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Tunable, Post-translational Hydroxylation of Collagen Domains in *Escherichia coli*

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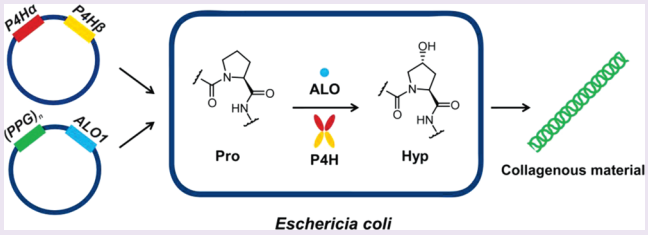
Supporting Information

ABSTRACT: Prolyl 4-hydroxylases are ascorbate-dependent oxygenases that play key roles in a variety of eukaryotic biological processes including oxygen sensing, siRNA regulation, and collagen folding. They perform their functions by catalyzing the post-translational hydroxylation of specific proline residues on target proteins to form (2*S*,4*R*)-4-hydroxyproline. Thus far, the study of these post-translational modifications has been limited by the lack of a prokaryotic recombinant expression system for producing hydroxylated proteins. By introducing a biosynthetic shunt to produce ascorbate-like molecules in *Eschericia coli* cells that heterologously express human prolyl 4-hydroxylase (P4H), we have created a strain of *E. coli* that produces collagenous proteins with high levels of (2*S*,4*R*)-4-hydroxyproline. Using this new system, we have observed hydroxylation patterns indicative of a processive catalytic mode for P4H that is active even in the absence of ascorbate. Our results provide insights into P4H enzymology and create a foundation for better understanding how post-translational hydroxylation affects proteins.



Dioxygenases dependent on α-ketoglutarate play diverse roles in a variety of eukaryotic biological processes, by catalyzing the irreversible post-translational hydroxylation of proteins.[1] They catalyze the insertion of oxygen atoms from molecular oxygen into methylene C−H bonds on the side chains of various amino acids in proteins, resulting in hydroxylated versions of genetically encoded amino acids at their sites of action.[2] Members of this enzyme family include lysyl and asparaginyl hydroxylases as well as various prolyl 3- and 4-hydroxylases with different peptidyl proline substrate preferences, including collagen prolyl 4-hydroxylase (P4H), which is the subject of this study.

P4H plays a critical role in the maturation and folding of collagen, the major component of vertebrate connective tissue. The folding of trimeric collagen, unlike canonical protein folding, is mediated by post-translational modification.[3,4] At the primary sequence level, collagens comprise long stretches of repeated X-Y-glycine amino acid triplets, where the most common residue in the X position is proline and in the Y position of mature collagen is (2*S*,4*R*)-4-hydroxyproline (Hyp).[5] P4H-mediated post-translational modification of Pro to Hyp in the Y position allows collagen to adopt its signature triple-helical fold by changing preferred bond angles of modified proline residues through stereoelectronic effects,[6,7] allowing collagen monomers to align. Despite successful transfer of human collagen biosynthesis into unicellular eukaryotes such as yeast,[8−10] many details of the collagen folding pathway and the relevant enzyme mechanisms remain unknown and are difficult to elucidate in host cells expressing a large diversity of potentially interfering native endoplasmic reticulum enzymes and chaperones. Thus, reconstitution of collagen folding pathways in a prokaryotic host will greatly increase our understanding of how these essential biomolecules assemble. We therefore embarked on a bottom-up approach to biosynthesizing collageous materials in *Escherichia coli.*

Expression and purification of properly assembled P4H tetramers in the Origami B strain of *E. coli* was previously described by Kersteen *et al.*,[11] opening the possibility of producing folded collagen trimers in *E. coli* if P4H could be activated in the cytosol. Still, because P4H is dependent on both L-ascorbate and molecular oxygen for catalysis (Figure 1, panels a and b), significant obstacles to activating P4H in the cytosol of *E. coli* remained. *E. coli* does not biosynthesize L-ascorbate, and although *E. coli* does harbor genes for an ascorbate transporter system, the transporter is strictly regulated for expression under anaerobic conditions.[12] However, it had been demonstrated that L-ascorbate and analogues could be biosynthesized in certain strains of *E. coli* by heterologously overexpressing the gene for D-arabinono-1,4-lactone oxidase (ALO) from *Saccharomyces cerevisae* (Figure 1, panel c) and feeding the bacteria sugar 1,4-lactones as a nutrient source.[13] Moreover, P4H is known to be somewhat flexible with respect to accepting reducing agents that are structurally related to L-ascorbate.[14] Because *E. coli* harbors at least one aldose sugar dehydrogenase,[15] which has the potential of producing lactones capable of acting as ALO substrates, we reasoned that ALO could be used to biosynthesize ascorbate-like

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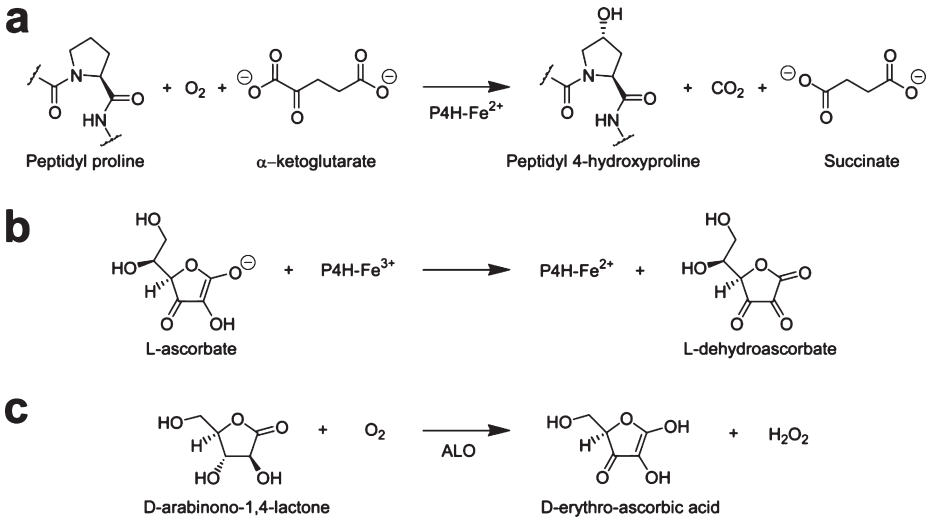

Figure 1. Reactions catalyzed by P4H and ALO. (a) P4H catalyzes the formation of peptidyl 4-hydroxyproline from peptidyl proline and molecular oxygen. (b) In the process of catalysis, the catalytic Fe^{2+} ion of P4H is oxidized occasionally to Fe^{3+}, which requires reduction by L-ascorbate for catalysis to continue. (c) ALO catalyzes the formation of ascorbate-like molecules from sugar-1,4-lactones. In the case shown, the reaction for which ALO is named, D-arabinono-1,4-lactone is reduced by ALO to D-erythro-ascorbic acid, a five-carbon analogue of L-ascorbate.

molecules that are capable of acting as P4H activators from endogenous substrates in the cytosol of *E. coli*.

To test the hypothesis that ALO could be used as an activator of P4H in *E. coli*, we first assembled a plasmid encoding both an expressable P4H activity reporter as well as ALO. The P4H activity reporter is composed of the P4H substrate peptide $(Pro\text{-}Pro\text{-}Gly)_5$ fused at its C terminus to the affinity purification tag glutathione *S*-transferase (GST), with an intervening thrombin cleavage sequence. The bifunctional plasmid (Supplementary Figure 1) was co-transformed into the Origami 2 strain of *E. coli* with the expression plasmid for heterotetrameric P4H previously described.[11]

Cultures expressing the two plasmids were suspended in buffer and fed various sugar 1,4-lactones as a sole nutrient source or no exogenous lactone. Resulting activity reporter peptides were purified from cell lysates by glutathione affinity, liberated from their purification tags by thrombin cleavage, and then analyzed for proline hydroxylation by liquid chromatography−mass spectrometry (LC−MS). We found that P4H was indeed cytosolically activated and that hydroxylation levels in all samples were nearly identical, even for the sample obtained after no lactone was fed (Supplementary Figure 2). These data indicate not only that there must be a cytosolic ALO substrate available in *E. coli* under these conditions but also that it is present in sufficient concentration to saturate the needs of P4H when converted by ALO in the collagen expression system.

We then optimized a simple shake flask culture methodology for producing $(Pro\text{-}Pro\text{-}Gly)_5$ repeats with high hydroxylation levels. The extent of hydroxylation observed for substrates produced in this manner was found to be strongly dependent on culture medium. We found that minimal medium yielded lower quantities of protein (Supplementary Table 1), but with higher hydroxylation levels than were obtained with rich medium (Figure 2, panels a and c−h). M9 minimal medium plus 0.4% w/v tryptone as the carbon source produced the highest hydroxylation levels, with 71 ± 6% of Y-position prolines modified. The trend of less rich medium leading to higher hydroxylation levels was seen reproducibly in experiments in which the amount of added tryptone was varied, while other medium parameters were held constant (Supplementary Figure 3).

On the basis of these results, we hypothesize that hydroxylation level is modulated by concentration of nutrient source in the medium for one of two reasons: either (1) gene expression differences resulting from media composition difference can affect the production of the ALO substrate, or (2) aerobic respiration creates competition for molecular oxygen that affects the activity of P4H and/or ALO in more nutritious medium. It is also possible that both of these factors play a role.

Cultures in which P4H was expressed without ALO in rich medium showed very low levels of hydroxylation, as expected. Yet when the experiments were performed again using optimized minimal medium conditions, we found that hydroxylation can occur with a surprising "all-or-none" pattern. That is, expressed $(Pro\text{-}Pro\text{-}Gly)_5$ peptides were found to be predominantly either nearly fully hydroxylated ($(Pro\text{-}Hyp\text{-}Gly)_5$) or to have remained unhydroxylated ($(Pro\text{-}Pro\text{-}Gly)_5$) (Figure 2, panel b). Experiments using longer P4H substrates showed a similar hydroxylation pattern (Supplementary Figure 4), showing the generality and reproducibility of these results. Our observations strongly suggest the existence of a mode of P4H processing of Pro-Pro-Gly repeats that is processive with respect to hydroxylation and sensitive to the presence of ascorbate-like reducing agents produced by ALO. Interestingly, no hydroxylation was observed in an *in vitro* experiment without supplemental ascorbate (Supplementary Figure 5), indicating that hydroxylation is mediated by factors present in the *E. coli* cytosol that are not present in the *in vitro* system. Models for nondistributive processing of collagenous substrates by P4H have been postulated,[16,17] but no evidence has previously been presented to support the existence of processive catalysis by P4H.[18] Additionally, the notion that an ascorbate-independent catalytic mode for human P4H could become active at low ascorbate levels has not, to our knowledge, been suggested. As ascorbate is an essential human nutrient;[19,20] it is tempting to speculate that such a mode could be active in humans under conditions in which ascorbate levels are deficient. More studies will be necessary to determine what *E. coli* cytosolic factors mediate this newly observed phenomenon and to establish if such an ascorbate-independent mode is active in human biology.

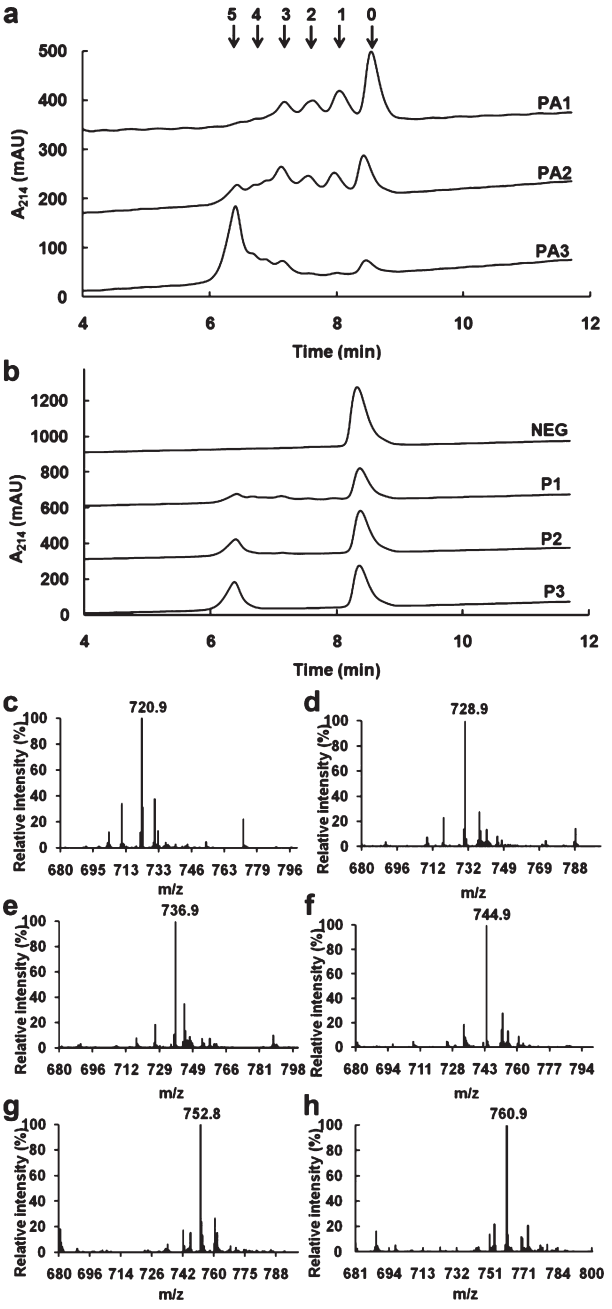

Figure 2. LC−MS analysis of $(Pro\text{-}Pro\text{-}Gly)_5$ peptides cytosolically hydroxylated in *E. coli* under various conditions. (a) UV absorbance chromatograms of $(Pro\text{-}Pro\text{-}Gly)_5$ peptides from the cultures expressing both P4H and ALO in (PA1) Terrific Broth, (PA2) M9 minimal medium plus 0.4% w/v tryptone and 0.4% v/v glycerol, and (PA3) M9 minimal medium plus 0.4% w/v tryptone. (b) Cultures not expressing ALO: (NEG) expressing neither P4H nor ALO in Terrific Broth, (P1) expressing P4H only in Terrific Broth, (P2) expressing P4H only in M9 minimal medium plus 0.4% w/v tryptone and 0.4% v/v glycerol, and (P3) expressing P4H only in M9 minimal medium plus 0.4% w/v tryptone. Arrows indicate number of hydroxylated prolines in the associated peaks as determined by quadrupole mass detection. Mass spectra of peaks with 0−5 hydroxyls are shown in panels c−h, respectively.

In eukaryotes, proper hydroxylation of collagen manifests itself as an increase in thermostability. We therefore wanted to validate that the P4H-mediated hydroxylation observed in the new prokaryotic system would function for that purpose. Thus, we fused our $(Pro\text{-}Pro\text{-}Gly)_5$ or $(Pro\text{-}Pro\text{-}Gly)_7$ constructs to the N-terminus of the 27-amino-acid T4-phage "foldon" domain, as was done previously for Pro-Pro-Gly synthetic peptide repeats in studies of collagen stability.[21,22] The foldon domain forms a tight, obligate trimer. By keeping three individual strands associated and aligned at one end, the presence of the foldon domain overcomes the entropic barrier of strand association and stabilizes the triple helix formed by attached collagenous domains.

We hydroxylated the $(Pro\text{-}Pro\text{-}Gly)_5$-foldon and $(Pro\text{-}Pro\text{-}Gly)_7$-foldon constructs to different extents by varying culture conditions. We then measured the effects on thermostability using circular dichroism (CD) spectroscopy by previously described methods.[23] Values of T_m, the midpoint temperature of the thermal transition between folded and unfolded states, were found to increase with hydroxylation levels for both constructs (Figure 3, panel a), verifying that P4H-mediated hydroxylation in our recombinant system does indeed have the desired functional effect on collagenous material.

Finally, we used the system to investigate how P4H interacts with collagen as it folds. We produced a series of $(Pro\text{-}Pro\text{-}Gly)_n$ constructs, where n = 5, 7, and 10, that were expressed either as fusions to a foldon domain or alone. The amino acid sequences and chain lengths of these constructs were chosen such that the triple helical domains in the resulting proteins would exhibit a wide range of T_m values; unhydroxylated $(Pro\text{-}Pro\text{-}Gly)_{10}$-foldon ($T_m$ = 63 ± 2 °C) forms a triple helix with a T_m value well above that of the bacterial culture condition, whereas another construct, $(Pro\text{-}Pro\text{-}Gly)_5$,[24] is incapable of forming trimers under culture conditions even when fully hydroxylated ($(Pro\text{-}Hyp\text{-}Gly)_5$). We produced this series of collagenous polypeptides by co-expressing P4H and ALO in M9 minimal medium plus 0.4% w/v tryptone and 0.4% v/v glycerol. We found that foldon-fused Pro-Pro-Gly repeats consistently exhibited extents of hydroxylation lower than those of their unfused counterparts (Figure 3, panel b). Also, the longer Pro-Pro-Gly repeats were disproportionately less hydroxylated (Figure 3, panel b). These data indicate that, on the whole, P4H-mediated hydroxylation is dependent on the folded state of the collagenous material in the *E. coli* expression system. That is, as the collagenous material folds to adopt a triple-helical structure, it loses its ability to act as a P4H substrate. This phenomenon is consistent with a previous report suggesting that P4H operates on unfolded collagen strands in eukaryotic cells.[25]

In conclusion, by introducing a biosynthetic shunt to produce ascorbate-like molecules, we have created a strain of *E. coli* that can produce recombinant proteins with a high fraction of hydroxylated proline residues. We have verified that the hydroxylation of collagenous materials produced in the new system provides an increase in protein thermostability and also that P4H interacts with collagenous materials as they fold in the *E. coli* cytosol in a manner similar to that of the eukaryotic system. Additionally, we present the first evidence for a processive mode of P4H catalysis that can operate in the absence of ascorbate. Last but not least, the technology we describe should prove useful for producing and studying proteins that are hydroxylated by other ascorbate-dependent hydroxylases.

METHODS

For complete experimental methods including details of constructions of plasmids, protein expression and purification, and all sequences of DNA oligomers and peptides, please see Supporting Information.

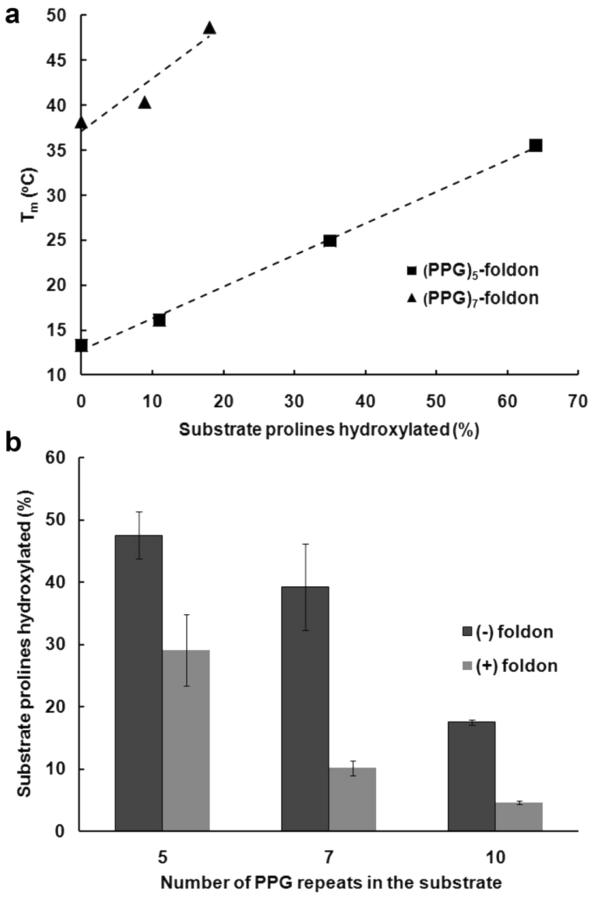

Figure 3. Triple helix formation by P4H-mediated hydroxylation of collagenous peptides in *E. coli*. (a) Relationship between the T_m values of triple helical (Pro-Pro-Gly)$_5$-foldon and (Pro-Pro-Gly)$_7$-foldon, and their hydroxylation levels. Squares represent (Pro-Pro-Gly)$_5$-foldon. Triangles represent (Pro-Pro-Gly)$_7$-foldon. (b) Hydroxylation levels of (Pro-Pro-Gly)$_5$, (Pro-Pro-Gly)$_5$-foldon, (Pro-Pro-Gly)$_7$, (Pro-Pro-Gly)$_7$-foldon, (Pro-Pro-Gly)$_{10}$, and (Pro-Pro-Gly)$_{10}$-foldon constructs co-expressed with both P4H and ALO in *E. coli*, using M9 minimal medium plus 0.4% w/v tryptone and 0.4% v/v glycerol. Hydroxylation level is reported as the percentage of substrate prolines (proline in the Y position of X-Y-glycine repeats) that were hydroxylated.

Lactone Feeding. Plasmids expressing GST-(Pro-Pro-Gly)$_5$, ALO, and P4H genes were co-transformed into Origami 2 (DE3) competent cells (Novagen). A starter culture was grown overnight in LB medium supplemented with 30 μg mL^{-1} Kanamycin and 200 μg mL^{-1} Ampicillin. The starter culture was used to inoculate flasks of Terrific Broth culture medium with appropriate antibiotics. The culture was incubated at 37 °C (250 rpm), induced with 500 μM of isopropyl-1-thio-β-D-galactopyranoside (IPTG, US Biological) at OD_{600} = 1.6−1.8, and expressed at 23 °C (250 rpm) for 14−18 h. Cell pellets were collected, washed three times with Dulbecco's phosphate buffered saline (DPBS), and then resuspended in DPBS for incubation with effectors.

These cell suspensions were split into 5 aliquots, and to each aliquot was added Fe(II)SO_4 at 250 μM, together with one of the following compounds at 10 mM: (1) D-arabinono-1,4-lactone, (2) L-galactono-1,4-lactone, (3) L-gulono-1,4-lactone, (4) L-ascorbic acid, or (5) nothing additional. The cell suspensions were incubated at 30 °C (250 rpm) for 3 h. Cell pellets were collected, washed three times with DPBS, resuspended in DPBS, and then lysed by sonication. The lysate supernatants were collected after centrifugation, and the expressed GST-(Pro-Pro-Gly)$_5$ was purified using glutathione affinity resin as described in the "protein expression and purification" section of Supporting Information.

GST-(Pro-Pro-Gly)$_5$ samples were then incubated with 50 U per mg-protein of thrombin to cleave GST tags from the (Pro-Pro-Gly)$_5$ peptides at RT. After 2 h, the cleaved peptide was separated from GST by applying the proteolysis mix to a 10 kDa cut off Amicon protein concentrator (Millipore) and collecting the flow through.

Characterization of Hydroxylation. The concentrations of the purified proteins were determined by $UV_{280\,nm}$ with a Nanodrop spectrophotometer (Thermo Scientific). In order to remove GST tags from the collagen like peptides, 4 units of thrombin (MP Biomedical) was incubated with 75 μg of protein at RT for 2 h in a final volume of 60 μL in DPBS; 1 mM benzamidine (Sigma-Aldrich) was added to the mixture to stop the cleavage reaction. The samples were analyzed by LC−MS (Waters) equipped with a diode array detector as well as a quadrupole mass spectrometer, with a gradient of 5−95% Acetonitrile over 1 h. Quantitative determination of hydroxylation fraction was calculated using the peak areas from extracted ion chromatograms. All relative ionization efficiencies (RIE) were set equal to 1 after verification that the RIE for (Pro-Hyp-Gly)$_5$ was greater than 80% of that for (Pro-Pro-Gly)$_5$ by comparing the ionization peak areas with $UV_{214\,nm}$ peak area in the chromatogram in Figure 2b. The percentage of substrate prolines hydroxylated (H.level %) was calculated using eq 1, in which n is the number of hydroxylated substrate prolines (note: only prolines in the Y position of X-Y-glycine repeats are considered as substrate prolines), n_{max} is the total number of substrate prolines, and A_n is the peak area in extracted ion chromatograms of peptide with hydroxylated proline number of n.

$$\text{H.level}\% = \frac{\sum_{n=0}^{n_{max}} n \times A_n}{n_{max} \sum_{n=0}^{n_{max}} A_n} \times 100\% \quad (1)$$

Characterization of Collagenous Material Thermostability. The proteins containing foldon were cleaved by Thrombin CleanCleave Kit (Sigma), and the cleaved products were separated from GST tag and uncleaved products by applying the mixture to glutathione affinity resin and collecting the flow through. The peptides were then concentrated using a 3 kDa cut off Amicon protein concentrator (Millipore), heated at 95 °C for 5 min, and applied to a 0.2 μm spin filter microcon (Millipore) to remove possible residual protein impurities. The purity of the products was checked by SDS-PAGE and analytical HPLC (Waters), and the final peptide concentration was determined by measuring $UV_{280\,nm}$ by Nanodrop spectrophotometer and analytical HPLC.

The spectra of the peptides (55 μM in DPBS buffer) were acquired with a Jasco J-815 CD spectrometer with a 1 mm path length quartz cell. The ellipticity at 210 nm was then monitored from −10 to 80 °C as the temperature was increased at a rate of 1 °C per min. The thermal transition curve was defined as three phases: folded state, melting state, and unfolded state, which were each linearly fit. The value of T_m was determined as the temperature at the midpoint of the intersections.

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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