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Synthesis and Assembly of Functional High Molecular Weight Adiponectin Multimers in an Engineered Strain of *Escherichia coli*

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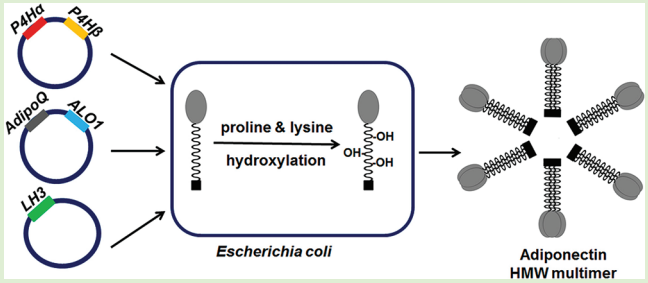
Supporting Information

ABSTRACT: Adiponectin has many beneficial effects on cardiovascular and obesity-related disorders. It is part of a class of proteins that contains short collagenous domains, along with surfactant proteins A and D, and complement protein C1q. This class of biomacromolecules requires post-translational modifications to form biologically active assemblies. By introducing a set of post-translational modifying enzymes into *Escherichia coli*, we have created a prokaryotic expression system that functionally assembles adiponectin, as assessed by the ability of produced adiponectin multimers to suppress human endothelial cell apoptosis. This study represents the first example of the assembly of functional high order multimers of any member of this class of proteins outside of eukaryotic cells. Furthermore, the results give fundamental insight into the process of assembly such as the necessity and sufficiency of various post-translational steps for functional assembly. We expect that fine-tuning of the expression system will allow for efficient production and functional assembly of biomolecules that assemble via short collagenous domains.



INTRODUCTION

Adiponectin is an adipokine that is abundantly present in plasma, typically at 2−30 μg/mL in humans.[1] Most other adipokines increase with Body Mass Index, whereas adiponectin mRNA and serum levels are found to be decreased in obese patients.[2] Adiponectin is reported to have vascular protective effects, and is shown to protect against hypertension, atherosclerosis, and heart failure.[3−5] In addition, adiponectin can increase insulin sensitivity, exerts a protective role against chronic inflammation, and can prevent nonalcoholic fatty liver disease (nonalcoholic steatohepatitis).[6−8] Due to its multiple beneficial effects, adiponectin has attracted increasing attention in the past decade.

Different oligomeric states of adiponectin forms have been found in circulation: low-molecular weight multimers (LMW), middle-molecular weight multimers (MMW), and high-molecular weight multimers (HMW). HMW adiponectin is the major active form for many of adiponectin's effects, such as inhibiting hepatic glucose production and suppressing apoptosis of endothelial cells.[9,10] Reduction in the amount of circulating HMW adiponectin has been shown to be associated with diseases like coronary artery disease and type II diabetes.[11,12]

Adiponectin is composed of four domains: an N-terminal signal peptide, a variable domain that is divergent among species, a collagenous domain comprising 22 Gly-Xaa-Yaa repeats, and a C-terminal globular domain that binds to adiponectin receptors and perhaps to other serum-binding partners as well.[13] Adiponectin is extensively post-translationally modified, and these modifications play important roles in multimerization. Proline hydroxylation contributes to stable association of triple-helical collagenous domains.[14] Hexamer and higher order multimer formation are mediated by the formation of disulfide bonds.[15] Hydroxylation and additional glycosylation of conserved lysines in collagenous domains have been reported to be necessary for the assembly and secretion of HMW multimers.[16] To date, functional recombinant human adiponectin has only been produced in eukaryotes, which is not ideal for potential biomedical applications due partly to yield and cost.

We recently developed an *Escherichia coli* system to post-translationally modify recombinantly expressed proteins. This system was shown to hydroxylate proline residues in collagenous sequences recombinantly coexpressed with D-arabinono-1,4-lactone oxidase (ALO) in *E. coli*,[17] causing the collagenous sequences to trimerize. Building on this expression platform that includes a functional human prolyl 4-hydroxylase (P4H), we expressed an active human lysyl hydroxylase 3 (LH3), capable of post-translationally modifying recombinant adiponectin. These two post-translational modifications proved necessary and sufficient in the formation of biologically active HMW adiponectin multimers, as assessed by the ability of the expressed adiponectin multimers to suppress endothelial cell apoptosis. Although the described hydroxylation platform is still

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in development, this work shows the potential of this expression platform to produce bioactive versions of the entire class of biomolecules that assemble via short collagenous domains such as complement protein C1q and surfactant proteins A and D.

EXPERIMENTAL SECTION

Materials. All restriction enzymes were purchased from New England Biolabs. Bacterial plasmid vectors and competent cells were obtained from EMD Chemicals, except pGEX4T-1 (GE Healthcare). All kits for molecular cloning were obtained from Qiagen. The DNA oligomers used in the gene construction as listed below were synthesized by the Protein and Nucleic Acid facility at Stanford University. Glutathione affinity resin and Fast Flow nickel-charged resin for protein purification were purchased from GE Healthcare. Protein concentrators were obtained from Millipore.

Construction of Plasmids. pET22b containing cDNAs encoding both the α and β subunits of human P4H were a generous gift from Prof. Ronald Raines (University of Wisconsin, Madison).

Human LH3 cDNA (gene accession number: NM_001084) was purchased from Origene. DNA encoding the mature peptide of LH3 was amplified by PCR using primers (forward: 5′-ACTGACATATGTCCGACCGGCCCCGGGGC-3′; reverse: 5′-ATCGACTCGAGTCAGGGGTCGACAAAGGACACCATGATG-3′), which introduced an *Nde*I site at the 5′ end and an *Xho*I site at the 3′ end. The PCR product was digested with *Nde*I and *Xho*I, and then ligated into the second multiple cloning site (MCS) of the coexpression vector pCDFDuet-1 using T4 ligase, resulting in the plasmid named pSD.CDFDuet-1.0.LH3.

DNA encoding ALO from *Saccharomyces cerevisiae* (strain EBY100) was inserted into the second MCS of coexpression vector pCOLADuet-1, resulting in a plasmid named pSD.COLADuet-1.0.ALO1, as previously described.[17]

Human adiponectin cDNA (gene accession number: NM_004797) was purchased from Thermo Fisher Scientific. Adiponectin fragment from its collagenous domain was amplified from adiponectin cDNA using PCR with primers (forward: 5′-ACTGAGGATCCGATCCAGGTCTTATTGGTCCTAAGGGAGAC-3′; reverse: 5′-CGATAGCGGCCGCTTAGCCTTGGATTCCCGGAAAGCCTCG-3′), which introduced a *Bam*HI restriction site at the 5′ end, and a stop codon and *Not*I site at the 3′ end. The PCR product was digested with *Bam*HI and *Not*I, and ligated into vector pGEX4T-1 in order to create the fusion of adiponectin fragment (AQfrag) to glutathione *S*-transferase (GST) with an intervening thrombin protease cleavage site (pSD.GEX4T-1.GST-(AQfrag)). To introduce appropriate restriction sites to ligate GST-(AQfrag) into the first MCS of pCOLADuet-1 vector, PCR was carried out on the plasmid pSD.GEX4T-1.GST-(AQfrag), using primers (forward: 5′- CAGCTACCATGGGTTCCCCTATACTAGGTTATTGGAAAATTAAGGGCC-3′; reverse: 5′-CGATAGCGGCCGCTTAGCCTTGGATTCCCGGAAAGCCTCG-3′) that introduced a *Nco*I site on the 5′ side of the translation initiation codon of GST-(AQfrag) and retain the *Not*I site after the 3′ side of the stop codon. The PCR fragment was digested with *Nco*I and *Not*I, and ligated into the first MCS of both the empty pCOLADuet-1 vector and the plasmid pSD.COLADuet-1.0.ALO1, which created plasmids pSD.COLADuet-1.GST-(AQfrag).0 and pSD.COLADuet-1.GST-(AQfrag).ALO1, respectively.

The gene for the mature adiponectin peptide was amplified from its cDNA by PCR with primers (forward: 5′-CAGTAGGCGCGCCCCGGTCATGACCAGGAAACCACG-3′; reverse: 5′-CTAGTGCGGCCGCTCAGTTGGTGTCATGGTAGAGAAGAAAGCCTG-3′), which introduced an *Asc*I restriction site at the 5′ end and a stop codon and *Not*I site at the 3′ end. The PCR product was digested with *Asc*I and *Not*I and ligated after the His_6 tag in the first MCS of both the empty pCOLADuet-1 vector and the plasmid pSD.COLADuet-1.0.ALO1, which created plasmids pSD.COLADuet-1.His-(AdipoQ).0 and pSD.COLADuet-1.His-(AdipoQ).ALO1, respectively.

Adiponectin Fragment Expression, Purification, and Characterization of Hydroxylation. The plasmid pSD.COLADuet-1.GST-(AQfrag).0 or pSD.COLADuet-1.GST-(AQfrag).ALO1 was transformed or cotransformed with pBK1.PDI1.P4H7 and pSD.CDFDuet-1.0.LH3 into Origami 2 (DE3) competent cells. In each case, a starter culture was grown overnight in LB broth supplemented with 30 μg/mL kanamycin when a pCOLADuet-1 vector construct was transformed, 200 μg/mL ampicillin when pBK1.PDI1.P4H7 was transformed, and 50 μg/mL spectinomycin when a pCDFDuet-1 vector construct was transformed. When all three antibiotics were added, the dose for each of the antibiotics was reduced by half. The starter culture was used to inoculate flasks of the LB broth with appropriate antibiotics. The culture was incubated at 37 °C (250 rpm) until OD_{600} 0.25−0.3, and then induced with 50 μM isopropyl thiogalactoside (IPTG; U.S. Biologicals) and simultaneously supplemented with 1 mM $Fe(II)SO_4$ (a prolyl hydroxylase cofactor; Sigma), and expressed at 23 °C (250 rpm) for 14−18 h. Cells were harvested, resuspended in lysis buffer (Dulbecco's Phosphate Buffered Saline (DPBS) plus 5 mM EDTA), and then lysed by sonication. The lysate supernatants were collected after centrifugation (30,000 g, 45 min, 4 °C) and incubated with glutathione affinity resin at 4 °C for 1 h. The resin was washed with DPBS, and then the GST tagged proteins were eluted with 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0. The proteins were then concentrated and buffer exchanged into DPBS using a 10 kDa cutoff Amicon protein concentrator.

The concentrations of the purified proteins were determined by $UV_{280\ nm}$ with a Nanodrop spectrophotometer (Thermo Scientific). The extinction coefficients of the proteins were calculated with the "ProtParam tool" at the ExPASy Web site. In order to remove GST tags from the collagenous peptides, 4 units of thrombin (MP Biomedical) were incubated with 75 μg of protein at room temperature for 2 h in a final volume of 60 μL in DPBS. The samples were then boiled to denature and precipitate the byproduct proteins, including free GST tag and uncleaved GST-AQfrag. The AQfrag peptide remained soluble after this process and was recovered by collecting the supernatant after centrifugation. The samples were analyzed by liquid chromatography−mass spectrometry (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.

Multimerization Analysis of Adiponectin Expressed in *E. coli* and Optimization for HMW Adiponectin in Small Scale Tests. The plasmid pSD.COLADuet-1.His-AdipoQ.0 or pSD.COLADuet-1.His-AdipoQ.ALO1 was transformed or cotransformed with pBK1.PDI1.P4H7 and pSD.CDFDuet-1.0.LH3 into Origami 2 (DE3) competent cells. After overnight culture of the cells in 2 mL of LB broth with appropriate antibiotics, 10 mL of the LB broth with appropriate antibiotics was inoculated with 250 μL of overnight culture for each culture condition. The cultures were incubated at 37 °C (250 rpm) until OD_{600} reached 0.25−0.3 and then induced with 50 μM IPTG, supplemented with 1 mM $Fe(II)SO_4$, and expressed at 23 °C (250 rpm) for 14−18 h. Cells were harvested, resuspended in lysis buffer (DPBS plus 5 mM EDTA) and then lysed by sonication. The lysate supernatants were collected and analyzed by SDS-PAGE and Western blot. Samples were 2× diluted with sample buffer (nonreducing buffer: 10% glycerol, 2% SDS, 20 mM Tris pH 6.8, and 0.002% bromophenol blue; reducing buffer: 10% glycerol, 2% SDS, 20 mM Tris pH 6.8, 0.002% bromophenol blue, and 10 mM 2-mercaptoethanol), and incubated for 5 min at 4 °C, followed by boiling or no boiling. The samples were separated with 4−12% gradient gels (Life Technologies) at 4 °C, followed by Western Blot analysis using antiadiponectin antibody (BD Biosciences) as the primary antibody.

Optimization of conditions for the expression of adiponectin was done under varied induction temperatures (16, 23, and 30 °C) and times (24 and 48 h), with the bacterial cells containing the plasmids pSD.COLADuet-1.His-AdipoQ.ALO1, pBK1.PDI1.P4H7, and pSD.CDFDuet-1.0.LH3.

Large Scale Recombinant Adiponectin Expression and Purification. The cultures for the Origami 2 bacterial cells were performed in 8 L of LB broth with appropriate antibiotics at 37 °C.

When OD_{600} reached 0.25−0.3, protein expression was induced with 50 μM IPTG and 1 mM $Fe(II)SO_4$ was supplemented into the medium. The cultures were shaken at 23 °C for 24 h, and then cell pellets were harvested by centrifugation. The cells were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and the proteins were purified with Chelating Sepharose Fast Flow nickel-charged resin under native conditions with imidazole-based competitive elution. The purified proteins were buffer exchanged into Hank's Buffered Salt Solution (HBSS) using 10 kDa cutoff Amicon protein concentrators and stored in HBSS with 15% glycerol at −80 °C after flash freezing in liquid nitrogen.

Cell Culture and Viability Assay. Human umbilical vein endothelial cells (HUVECs) were purchased from Cell Applications (San Diego, CA) and passages 3−5 were used in these studies. HUVECs were maintained in endothelial cell growth medium. Cell viability in the presence of adiponectin was studied by an MTS assay using the CellTiter 96 AQueous kit (Promega). Briefly, cells were plated at a density of 10^4 cells per well in a 96-well plate and incubated in endothelial cell growth medium for 20 h. The cells were washed with HBSS, and then fresh Dulbecco's Modified Eagle Medium (DMEM) containing 0.5% FBS was added to each well, supplemented with different concentrations of purified adiponectin. In positive controls, cells were incubated with endothelial cell growth medium. After 24 h, the cells were washed with HBSS, fresh DMEM containing 0.5% FBS was added to each well, and the 490 nm absorbance was measured after incubation for 2 h with MTS. Cell death was quantified by the percentage decrease in 490 nm absorbance relative to positive controls. At least three independent experiments were carried out at each condition studied. The difference in cell death rate between cells incubated with adiponectin expressed with and without enzyme coexpression was analyzed by one-way ANOVA. The difference was statistically significant with $P < 0.05$.

RESULTS

We hypothesized that by introducing the enzymatic machinery to post-translationally modify both proline and lysine residues into *E. coli*, adiponectin would be assembled to form functional HMW multimers. We have previously shown that P4H can be activated in the cytosol of *E. coli* by coexpressing the gene for D-arabinono-1,4-lactone oxidase (ALO) from *Saccharomyces cerevisae* to produce ascorbate-like molecules.[17] Because both P4H and LH3 are ascorbate-dependent oxygenases that share the same essential cofactors, we proposed to use the same strategy to activate LH3 in *E. coli*.

Hydroxylases Activated in the Cytosol of *E. coli*. Adiponectin multimer assembly (Figure 1C) depends on post-translational modifications, including proline and lysine hydroxylation, which are catalyzed by prolyl and lysyl hydroxylases. P4H hydroxylates proline residues on collagenous domains (Figure 1A), affecting collagen triple helix stability.[18] Among the human lysyl hydroxylases, LH3 is most likely involved in the post-translational modification of lysine residues in adiponectin. In addition to its lysyl hydroxylase activity (Figure 1B), LH3 has also been reported to possess a low level of glucosyltransferase and galactosyltransferase activities (Supporting Information, Figure S1),[19,20] suggesting that LH3 alone may be sufficient for catalyzing lysine hydroxylation and its further glycosylation in nascent adiponectin.

A reporter peptide sequence based on a fragment of the collagenous domain of adiponectin (AQfrag, sequence: DPGLIGPKGDIGETGVPGAEGPRGFPGIQG) was constructed to enable assessment of the activities of the hydroxylases on their natural substrates. This fragment comprises two canonical sites for proline hydroxylation and one canonical site for lysine hydroxylation and subsequent

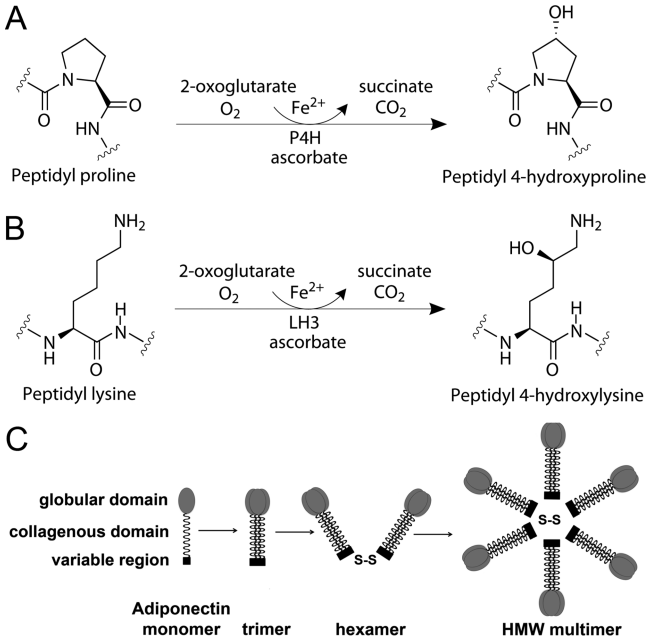

Figure 1. Adiponectin requires hydroxylation for assembly. (A) P4H catalyzes the formation of peptidyl (2*S*-4*R*)-4-hydroxyproline from peptidyl L-proline. (B) LH3 catalyzes the formation of peptidyl 5-hydroxy-L-lysine from peptidyl L-lysine. (C) Schematic representation of adiponectin domains and multimer formation. S−S represents disulfide bonds.

glycosylation. The gene for the AQfrag was C-terminally fused to the purification tag glutathione-S-transferase (GST), with an intervening thrombin cleavage sequence. A map of the resulting plasmid pSD.COLADuet-1.GST-(AQfrag).ALO1 is provided in Supporting Information, Figure S2.

The Origami 2 strain of *E. coli* was chosen for this study, providing the oxidative cytosolic environment necessary for P4H activity.[21] As an initial check on the feasibility of P4H/LH3 coactivation, N-terminally His-tagged LH3 was expressed and purified using the Origami 2 strain of *E. coli*, and hydroxylase activity was measured in vitro (Supplemental Methods in SI). After incubating purified LH3 with the AQfrag peptide and appropriate supplements with and without ascorbate, the substrate peptide was analyzed by liquid chromatography−mass spectrometry (LC-MS). We observed that the peptide was nearly completely monohydroxylated after incubation (Supporting Information, Figure S3), confirming LH3 activity.

The hydroxylation pattern of AQfrag was then studied in the context of in vivo hydroxylase activities in *E. coli* under different coexpression conditions. Plasmids encoding GST-(AQfrag), P4H, LH3 (untagged LH3 was used in all coexpression experiments), and ALO were cotransformed in different combinations into the Origami 2 strain of *E. coli*. After expressing the protein, GST-tagged AQfrag peptides were purified from cell lysates by glutathione affinity, liberated from their purification tags by thrombin cleavage, and analyzed by LC-MS (Figure 2). When AQfrag was expressed by itself or was coexpressed with ALO only, the product was found to be unhydroxylated, with a single observed mass of 1505 Da ($+H^+ + Na^+$ doubly charged species; Figure 2A,B). When the peptide was coexpressed with P4H only, the majority of the peptide was unhydroxylated, with a small population of monohydroxylated product (1513 Da, m/z; Figure 2C). However, when AQfrag

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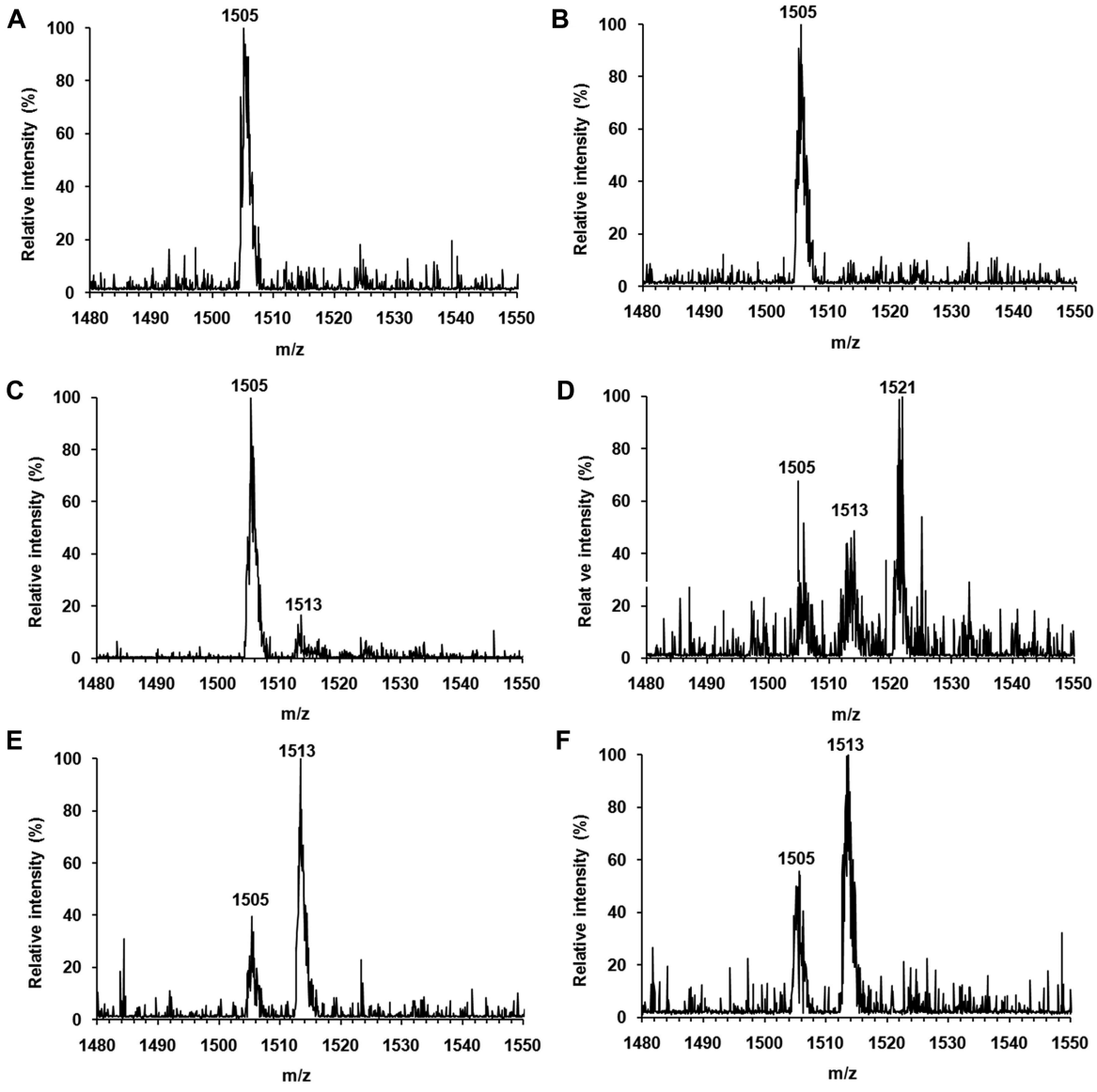

Figure 2. LC-MS analysis of adiponectin fragment hydroxylation. GST-(AQfrag) was expressed in *E. coli* with different coexpressed enzymes conditions: (A) GST-(AQfrag) only; GST-(AQfrag) coexpressed with (B) ALO, (C) P4H, (D) both P4H and ALO, (E) LH3, and (F) both LH3 and ALO. The AQfrag peptides were obtained after thrombin cleavage and analyzed by LC-MS. The observed mass of 1505 corresponds to the doubly charged species ($+H^+ + Na^+$).

was coexpressed with both P4H and ALO, the product showed multiple peaks at 1505, 1513, and 1521 Da (m/z), confirming that substantial hydroxylation of the peptide was obtained when P4H was activated in the *E. coli* cytosol by ALO coexpression (Figure 2D). Coexpression of AQfrag with LH3, with and without ALO coexpression, showed very similar hydroxylation patterns, indicating that LH3 was cytosolically activated to a similar extent with (Figure 2E) and without (Figure 2F) ALO coexpression.

It has been reported that lysyl hydroxylases can be activated by several reducing agents other than ascorbate in vitro, although with lower observed efficiencies, whereas prolyl hydroxylase exhibits a much more stringent dependence on ascorbate.[22] This difference between P4H and LH3 is thus confirmed in our *E. coli* expression system, and our result indicates the existence of certain reducing agents in the Origami strain of *E. coli*, which are sufficient for the activation of LH3 in the bacterial cytosol.

Formation of Adiponectin HMW Multimers in *E. coli* by Activating both LH3 and P4H. After confirming the competence of both P4H and LH3 to hydroxylate adiponectin's collagenous domain in *E. coli*'s cytosol, we used this coexpression system to introduce proline and lysine hydroxylation into human adiponectin. In the natural adiponectin assembly process, the protein first forms homotrimers via the noncovalent interactions in the triple helix structure of the collagenous domains and the hydrophobic interactions within globular domains, which further assembles into higher order complexes via intertrimer disulfide bonds.[14] Post-translational modifications of proline and lysine residues in the collagenous domain have been reported to play an important role in adiponectin assembly.[13] With the novel *E. coli* expression platform developed in this study, we investigated how these two post-translational modifications affect adiponectin multimerization using a bottom-up approach.

The mature adiponectin constituent peptide (full-length adiponectin without its N-terminal signal peptide) was fused to an N-terminal His tag and then inserted into the MCS1 site of the pCOLADuet-1 vector, with or without concomitant insertion of the ALO gene in the MCS2 site. The plasmids were transformed into *E. coli* along with plasmids encoding P4H and LH3. We expressed adiponectin in small-scale cultures to investigate the multimer distribution that was obtained under various coexpression conditions. Bacterial lysate supernatants were analyzed by partially denaturing SDS-PAGE and Western blot. Unheated, nonreducing SDS-PAGE has been used widely to study adiponectin multimerization.[12,14,23] Western blots using antiadiponectin antibody were performed to visualize the various adiponectin multimers (Figure 3A).

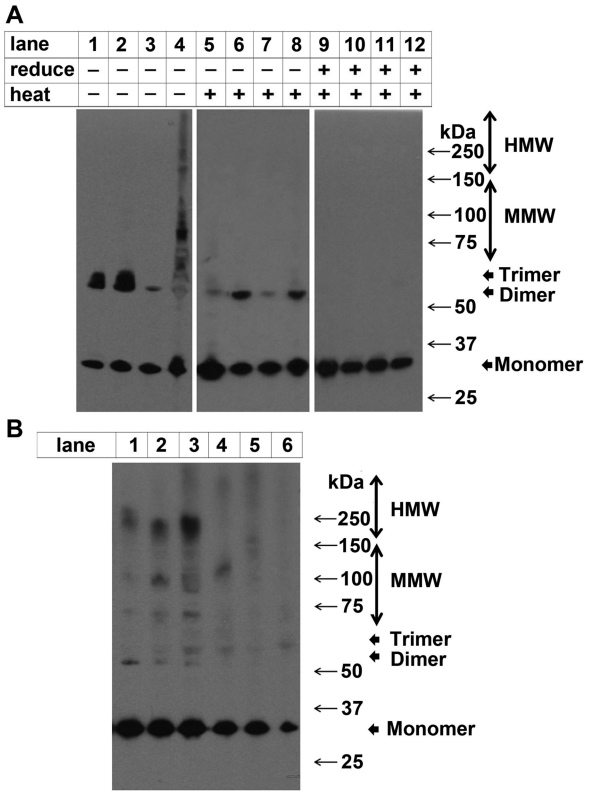

Figure 3. Adiponectin multimer composition analysis by Western blot. (A) Adiponectin was expressed in *E. coli* (23 °C overnight) with different coexpressed enzymes: adiponectin only (lanes 1, 5, 9), adiponectin coexpressed with P4H and ALO (lanes 2, 6, 10), adiponectin coexpressed with LH3 and ALO (lanes 3, 7, 11), adiponectin coexpressed with P4H, LH3, and ALO (lanes 4, 8, 12). After lysing by sonication in DPBS buffer, lysate supernatants were analyzed by Western blot under three different conditions: unboiled, nonreducing; boiled, nonreducing; and boiled, reducing. (B) Adiponectin was coexpressed with P4H, LH3, and ALO under different conditions: 16 °C expression for 24 h (lane 1), 16 °C expression for 48 h (lane 2), 23 °C expression for 24 h (lane 3), 23 °C expression for 48 h (lane 4), 30 °C expression for 24 h (lane 5), 30 °C expression for 48 h (lane 6).

Under unheated, nonreducing conditions, adiponectin assemblies showed a wide range of electrophoretic mobilities when coexpressed with LH3, P4H, and ALO, indicating the formation of a range of different multimeric states (lane 4, Figure 3A). We have observed monomers at about 28 kDa, dimers at about 56 kDa, and other higher molecular weight multimers up to above 250 kDa. Unlike monomers and dimers, larger adiponectin multimers fail to migrate proportionally to their molecular masses in SDS-PAGE,[12] as they retain certain folded structures under SDS-PAGE conditions. Usually, adiponectin trimers migrate only slightly more slowly than dimers, followed by MMW multimers (started with hexamers), and then HMW multimers (12−18mers).[12−14] On the other hand, when adiponectin was expressed without either the prolyl or the lysyl hydroxylase (lanes 3 and 2, respectively, Figure 3A), adiponectin multimers were present only in the low molar mass range. The majority of the protein migrated as monomers, with some dimers/trimers present. To characterize the nature of the different species, we also analyzed the samples under heated/nonreducing and heated/reducing conditions. When the samples were both boiled and reduced, all of the multimers were denatured, and only the monomer bands were detected, for all coexpression conditions (lanes 9−12, Figure 3A). When samples were heat-denatured but not reduced, higher order multimers requiring collagen domain mediated trimerization were dissociated into monomers and dimers (lane 8, Figure 3A). These dimers are likely a result of disulfide-bridged dimerization due to the absence of these species under reducing conditions. HMW adiponectin assemblies were observed only when both P4H and LH3 were activated (lane 4, Figure 3A), which confirmed that both proline and lysine hydroxylation are necessary for adiponectin HMW formation.

We then optimized the expression conditions for HMW adiponectin formation. Since enzyme activities and protein solubility are usually strongly dependent on temperature, we began by coexpressing adiponectin with P4H, LH3, and ALO at three different temperatures (16, 23, and 30 °C). Bacterial cells were collected either 24 or 48 h after protein induction. The resultant multimer distribution patterns were assessed using unheated, nonreducing SDS-PAGE (Figure 3B). We detected HMW adiponectin assemblies only when expression was performed at the two lower temperatures (16 and 23 °C, lanes 1−4, Figure 3B). Induction time also affected the multimer distribution: extended induction at 23 °C led to the dissociation of most of the HMW adiponectin (lane 4, Figure 3B). Of all conditions tested, we found that a 23 °C induction for 24 h generated products with the highest ratio of HMW adiponectin multimers (lane 3, Figure 3B).

Production and Characterization of Recombinant Adiponectin in *E. coli*. To produce and characterize adiponectin using our novel expression system, we expressed N-terminally His-tagged adiponectin under the optimized conditions determined in the small scale test. Adiponectin was either produced without enzyme coexpression or coexpressed with P4H, LH3, and ALO. The proteins were subsequently purified with nickel affinity chromatography. The resulting protein multimer distribution was analyzed by SDS-PAGE (Figure 4). The yields and fractions of different multimers are summarized in Table S1. The results were consistent with what was obtained in the small-scale test expressions. When P4H and LH3 were both activated in the expression system, adiponectin was found to be assembled into higher order structures (lane 2, Figure 4), including HMW multimers, while in the absence of coexpressed hydroxylases the expressed adiponectin was in the form of monomers, dimers, and other multimers in the low molecular weight range (lane 1, Figure 4).

After we obtained HMW multimers of adiponectin in the *E. coli* system, we then studied their bioactivity. Different

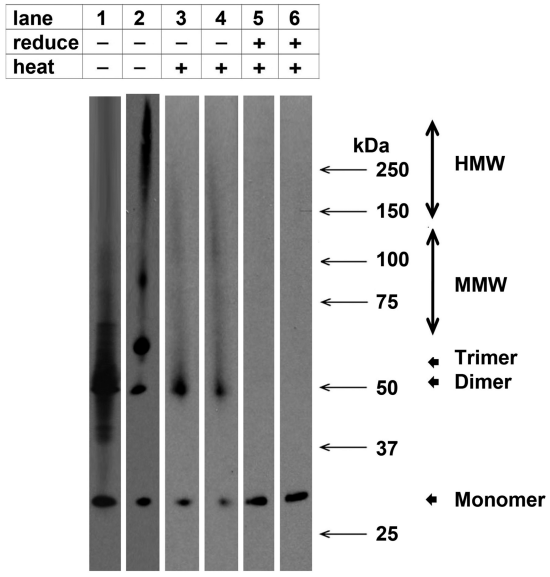

Figure 4. Multimer composition analysis of purified adiponectin. Adiponectin was expressed in *E. coli* with different coexpressed enzymes: adiponectin only (lanes 1, 3, 5) and adiponectin coexpressed with P4H, LH3, and ALO (lanes 2, 4, 6). Purified proteins were analyzed by SDS-PAGE under three different conditions: unboiled, nonreducing; boiled, nonreducing; and boiled, reducing.

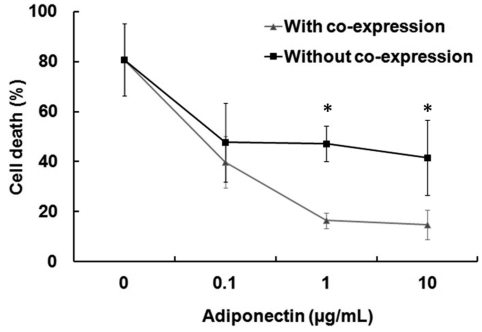

Figure 5. Effects of recombinant adiponectin on HUVEC viability. Cell death was induced by serum starvation in the presence of different concentrations of adiponectin that was produced with or without coexpressed post-translational modification enzymes (P4H, LH3, and ALO). Cell viability was determined by MTS assay. The error bars represent standard deviation. *P < 0.05 of cell death rate between adiponectin with and without enzyme coexpression at indicated concentrations (P = 0.002 at 1 μg/mL and P = 0.046 at 10 μg/mL).

oligomeric forms of adiponectin have been reported to exert distinct biological functions. For example, the HMW form of adiponectin was found to confer, specifically, the vascular-protective activities of this adipocytokine and to suppress endothelial cell apoptosis.[9] Using this previously described cell-based assay, we investigated whether our recombinant adiponectin with HMW multimers can suppress endothelial cell apoptosis in a similar manner. To do this, HUVEC apoptosis was induced by serum deprivation, and the protective effect of adiponectin was investigated by adding different concentrations of adiponectin multimers to the cells. Cell viability was quantified by MTS assay, and cell death percentage was calculated by comparing to counts of cells cultured in complete medium. When no adiponectin was added to the cells cultured in serum-deprived medium, about 80% of the cells were dead after 24 h (Figure 5). However, upon addition of the purified adiponectin that we coexpressed with the essential post-translational modification enzymes, the HUVECs were substantially protected from apoptosis, as compared to results obtained with the addition of adiponectin that was expressed without post-translational enzyme coexpression. For example, at 1 μg/mL of adiponectin concentration, 47.2% of cells were dead at 20 h when incubated with adiponectin without coexpression, while the cell death rate was only 16.5% at 20 h when the cells were incubated with adiponectin that was coexpressed with P4H, LH3, and ALO.

These results indicate that the recombinant adiponectin modified in our *E. coli* system not only assembles into HMW multimers but also that these multimers show the promising ability to exert their expected biological functions in cell culture.

DISCUSSION

Adiponectin has been shown to exert beneficial effects that can significantly reverse metabolic disease, and is a promising drug candidate for treating metabolic disorder-related diseases such as cardiovascular dysfunction, prediabetic conditions such as insulin insensitivity and high blood sugar, as well as diabetes mellitus type II. However, significant hurdles remain to its use as a therapy. In particular, inexpensive production of active material would need to be achieved, and functional expression of adiponectin in *E. coli* is a plausible route to this aim.

Adiponectin's globular domain, which presumably comprises its key binding and recognition interface, was previously expressed in *E. coli* and reported to exert some potentially beneficial functions, that is, to increase the oxidation of fatty acids by muscle.[24] However, the globular domain does not assemble into oligomeric species and is deficient and nonbiomimetic in key aspects. In particular, it fails to affect the glucose production of isolated hepatocytes and it does not decrease serum glucose concentration upon injection in mice.[25]

Previous to this report, assembled multimers of adiponectin had not been produced in *E. coli* as the post-translational machinery enabling key hydroxylations necessary for multimer assembly were not achieved. Bioactive/biomimetic adiponectin multimers had only been obtained through expression in mammalian cell systems. Our novel method of synthesizing biomimetically assembled adiponectin in *E. coli* has the potential to reduce the cost of adiponectin production. Importantly, we expect that expressability in *E. coli* will accelerate research efforts aimed at understanding adiponectin biology, as well as facilitate its development and engineering into a novel and powerful treatment for metabolic diseases.

Comparison of the adiponectin produced in our *E. coli* system with those existing in healthy human serum[12] reveals certain differences in their multimer distributions. For example, adiponectin monomers and dimers are present in the recombinant adiponectin expressed under the current coexpression conditions, while the smallest form of adiponectin present in human serum is trimeric. HMW multimer formation requires post-translational modifications and disulfide bond formation. In addition, processes in the endoplasmic reticulum (ER) or the presence of chaperones may also play a role in the multimerization of adiponectin. For example, ERp44, DsbL-A, and the oxidoreductase Ero1-Lα were reported to delay secretion and facilitate post-translational modification and multimerization of adiponectin by thiol-mediated ER-retention.[26−28] We used an Origami strain of *E. coli* for this study to facilitate disulfide bond formation. However, in our current plasmid constructs, adiponectin, P4H, LH3, and ALO

expression are all under the control of the T7 promoter. Therefore, after IPTG induction, adiponectin (without post-translational modifications) is produced simultaneously with these hydroxylases. Therefore, a certain amount of adiponectin would be unhydroxylated or under-hydroxylated when harvested. This is also supported by the hydroxylation pattern observed for AQfrag peptide (Figure 2). Although it is not necessarily critical for a therapeutic agent to mimic the natural multimerization state of adiponectin, a higher yield of HMW multimers would likely increase the potency of a multimer mixture, as HMW multimers were previously observed to suppress endothelial cell apoptosis.[9] Further engineering of the *E. coli* expression system could be done to allow sufficient time for post-translational modifications and correct assembly of adiponectin. For example, independent control of the expression start and end points for the post-translational modification enzymes could be realized through the introduction of orthogonal promoter induction and shutdown mechanisms for each gene.

In addition to hydroxylation, glycosylation is also found in the collagen domain of natural adiponectin, which may play an important role in its conformation and stability.[29] LH3 was reported to possess a low level of glucosyltransferase and galactosyltransferase activities;[19,20] however, in our system we did not observe any glycosylation of the AQfrag peptide by LC-MS. Although further studies will be necessary to rule out the possibility that the recombinant adiponectin that is produced in our system is glycosylated, our results suggest that our recombinant adiponectin is not glycosylated. There are differences in the multimer distribution pattern, however, between adiponectin produced in our system and that from healthy human serum, as discussed above, and it is reasonable to believe that, although functionally biomimetic, there remain some structural differences between the recombinant HMW adiponectin produced in this system compared to natural adiponectin. Answering these questions definitively, however, will require further study.

We also investigated the biofunction of recombinant adiponectin with a cell based assay. Previous literature has shown that adiponectin produced in mammalian cells (1−30 μg/mL) could reduce HUVEC death, while heat treated adiponectin at 30 μg/mL did not affect HUVEC viability. Further investigation demonstrated that this effect was mainly contributed to by the HMW fraction of adiponectin, instead of the trimer or hexamer form of adiponectin.[9] Our preliminary biofunction assay is consistent with the previous literature[9] in showing that adiponectin with HMW multimers could suppress endothelial cell apoptosis more efficiently than adiponectin lacking HMW multimers, which suggests the effectiveness of HMW multimers produced in our novel *E. coli* system. Vascular protective function is one of the beneficial effects of adiponectin, and our result suggests the potential application of our recombinant adiponectin in preventing vascular diseases. Future work could further expand the biofunction study of adiponectin produced in this system to investigate its effect in protecting against other cardiovascular and obesity-related metabolic dysfunctions.

The technology we describe here provides not only a facile engineering system for the production of proteins assembled via collagen domains, but also a research platform to generate new biological insights. Our novel *E. coli* expression system allows specific activation of certain post-translational modifications. This bottom-up approach opens up a new route to more easily elucidate the in vivo folding and assembly pathway of proteins assembled through collagen domains.

CONCLUSIONS

In summary, we have successfully transferred the post-translational machinery for hydroxylating lysine and proline residues in collagen domain containing proteins from a eukaryotic system into a simple prokaryotic system. Adiponectin was functionally assembled into different multimeric forms including HMW multimers when it was coexpressed with both P4H and LH3, while no HMW adiponectin was generated in the absence of either hydroxylase. To our knowledge, this is the first report demonstrating biological activity conferred to a recombinant biomolecule by post-translational hydroxylation in an engineered bacterial host. The bottom-up approach that we have undertaken to biosynthesize and assemble adiponectin also opens up the possibility of studying the roles of each post-translational modification on bioactivity. This new system allows the production of assembled recombinant adiponectin in a robust and fully characterized expression host, enabling future studies of adiponectin, and is a starting point for a protein engineering approach to develop a drug to treat and prevent cardiovascular and obesity-related diseases.

ASSOCIATED CONTENT

Supporting Information

Methods of His-LH3 gene construction, expression, purification and characterization; (Figure S1) LH3 is a multifunctional enzyme; (Figure S2) plasmid map of activator/reporter plasmid and LH3 plasmid; (Figure S3) in vitro His-LH3 activity assay; and (Table S1) summary of yield and multimer fractions of purified adiponectins. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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