic interface for thermodynamically stable orientation of amphiphilic hyperpolarizable moieties, the PS layer can also act as a protective coating for the dye; as a substrate upon which to deposit further PE*co*MA–dye–PS layers for multiplied SHG efficiency across the laminate; and as a medium for longitudinal propagation/SHG of IR/visible light ("organic nonlinear waveguide"). Further physical characterization of nonlinear monolayers, multilayers, and waveguides is in progress.

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Tandem Stereochemistry of Peptidylglycine α-Monooxygenase and Peptidylamidoglycolate Lyase, the Two Enzymes Involved in Peptide Amidation

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Carboxy-terminal amidation is a prevalent post-translational modification essential to the bioactivity of many neuropeptides.[1,2] Recently, we and others have demonstrated that formation of peptide amides from their glycine-extended precursors is not catalyzed by a single enzyme, as previously thought, but is a two-step process resulting from the sequential action of two enzymatic activities.[3-8] The first enzyme, peptidylglycine α-monooxygenase (PAM, EC 1.14.17.3), catalyzes formation of the α-hydroxyglycine derivative of the glycine-extended precursor in a process dependent upon ascorbate, copper, and molecular oxygen.[3,4,9] The second enzyme, peptidylamidoglycolate lyase (PGL, EC 4.3.2.5), catalyzes breakdown of the α-hydroxyglycine derivative to produce the amidated peptide and glyoxylate.[3,4,8]

While it has been shown that the *pro-S* glycine hydrogen is stereospecifically abstracted during PAM catalysis,[10] the stereochemistry of enzymatic amidation remains unknown. Elucidation of the stereochemical course of the amidation process requires determination both of the configuration of the α-hydroxyglycine species produced from PAM-catalyzed oxygenation and of the stereospecificity of PGL-catalyzed dealkylation. These determinations are experimentally difficult due to the instability of α-hydroxyglycine derivatives under many conditions[8] and to the absence of an appropriate chromophore in typical amidation substrates. Yet, knowledge of the stereochemistry for the two enzymatic steps in amidation is essential for the design of pseudosubstrates and inhibitors directed selectively at PGL, and for exploring the very important question of whether α-hydroxyglycine peptides have a biological function.[3,8] We report herein a series of experiments which demonstrate that PAM and PGL exhibit tandem stereospecificities in carrying out the two requisite steps in carboxy-terminal amidation.

PAM and PGL (homogeneous on SDS–PAGE) were isolated from bovine pituitaries as described previously.[8] Incubation of purified PAM with the substrate TNP-D-Tyr-Val-Gly under standard reaction conditions[8] resulted in *complete* conversion of substrate to TNP-D-Tyr-Val-α-hydroxy-Gly, with no detectable formation of TNP-D-Tyr-Val-NH_2. Figure 1 shows HPLC traces of synthetic[11] and enzymatically-produced TNP-D-Tyr-Val-α-hydroxy-Gly. As is evident from panel A, the two diastereomers

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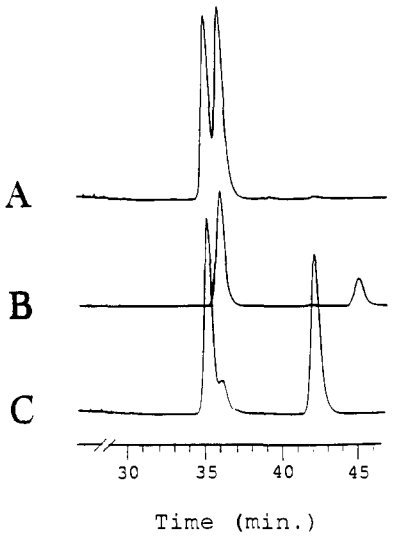

Figure 1. Tandem stereospecificities of PAM and PGL. (A) HPLC chromatogram of synthetic TNP-D-Tyr-Val-α-hydroxy-Gly. (B) HPLC chromatogram of TNP-D-Tyr-Val-α-hydroxy-Gly produced by PAM-catalyzed oxygenation of TNP-D-Tyr-Val-Gly. This panel shows the chromatogram obtained after the PAM reaction had gone to ca. 80% conversion, in order to illustrate the TNP-D-Tyr-Val-Gly substrate peak at 45.4 min. (C) HPLC chromatogram obtained after a 15-min incubation of synthetic TNP-D-Tyr-Val-α-hydroxy-Gly with PGL, showing conversion of only the slower-eluting diastereomer to TNP-D-Tyr-Val-NH_2 (peak at 42.2 min). HPLC analyses were performed on a C18 reverse-phase column using a gradient from 40% to 60% of the two eluent mixtures 10% acetonitrile/90% water/0.1% TFA and 90% acetonitrile/10% water/0.1% TFA. Peak assignments were confirmed by FAB-MS analyses.

of the synthetic material are well-separated on C18 RP-HPLC using a slow gradient. (Peak assignments were confirmed by FAB-MS analyses.) Comparison of panels A and B reveals that PAM-catalyzed oxygenation produces only one diastereomer of TNP-D-Tyr-Val-α-hydroxy-Gly, whose retention time corresponds to the slower-eluting peak in panel A. Panel C shows the chromatogram obtained after reaction of synthetic TNP-D-Tyr-Val-α-hydroxy-Gly with PGL; it is clearly evident that PGL converts *only* the slower-eluting diastereomer to the amide product, TNP-D-Tyr-Val-NH_2. Thus, these results demonstrate the tandem stereospecificities of PAM and PGL; PAM produces only α-hydroxyglycine of the chirality accepted by PGL.

In order to determine the configuration of the α-hydroxyglycine moiety processed by PGL, we decided to employ the enzyme acylase I (aminoacylase; *N*-acyl-L-amino acid amidohydrolase, EC.3.5.1.14), which catalyzes the hydrolysis of *N*-acyl-L-amino acids with high (>99% ee) enantioselectivity.[14] Acylase I has been widely used to resolve natural and unnatural amino acid enantiomers from their racemic mixtures.[15] In order to utilize this approach, it was first necessary to find a substrate which would undergo facile reaction with both PGL and acylase I. We therefore carried out a systematic specificity study with both enzymes. We found that *N*-cinnamoyl-α-hydroxy-Gly is an active substrate for both PGL and acylase I; PGL catalyzes N-dealkylation to produce cinnamamide, whereas acylase I catalyzes hydrolysis to produce cinnamic acid.

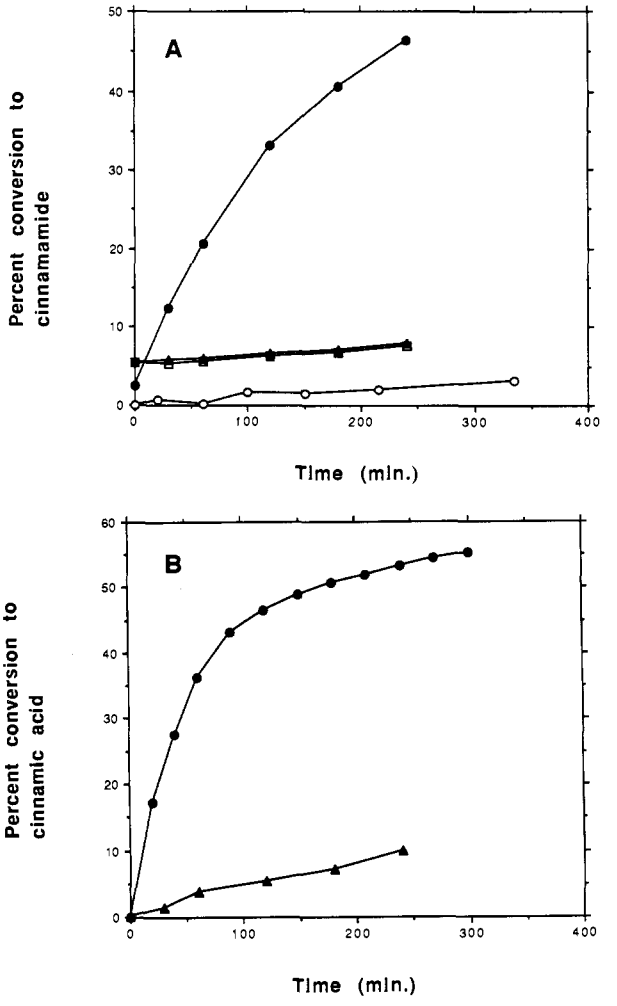

Figure 2. Time-dependent conversion of *N*-cinnamoyl-α-hydroxy-Gly to cinnamamide by PGL (A) and to cinnamic acid by acylase I (B). (A) Time course of cinnamamide formation from racemic *N*-cinnamoyl-α-hydroxy-Gly (46 μM; 100 mM MES, pH 6.5, 37 °C), in the presence (●) or absence (○) of PGL. In a separate experiment, 109 μM *N*-cinnamoyl-α-hydroxy-Gly was first reacted with acylase I. After formation of 65 μM cinnamic acid, acylase I was removed by ultrafiltration, the unreacted *N*-cinnamoyl-α-hydroxy-Gly was incubated with (▲) or without (□) PGL, and formation of cinnamamide with time was monitored. (B) Time course of cinnamic acid formation from racemic *N*-cinnamoyl-α-hydroxy-Gly (55 μM; 100 mM MES, pH 6.5, 37 °C) in the presence (●) of acylase I (Sigma porcine kidney grade III, 0.75 mg/mL). In a separate experiment, 109 μM *N*-cinnamoyl-α-hydroxy-Gly was first reacted with PGL. After formation of 56 μM cinnamamide, PGL was removed by ultrafiltration, the unreacted *N*-cinnamoyl-α-hydroxy-Gly was incubated with acylase I, and formation of cinnamic acid (▲) with time was monitored. HPLC analyses were performed on a C8 RP-HPLC column, with a mobile phase of 30% acetonitrile/70% water/0.2% phosphate.

A series of experiments were performed in which racemic *N*-cinnamoyl-α-hydroxy-Gly was reacted sequentially with these two enzymes in either order. The results, shown in Figure 2, establish that PGL reacts only with the same enantiomer of *N*-cinnamoyl-α-hydroxy-Gly which shows preferential reactivity toward acylase I.[16] Since (*S*)-*N*-cinnamoyl-α-hydroxy-Gly is the acylase-reactive enantiomer (corresponding stereotopically to an

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(16) The commercial acylase used in this work exhibited specific activities of 7 μM mg^{-1} min^{-1} and 0.027 μM mg^{-1} min^{-1} toward the two enantiomers of *N*-cinnamoyl-α-hydroxyl-Gly, respectively. The latter is in excellent agreement with the specific activity of 0.025 μM mg^{-1} min^{-1} exhibited by acylase for the unreacted *N*-cinnamoyl-α-hydroxy-Gly remaining after incubation of a racemic mixture with PGL.

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.

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(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.

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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).

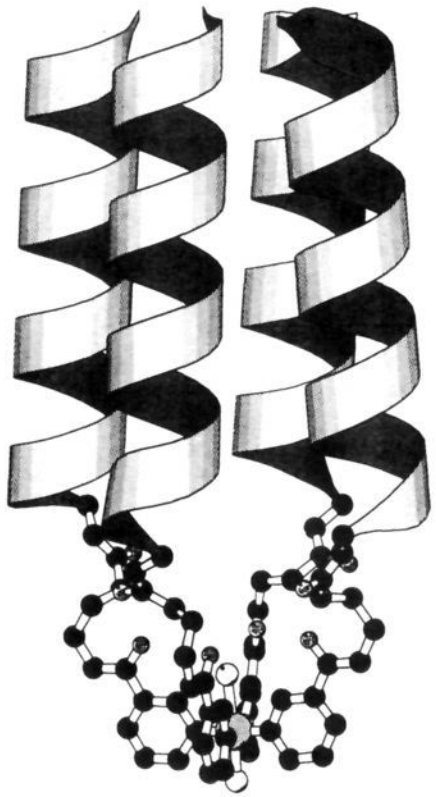

Figure 1. Computer-generated model of the parallel four-helix bundle metalloprotein. The polypeptide sequence is shown on the bottom.

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,

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(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.

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