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Disulfide-Dependent Self-Assembly of Adiponectin Octadecamers from Trimers and Presence of Stable Octadecameric Adiponectin Lacking Disulfide Bonds *in Vitro*[†]

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ABSTRACT: Adiponectin is a circulating insulin-sensitizing hormone that homooligomerizes into trimers, hexamers, and higher molecular weight (HMW) species. Low levels of circulating HMW adiponectin appear to increase the risk for insulin resistance. Currently, assembly of adiponectin oligomers and, consequently, mechanisms responsible for decreased HMW adiponectin in insulin resistance are not well understood. In the work reported here, we analyzed the reassembly of the most abundant HMW adiponectin species, the octadecamer, following its collapse to smaller oligomers *in vitro*. Purified bovine serum adiponectin octadecamer was treated with reducing agents at pH 5 to obtain trimers. These reduced trimers partially and spontaneously reassembled into octadecamers upon oxidative formation of disulfide bonds. Disulfide bonds appear to occupy a greater role in the process of oligomerization than in the structural stabilization of mature octadecamer. Stable octadecamers lacking virtually all disulfide bonds could be observed in abundance using native gel electrophoresis, dynamic light scattering, and collision-induced dissociation nanoelectrospray ionization mass spectrometry. These findings indicate that while disulfide bonds help to maintain the mature octadecameric adiponectin structure, their more important function is to stabilize intermediates during the assembly of octadecamer. Adiponectin oligomerization must proceed through intermediates that are at least partially reduced. Accordingly, fully oxidized adiponectin hexamers failed to reassemble into octadecamers at a rate comparable to that of reduced trimers. As the findings from the present study are based on *in vitro* experiments, their *in vivo* relevance remains unclear. Nevertheless, they describe a redox environment-dependent model of adiponectin oligomerization that can be tested using cell-based approaches.

Adiponectin is a peptide hormone secreted from adipocytes with insulin-sensitizing and vascular and cardiac protective functions (*1*−*4*). Expression of the adiponectin gene and circulating adiponectin levels are subjected to regulation by a variety of hormones, cytokines, and transcription factors (*1*). Low levels of circulating adiponectin are associated with insulin resistance, coronary artery disease, and obesity, especially visceral obesity, in humans and animals (*5*, *6*). Mice lacking adiponectin display increased proliferation of vascular smooth muscle cells (*7*) and hypertrophic cardiomyopathy (*8*) and are predisposed to develop insulin resistance (*9*−*11*). Similarly, hypoadiponectinemia appears to be a risk factor for developing insulin resistance or type 2 diabetes in populations from diverse ethnic backgrounds (*6*).

The primary sequence of adiponectin consists of three domains: an N-terminal region, a collagenous domain, and a C-terminal globular domain (*12*, *13*). Through hydrophobic interactions, three individual globular domains form a globular head that is evidenced by X-ray crystallography (*14*). The three collagenous domains extending from the globular head presumably adopt a triple-helical structure that appears on electron micrographs as the stick on the lollipop-shaped adiponectin trimer (*15*, *16*). The N-terminal region contains a conserved cysteine at position 22 of mature mouse adiponectin (position 39 including signal peptide) that is required for oligomerization of adiponectin trimers into hexamers and higher molecular weight (HMW)[1] oligomers (*15*−*17*). Using different assay methods, different groups have alternatively referred to trimers as low molecular weight (LMW) adiponectin (*17*) and hexamers as either medium molecular weight (MMW) (*17*, *18*) or LMW (*15*, *19*) adiponectin. Trimers, hexamers, and the largest HMW species, an octadecamer (*20*), are the three major adiponectin oligomers present in mouse or human serum (*15*, *17*, *21*).

Although a consensus is yet to be reached, accumulating evidence indicates that levels of circulating HMW adiponectin correlate more closely with insulin action than total

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[1]Abbreviations: IAA, iodoacetamide; NEM, *N*-ethylmaleimide; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; DTT, dithiothreitol; βME, β-mercaptoethanol; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfiate; PAGE, polyacrylamide gel electrophoresis; MS, mass spectrometry; CID, collision-induced dissociation; HMW, higher molecular weight; PBS, phosphate-buffered saline; kDa, kilodaltons; ER, endoplasmic reticulum.

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adiponectin (*22*−*26*). The cause for the selective decrease of HMW adiponectin in insulin resistance is currently not understood. It is critical to study the biogenesis of adiponectin oligomers because defining the assembly pathways of different oligomers may help us understand the cause of the reduced circulating levels of HMW adiponectin in insulin resistance. In addition, numerous studies have demonstrated discrepancies between changes in adipose tissue adiponectin mRNA levels and circulating adiponectin concentrations (*27*−*29*). This suggests that factors other than adiponectin gene expression strongly influence circulating adiponectin levels (*30*−*34*). The ability of the endoplasmic reticulum (ER) to undergo oxidative protein folding and properly assemble adiponectin oligomers likely represents one such factor.

Assembly of adiponectin oligomers is poorly understood. The C22 residues in hexameric and HMW adiponectin are fully oxidized to disulfides (*16*). In addition, replacement of the C22 residue near the N-terminus of adiponectin with alanine or serine precluded formation of hexamers or HMW species (*15*−*17*), indicating the importance of disulfide bonds in adiponectin oligomerization beyond trimers. However, while HMW adiponectin is extremely stable under conditions of high salt or pH, it is readily collapsed to hexamers upon modest reduction in pH to 4 or 5 (*15*, *17*). This indicates strong intermolecular forces other than disulfide bonds are needed to maintain the structure of HMW adiponectin. An ER chaperone, ERp44, has been shown to form mixed disulfides with adiponectin via the cysteine residue near the N-terminus (*35*). Downregulation of ERp44 in cultured adipocytes led to increased secretion of trimeric adiponectin and decreased secretion of HMW adiponectin (*35*). Another recently discovered ER chaperone, DsbA-L, has also been shown to promote formation of HMW adiponectin (*36*). However, the molecular mechanisms by which these chaperones promote HMW adiponectin formation are unclear because a basic understanding of the adiponectin oligomer assembly pathway remains lacking.

Studying adiponectin oligomerization *in vivo* entails labeling nascent adiponectin followed by isolation of labeled adiponectin in intact oligomeric complexes. Due to the technical difficulties surrounding these types of studies, we have developed an *in vitro* assay to analyze adiponectin oligomerization using purified bovine serum adiponectin. We show that octadecameric adiponectin could be assembled spontaneously from reduced trimers, but not from fully oxidized hexamers, indicating formation of mature hexamers is a process that is distinct from that of HMW adiponectin. We also identified a non-disulfide-bonded octadecameric complex whose oligomerization state was confirmed by multiple analytical methods. This finding underscores the importance of disulfide bond formation in adiponectin assembly because it is not critical for maintaining the octadecameric structure. Indeed, disulfide bond formation accompanied the appearance of octadecameric adiponectin, and alkylation of cysteines blocked the assembly of octadecameric adiponectin. Taken together, these findings suggest that free sulfhydryls must be available on adiponectin intermediates in order for disulfide bonds to form between distinct trimer subunits during the oligomerization process. The present study provides a framework for defining the assembly process of adiponectin oligomers, which may assist in understanding the decreased levels of HMW adiponectin observed in insulin-resistant states.

EXPERIMENTAL PROCEDURES

Purification of Adiponectin Octadecamers. Adiponectin octadecamers were purified to homogeneity from fetal bovine serum (Atlanta Biologicals, Atlanta, GA) or calf serum (Invitrogen, Carlsbad, CA) as described previously (*37*, *38*) with one additional chromatography step. The eluate from the zinc chelation column was added to reactive green 19 resin (Sigma, St. Louis, MO) equilibrated with PBS, pH 7.6. This mixture was allowed to rock overnight at 4 °C, and the supernatant was recovered and applied to the next chromatography step. The final preparations contained predominantly octadecamers with small amounts of hexamers. The oligomerization state of purified adiponectin octadecamer was confirmed by established gel filtration chromatography and equilibrium sedimentation techniques (*20*, *21*).

Native and Denaturing PAGE Analysis of Adiponectin Oligomers. To analyze oligomerization states of adiponectin under native conditions, samples were diluted with a concentrated native loading buffer to a final composition of 31.25 mM Tris, pH 6.8, 12% glycerol, and 0.05% Orange G. Adiponectin oligomers were fractionated in 7% native Tris−acetate gels that were either purchased (Invitrogen, Carlsbad, CA) or prepared from 30% acrylamide stock solution (37.5:1 acrylamide:bisacrylamide; Bio-Rad Laboratories, Hercules, CA) buffered with 375 mM Tris base titrated to pH 8.5 with glacial acetic acid. The composition of the native running buffer was 25 mM Tris base and 192 mM glycine at pH 8.3. Gels were run at 18 V/cm for 2 h. The oxidation states of the cysteine residue near the N-terminus of adiponectin were determined by nonreducing denaturing PAGE. Samples were denatured by heating at 85 °C for 10−20 min in 246 mM Tris, pH 8.5, 10% glycerol, 0.51 mM EDTA, 0.2 mM Serva Blue R, 0.175 mM Phenol Red, and 3% SDS. Monomeric (reduced) and dimeric (oxidized) adiponectin molecules were separated by discontinuous (11% separating, 5% stacking) SDS−PAGE (*39*) or precast 10% Bis-Tris gels in either MOPS- or MES-based SDS running buffer (Invitrogen, Carlsbad, CA). Gels were stained with Krypton IR (Pierce, Rockford, IL) or Coomassie and visualized using the LI-COR Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). Densitometry was performed using the Odyssey software to quantify the intensity of the bands corresponding to adiponectin oligomers.

Reoligomerization of Adiponectin following 100 mM Dithiothreitol (DTT) and 200 mM β-Mercaptoethanol (βME) Treatment. Purified adiponectin octadecamers (0.25 mg/mL) dissolved in PBS were treated with 100 mM DTT or 200 mM βME and incubated at 37 °C for 60 min. The reaction was then dialyzed against PBS at pH 7.6 using MINI-Dialysis units (Pierce, Rockford, IL) for 3 h. Aliquots were removed at 0, 30, 60, 120, and 180 min and placed on ice after addition of concentrated native loading buffer. At each time point, the remaining sample was placed in fresh dialysis buffer. Separate aliquots of octadecamers were kept in 100 mM DTT or 200 mM βME for the duration of the experiment to assess the requirement for dialysis in reoligomerization. The oligomerization states of the samples were subsequently analyzed using native PAGE.

Effect of Reducing Agent Concentration and pH on Collapse of Adiponectin Octadecamers to Smaller Oligomers. Purified adiponectin octadecamers in PBS (0.25 mg/mL) were treated with 1, 10, or 100 mM DTT for 30 min at 50 °C followed by further incubation at 37 °C for 90 min. Alternatively,

octadecamers were treated with 2, 20, 50, or 200 mM βME for 15 min at 37 °C. At that time an aliquot of each reaction was removed and brought to 50 mM glycine and pH 5. Reactions were further incubated for 15 min at 37 °C. The remaining samples were incubated for 15 min at 37 °C without glycine and at neutral pH. All samples were immediately analyzed using native PAGE as described above.

Reoligomerization of Octadecameric Adiponectin in the Presence of Hydrogen Peroxide. Purified adiponectin octadecamers (0.25 mg/mL) were incubated with 25 mM DTT at 4 °C overnight to reduce the disulfide bonds in adiponectin. The following morning, the pH of the reactions was adjusted to 5 by adding glycine, *N*-acetylneuraminic acid, and glucose to final concentrations of 32.5, 5, and 5 mM, respectively. *N*-Acetylneuraminic acids and glucose were added to reduce aggregation of adiponectin. The reactions were further incubated for 20 min at 37 °C to collapse a majority of adiponectin octadecamers to trimers. The reactions were dialyzed against PBS at pH 7.6 supplemented with 10 μM zinc chloride in the presence or absence of 5 mM hydrogen peroxide in MINI-Dialysis units (Pierce, Rockford, IL). Divalent cations were added to facilitate peroxide-mediated formation of disulfide bonds (*40*). Aliquots were removed and fresh buffer replaced at 0, 30, 60, 120, and 180 min into the dialysis. Half of the aliquots were placed on ice after addition of concentrated native loading buffer while Tris-HCl at pH 7.4 and *N*-ethylmaleimide (NEM) were added to the other half of the aliquots at final concentrations of 100 mM each and incubated at 37 °C for 20 min to fix the redox state of adiponectin. Oligomerization and oxidation states of adiponectin were analyzed by gel electrophoresis as described above.

Iodoacetamide Alkylation of Adiponectin following βME and Low pH Treatment. Purified adiponectin octadecamers (0.16 mg/mL) were treated in 50 mM βME for 15 min at 37 °C. The pH of the reactions was then lowered to approximately 5 with the addition of glycine, *N*-acetylneuraminic acid, and glucose to final concentrations of 32.5, 5, and 5 mM, respectively. After an additional incubation for 20 min at 37 °C to collapse octadecamers to mostly trimers, the pHs of the samples were titrated back to approximately 7 following addition of an equal volume of a solution containing 100 mM Tris base and 100 mM iodoacetamide (IAA). The samples were incubated in the presence or absence of 50 mM iodoacetamide (IAA) at 37 °C for 45 min. The purpose for using high concentrations of IAA was to quench βME and to alkylate the cysteines on reduced adiponectin. Reactions with or without IAA were all dialyzed extensively into PBS containing 25 mM βME to remove excess IAA and the highly acidic byproduct of the alkylation reaction hydroiodic acid. Prior to initiation of reoligomerization by dialysis, the pH of the reactions was measured to ensure they were neutralized. Samples were then dialyzed against PBS at pH 7.6 supplemented with 10 μM zinc chloride and 5 mM hydrogen peroxide for 3 h. At 0, 30, 60, 120, and 180 min into dialysis, aliquots of reactions were removed, and dialysis buffer was replaced with fresh buffer of the same composition. Oxidation states of adiponectin were fixed with NEM as described above. Oligomerization and oxidation states of adiponectin were analyzed by gel electrophoresis also as described above, except the samples for nonreducing SDS−PAGE analysis were heated at 50 °C for 15 min instead of 85 °C. While this lower temperature was unable to convert much of the mature octadecamers to smaller subunits, it was sufficient to collapse the vast majority of intermediates to dimers or monomers.

Reduction of Adiponectin Octadecamers without Significant Conversion to Smaller Oligomers. Purified adiponectin octadecamers in PBS (0.16 mg/mL) were incubated in the presence or absence of 4 mM DTT overnight at 4 °C. The oligomerization state after treatment was assessed using native PAGE as described above and dynamic light scattering and mass spectrometry as described below. An aliquot of the reaction was removed for redox state assessment using nonreducing denaturing gel electrophoresis. Prior to fractionation in SDS−PAGE, the DTT-treated reaction was incubated at 37 °C for 1 h in the presence of 10 mM NEM to alkylate the reduced cysteines of adiponectin and DTT. This procedure served to prevent reoxidation of adiponectin and, more importantly, a false positive result due to artificial reduction of adiponectin by DTT during heat and detergent denaturation prior to gel loading. To determine if DTT was completely inactivated by NEM, levels of DTT remaining in DTT- and NEM-treated samples were determined using Ellman's reagent (*41*) with 2-fold serial dilutions of DTT (from 5 mM to 39 μM) as standards. No DTT was detected in NEM-treated adiponectin samples.

Dynamic Light Scattering Analysis of Adiponectin Oligomerization State. Purified adiponectin octadecamer dissolved in PBS (0.16 mg/mL) was treated with or without 4 mM DTT at 4 °C overnight. Samples were analyzed at 20 °C in a Zetasizer Nano instrument equipped with a 4 mW, 633 nm He−Ne laser (Malvern Instruments, Worcestershire, U.K.). The apparent molecular mass was estimated from empirical relationships between the hydrodynamic radii and known molecular masses of a series of globular proteins. Following data collection, the samples were retrieved from light scattering cuvettes. The oligomerization state of adiponectin was analyzed again using native PAGE. The oxidation state of adiponectin in the samples were fixed by treating them first with 2% SDS for 2 h at 4 °C and then with 25 mM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) for 15 min at 50 °C. Nonreducing SDS−PAGE was used to assess oxidation states as described above.

Mass Spectrometry of Octadecameric Adiponectin. The monomeric molecular mass within bovine adiponectin octadecamers was determined by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) through the University of Arizona Proteomics Consortium facilities. MALDI-TOF mass spectra were acquired using an Applied Biosystems Voyager DE-STR (Framingham, MA), operating a 337 nm nitrogen laser. An aliquot of sample was mixed with an equal volume of a saturated α-cyano-4-hydroxycinnamic acid solution in 50% acetonitrile/50% water containing 0.1% TFA, and then 1 mL was spotted on the target plate and allowed to air-dry prior to mass analysis. Mass spectra were collected in linear mode with an accelerating voltage of 25000 V. The grid voltage was set at 92.5% with an extraction delay time of 1050 ns. A minimum of 400 laser shots at 20 Hz were combined per mass spectra recorded. Octadecamers were partially heat denatured at 75 °C in 1% SDS and 50 mM DTT in PBS and subsequently dialyzed into deionized water supplemented with 0.1% SDS and 50 mM DTT before being mixed with matrix. The oligomerization state of adiponectin was determined by native nanoelectrospray mass spectrometry. Purified HMW adiponectin was buffer exchanged into 200 mM ammonium acetate at pH 7 using microconcentrators (Bio-Rad, Hercules, CA). An aliquot of purified octadecameric adiponectin was also treated with 1 mM DTT at 4 °C for 24 h. Samples were ionized via the nanospray method and analyzed in a Q-Tof 2 quadrupole-time-of-flight mass

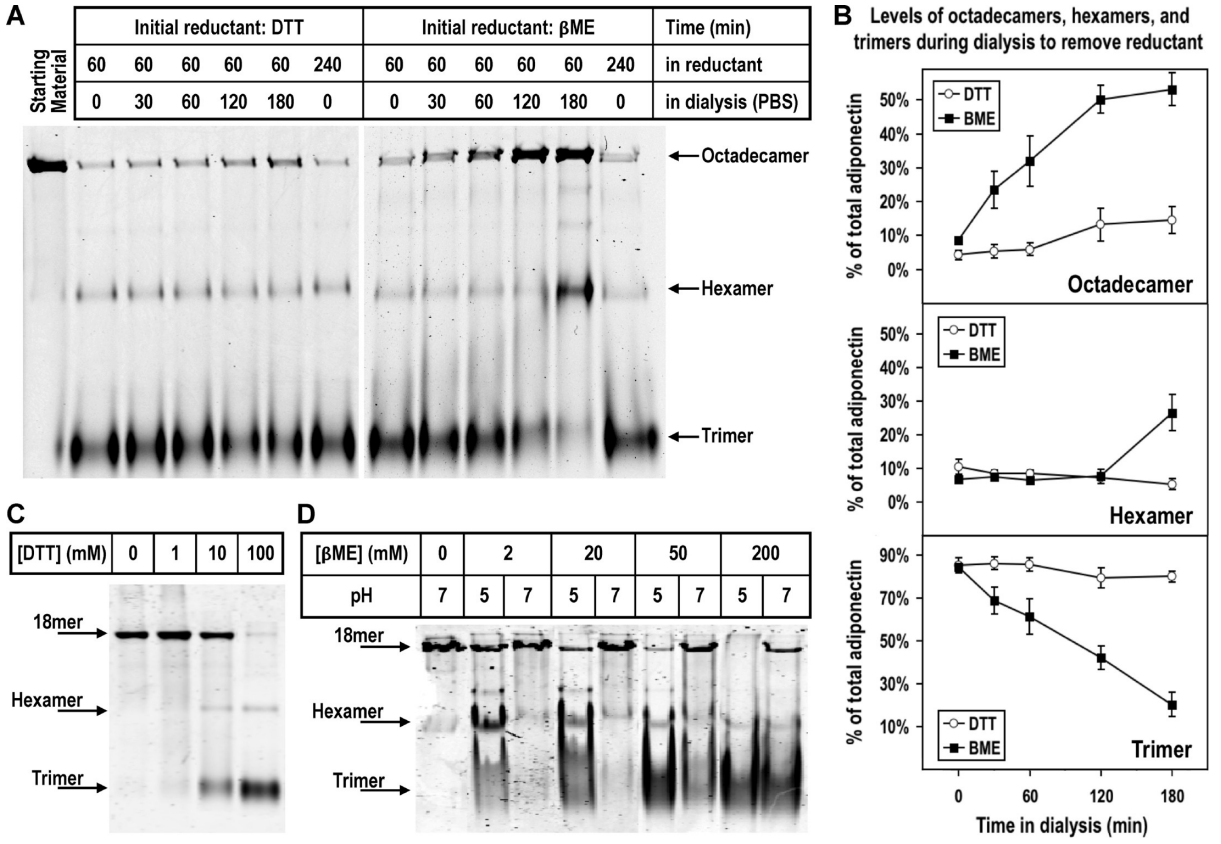

FIGURE 1: (A) Native PAGE analysis of adiponectin oligomeric state following reduction and collapse of octadecamers and subsequent removal of reductant by dialysis. Purified bovine serum octadecamers were collapsed to trimers in 100 mM DTT or 200 mM βME and subsequently dialyzed against PBS over a 3 h period to remove DTT or βME. Following fractionation in PAGE, the proteins were detected by fluorescence from an infrared stain as described in Experimental Procedures. Two samples, one in DTT and the other in βME, were not dialyzed against PBS to assess if reoligomerization to octadecamers depended upon removal of reducing agents. (B) Densitometric analysis of adiponectin octadecamer (top panel), hexamer (middle panel), and trimer (bottom panel) levels during removal of reductant by dialysis following collapse of purified octadecamers. The integrated intensity of each band corresponding to a specific oligomer at a particular time point was normalized to the sum of the integrated intensities of all three major oligomers at the same time point to obtain a value for the percentage of total adiponectin. Average percentage values and standard error of the mean from four and five independent experiments whose initial reductant was respectively DTT (open circle) and βME (closed square) were plotted. (C) Oligomerization state of adiponectin following titration with different concentrations of DTT. Purified octadecamers were treated with 1, 10, and 100 mM DTT and resulting products' oligomerization states were analyzed using native PAGE as described above and in Experimental Procedures. (D) Effect of pH and βME concentration on adiponectin oligomerization state. Adiponectin octadecamers were treated with 2, 20, 50, or 200 mM βME at pH 5 or 7 as described in Experimental Procedures. Native PAGE was used to analyze oligomerization states following the treatments.

spectrometer (Waters Corp., Milford, MA). The cone voltage was varied between 150 and 200 V DC. Charge state distributions were analyzed using the MassLynx software system. For collision-induced dissociation (CID) experiments, ions with a given mass-to-charge ratio were selected with the quadrupole mass analyzer and fragmented through multiple low-energy collisions with argon gas in the collision cell, and the product ions were analyzed in the TOF. The voltage difference between the source hexpole and the collision cell was 175−200 V and analyzer pressure was 3.5×10^{-4} mbar. Additional details for native mass spectrometry and CID have been described previously (*42*).

Collapse of Adiponectin Octadecamers to Hexamers and Subsequent Reoligomerization Studies. Purified adiponectin octadecamers (0.16 mg/mL) were converted to hexamers by reducing the pH of the samples to 5 with addition of glycine to a final concentration of 50 mM and incubated for 30−40 min at 37 °C. Duplicate samples were also treated in 50 mM βME at 37 °C for 15 min to obtain trimers. Both samples were dialyzed against PBS supplemented with 10 μM zinc chloride and 5 mM hydrogen peroxide at pH 7.6 as described above for 1−2 h in a microdialyzer system (Pierce, Rockford, IL). Samples for native PAGE were removed at 0, 30, and 60 min into dialysis. Buffer chamber was replenished with fresh dialysis buffer every 10−20 min. To determine if the hexamers could reoligomerize into octadecameric adiponectin following reduction to trimers, the once glycine-treated hexamer samples were placed in 50 mM βME at the end of a 2 h dialysis period and incubated for 30 min at 37 °C. The samples were again dialyzed for 2 h against the same buffer as the first dialysis. Oligomerization and oxidation states of adiponectin were analyzed by gel electrophoresis as described above. Oxidation states of adiponectin were fixed in 25 mM NEM at 37 °C for 45 min.

RESULTS

Octadecameric Adiponectin Reoligomerizes from Reduced Trimer. In order to resolve the molecular details of adiponectin oligomerization from trimers to hexamers and HMW species, we used trimers from purified adiponectin octadecamers as starting substrates for *in vitro* oligomerization assays. As shown in Figure 1A, DTT (100 mM) and βME (200 mM) treatment at 37 °C for 60 min converted the majority of octadecamers to mostly trimers and a small amount of hexamers. Titration experiments showed that high concentrations of

reducing agents were needed to significantly collapse octadecamers to trimers at neutral pH (Figure 1C,D). The collapsing activities of βME were more pronounced at pH 5 (Figure 1D), indicating the effects of reducing agents and the acidic pH treatment are additive. Following octadecamer breakdown by reducing agents, we attempted to generate stable trimers by removing the reducing agents. Surprisingly, as either reducing agent was gradually removed by dialysis against PBS at pH 7.6, increasing amounts of octadecamers were observed along with decreasing amounts of trimers (Figure 1A,B). Our experimental procedures did not allow us to determine if trimers were stoichiometrically converted to octadecamers because of unequal accessibility to the protein stain dyes among different oligomers in native gels. To determine if removal of the reductants is necessary for oligomerization, an aliquot of octadecamer was kept in 100 mM DTT or 200 mM βME for the duration of the entire experiment including the dialysis period. Unlike the samples that were dialyzed against PBS, neither sample exhibited reoligomerization to octadecamer (Figure 1A). These data indicate that octadecameric adiponectin can be spontaneously assembled from reduced trimers, but removal of the reductant is necessary for reoligomerization to occur.

Formation of Disulfide Bonds Accompanies Assembly of Octadecameric Adiponectin. The secreted HMW and hexameric isoforms of adiponectin are composed of disulfide-bonded dimers (*16*). Studies of adiponectin in which the cysteine residue near the N-terminus was replaced with alanine or serine have demonstrated that oxidation of that cysteine residue is critical for the existence of hexameric and HMW adiponectin (*15*−*17*). To understand the role of cysteine oxidation in adiponectin assembly *in vitro*, we examined whether the reoligomerization of octadecameric adiponectin from reduced trimers is accompanied by formation of disulfide bonds. Nonreducing denaturing PAGE was the method of choice for assessing the oxidation states of adiponectin oligomers, with reduced adiponectin migrating as monomers and oxidized adiponectin as dimers. Prior to denaturation by heat and detergent, the sulfhydryl-alkylating agent NEM was added to protect the oxidation states of adiponectin and to quench the remaining reducing agents that had not been removed by dialysis. In order to maintain a sufficient ratio of NEM to the reductant, octadecameric adiponectin was collapsed to trimers by a decreased DTT concentration of 25 mM combined with a mild acid treatment at pH 5. Data in Figure 1D showed that octadecamers could be collapsed to trimers using lower reducing agent concentrations at pH 5 than at pH 7. As shown in Figure 2, while 25 mM DTT was able to reduce nearly all adiponectin to monomers from dimers, significant amounts of adiponectin remained as octadecamers. Further incubation of DTT-treated octadecamer at pH 5 resulted in near complete collapse to trimers with a minor amount of hexamers (Figure 2A). The hexamers that remained likely represent adiponectin cross-linked at the collagenous domain, a known phenomenon in collagens. The putatively cross-linked adiponectin is also the probable source of the dimers in Figure 2B that could not be converted to monomers by 25 mM DTT in denaturing gels. Figure 2A also shows that as DTT was slowly removed by dialysis, increasing amounts of octadecamers reappeared. This was associated with increasing levels of oxidized adiponectin that migrated as dimers in nonreducing denaturing gel (Figure 2B). Currently, the identity of the electron acceptor for the oxidation of adiponectin is unclear. To further address the role of oxidation in adiponectin oligomerization, we examined

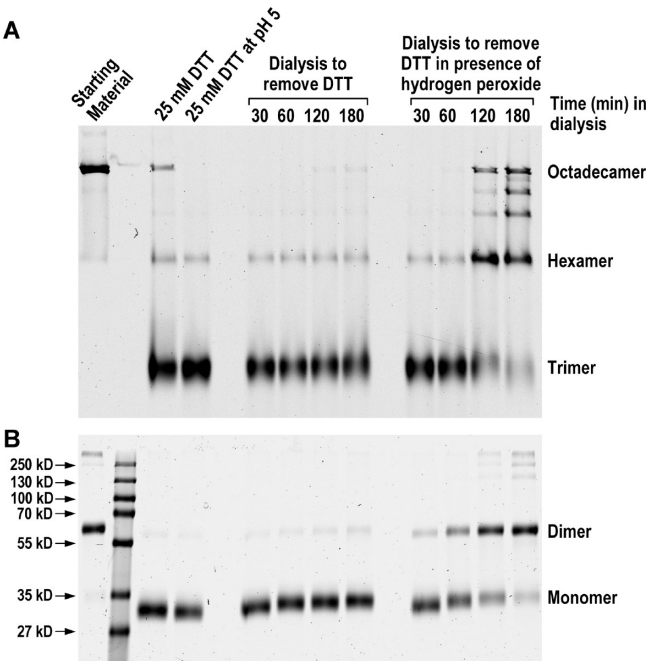

FIGURE 2: Effect of oxidizing agent hydrogen peroxide on (A) reoligomerization of octadecameric adiponectin and (B) oxidation of cysteines near the N-terminus to form disulfide bonds. Purified adiponectin octadecamers were first reduced by DTT followed by lowering the pH to 5 as described in Experimental Procedures to produce trimers. The samples were subsequently dialyzed against PBS at pH 7.6 to remove DTT in the absence or presence of 5 mM hydrogen peroxide over a period for 3 h as indicated. Samples were removed at different time points during the dialysis and fractionated in native gels to monitor oligomerization states and in nonreducing denaturing gels to distinguish the oxidized (dimer) and reduced (monomer) forms of adiponectin. Oxidation states were fixed by NEM treatment.

whether inclusion of a strong oxidizer could accelerate adiponectin oxidation and re-formation of octadecameric adiponectin. Hydrogen peroxide causes the oxidation of cysteines to cystines through a cysteinesulfenic acid intermediate (*40*). Addition of 5 mM hydrogen peroxide during dialysis increased the rate of octadecamer assembly (Figure 2A) and formation of disulfide-bonded adiponectin that was reflected in the increasing ratios of dimers to monomers (Figure 2B). Intermediate oligomers between hexamers and octadecamers were also observed in the presence of hydrogen peroxide (Figure 2A). These results indicate that disulfide bond formation is an important feature in the assembly of adiponectin octadecamer.

Assembly of Octadecameric Adiponectin Requires Formation of Disulfide Bonds. To test if disulfide bond formation is required for octadecamer reoligomerization, we used the thiol-specific alkylating agent iodoacetamide (IAA) to block disulfide formation and examined the effects on assembly of octadecameric adiponectin. Octadecamers were collapsed to trimers by treatment with βME under mildly acidic conditions as described in Experimental Procedures. IAA and βME were chosen because of their relatively low reactivity with each other (*43*). Following near complete collapse of octadecamers, the samples were neutralized and alkylated with 50 mM IAA at 37 °C for 45 min. Reaction of IAA with thiol groups (in cysteine residues and in βME) results in formation of hydroiodic acid, a strong acid that may interfere with the subsequent reoligomerization of octadecamers. The samples were therefore further dialyzed against PBS at pH 7.6 supplemented with 25 mM βME to

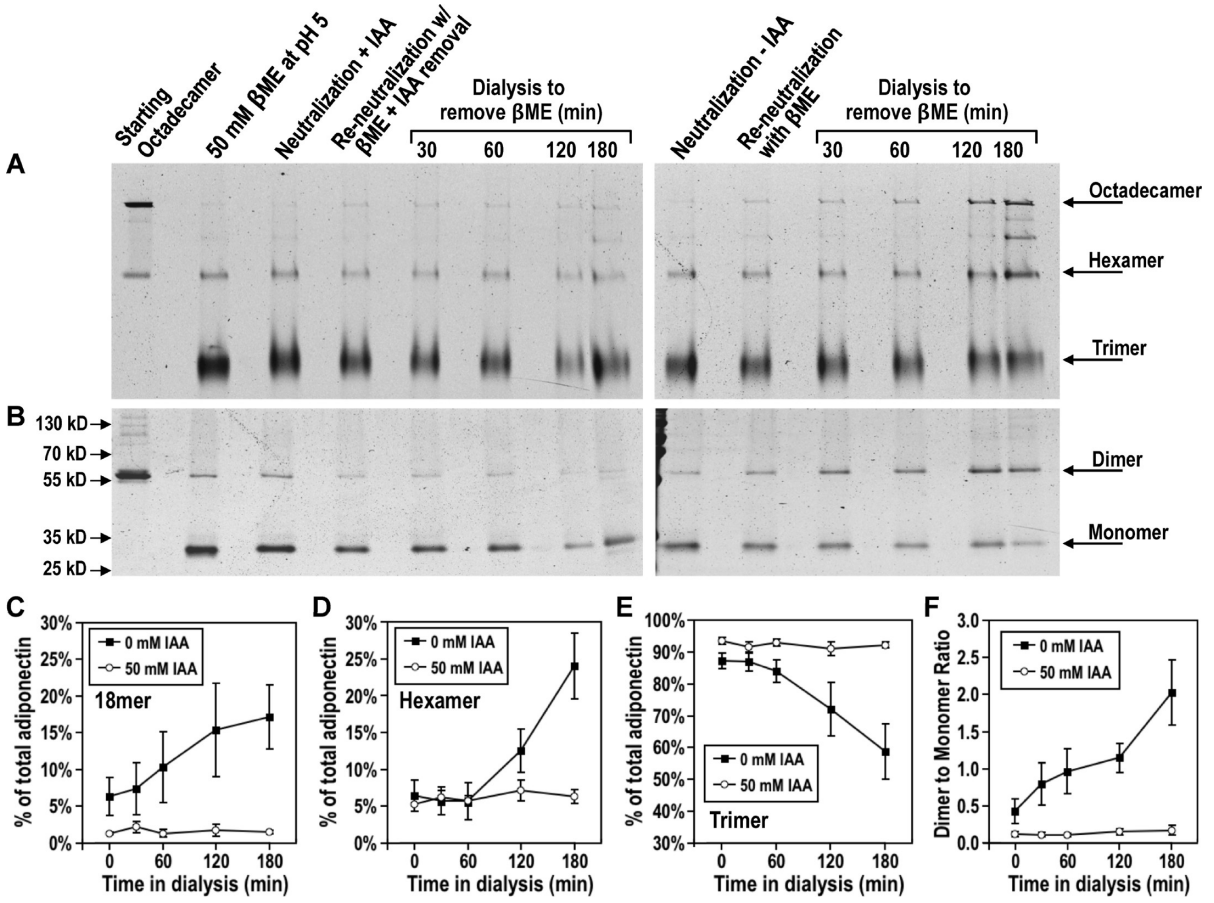

FIGURE 3: Effect of sulfhydryl alkylating agent iodoacetamide (IAA) on (A) reoligomerization and (B) oxidation of adiponectin. Purified adiponectin octadecamers were first reduced by βME followed by lowering of pH to 5 as described in Experimental Procedures to produce trimers. Samples were neutralized in the absence or presence of IAA as indicated and dialyzed against PBS containing 25 mM βME to quench IAA and remove hydroiodic acid, a product of the alkylation reaction. Samples were then dialyzed against PBS to remove βME and allow reoligomerization to occur. Native PAGE analysis was performed to distinguish between the different oligomeric states of adiponectin and assess the extent to which oligomerization was inhibited by lack of disulfide formation following alkylation of reduced cysteines. Oxidation states were fixed by NEM treatment and determined using nonreducing SDS−PAGE. (C−E) Densitometric analysis of (C) octadecameric, (D) hexameric, and (E) trimeric adiponectin during removal of βME following alkylation of cysteines by IAA. Levels of each oligomer as percentages of total adiponectin were calculated as described in legend to Figure 1B. Average percentage values and standard error of the mean from three independent experiments in which collapsed octadecamers were treated with (open circle) or without (closed square) IAA were plotted. (F) Densitometric analysis of disulfide bond formation in adiponectin complexes. The integrated intensities of protein bands corresponding to adiponectin dimers in nonreducing denaturing gels were normalized to the integrated intensities of bands corresponding to monomers in order to obtain a dimer to monomer ratio. Average ratios and standard error of the mean from three independent experiments in which collapsed octadecamers were treated with (open circle) or without (closed square) IAA were plotted.

remove hydroiodic acid and unreacted IAA. Upon returning the pH to neutral, samples were placed in the same dialysis buffer except the 25 mM βME was replaced with 5 mM hydrogen peroxide in order for reoligomerization to proceed. Similar to the results shown in Figures 1 and 2, reduced adiponectin trimers that had not been treated with IAA were able to reoligomerize into octadecamers (Figure 3A, right panel, Figure 3C, and Figure 3E) and to re-form disulfide-bonded dimers as evidenced by an increase in the dimer to monomer ratio (Figure 3B, right panel, and Figure 3F). In contrast, IAA-treated adiponectin trimers showed a greatly diminished propensity to undergo oxidation (Figure 3B, left panel, and Figure 3F). This resulted in a lack of reoligomerization from reduced trimers to octadecamers in samples treated with IAA (Figure 3A, left panel, Figure 3C, and Figure 3E). These findings indicate that assembly of octadecameric adiponectin requires disulfide formation.

Stable Octadecameric Adiponectin in Native Gels following Reduction of Disulfide Bonds. Disulfide bonds between correct pairs of cysteine residues are known to have stabilizing effects on the structures of many proteins. It is possible that disulfide formation is critical for adiponectin oligomerization because it is needed to stabilize the structure of hexameric and octadecameric adiponectin. However, extreme molar excesses of DTT and βME were needed to collapse most octadecamers to trimers (Figure 1), raising the possibility that octadecameric adiponectin lacking disulfide bonds could be stable in solution. The presence of reduced octadecamers argues against a vital structural role for disulfide bonds and instead supports a role in the assembly process *per se*. To examine the possible existence of non-disulfide-bonded octadecameric adiponectin, we treated purified octadecamers with or without 4 mM DTT at 4 °C overnight. Native PAGE analysis of the DTT-treated sample revealed that a majority of the adiponectin remained as octadecamers along with minor amounts as hexamers and trimers (Figure 4A). Following complete inactivation of DTT by NEM, as detailed in Experimental Procedures, analysis of the oxidation states of adiponectin by nonreducing denaturing PAGE showed that the vast majority of the DTT-treated octadecameric adiponectin existed in the reduced state lacking disulfide bonds (Figure 4B). As controls, the non-DTT-treated

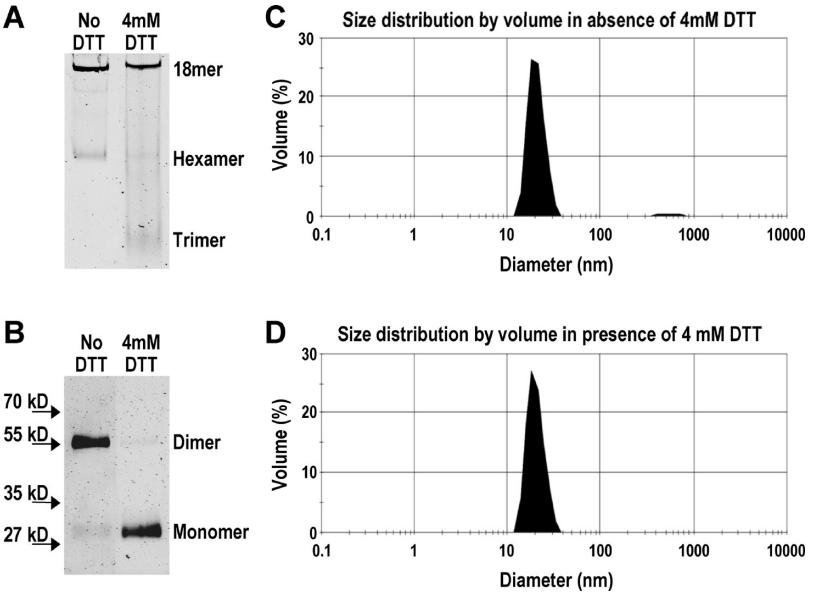

FIGURE 4: Effect of low concentrations of DTT on (A) oligomerization and (B) oxidation states of adiponectin octadecamers using gel electrophoresis and confirmation of oligomerization state in solution by dynamic light scattering under (C) oxidized and (D) reduced conditions. Purified adiponectin octadecamers in PBS were treated with or without 4 mM DTT at 4 °C overnight. Oligomerization state was assessed using native and oxidation state using nonreducing denaturing PAGE. AMS was used to quench DTT and alkylate reduced cysteines to prevent their oxidation during sample denaturation. Samples identically treated as those loaded onto native gels were analyzed using dynamic light scattering as described in Experimental Procedures.

Table 1: Hydrodynamic Parameters of Native and Reduced Adiponectin Octadecamer

sample	% mass	mean diameter (nm)	mode diameter (nm)	standard deviation (nm)	% polydispersity	predicted mass (kDa)[a]
18mer	97.6	20.36	18.17	4.163	20.5	767
18mer + DTT	99.2	20.02	18.17	4.281	21.4	738

[a]The apparent molecular mass was predicted from the known hydrodynamic sizes of a series of globular protein standards.

octadecamers consisted mostly of dimers, indicating they were disulfide-bonded and oxidized (Figure 4B).

Stable Octadecameric Adiponectin in Solution Following Reduction of Disulfide Bonds. To eliminate the possibility that the presence of reduced octadecameric adiponectin in native gels was an artifact caused by aberrant migration of a smaller oligomer, the oligomerization state of DTT-treated adiponectin in solution was assessed using dynamic light scattering. Purified adiponectin octadecamers were treated with 4 mM DTT at 4 °C overnight in the same manner as described above for comparative native and nonreducing denaturing electrophoresis. The hydrodynamic parameters of octadecamers in the absence and presence of DTT are listed in Table 1. The hydrodynamic sizes of oxidized and reduced adiponectin were virtually identical and also in agreement with previous reports (*20*). With the appearance of small amounts of trimers and hexamers following DTT treatment (Figure 4A), the mean diameter of the reduced adiponectin sample was expectedly lower (by 1.7%) than that of oxidized adiponectin octadecamer. The increased oligomer heterogeneity in the reduced sample also led to a slightly higher standard deviation and polydispersity as well as slightly lower apparent molecular mass (Table 1). The mode diameter, a measurement that is less sensitive to the presence of small amounts of trimers and hexamers, was identical between DTT-treated adiponectin and untreated octadecamer (Table 1). The size distributions of both oxidized and reduced adiponectin samples were relatively narrow (Figure 4C,D), reflecting the overall low degree of polydispersity exhibited by both samples (Table 1). Following light scattering analysis, the oligomerization and oxidation states of the samples were confirmed again using respectively native and nonreducing denaturing PAGE (data not shown). These results indicate the presence of abundant and fully reduced octadecameric adiponectin lacking disulfide bonds in a physiological solution.

Simultaneous Demonstration of Reduced and Octadecameric Adiponectin by Mass Spectrometry. While native electrophoresis and dynamic light scattering experiments demonstrated the presence of abundant octadecameric adiponectin following reduction by DTT, the redox state of adiponectin had to be verified separately using nonreducing denaturing PAGE. Ideally, proof of reduced octadecameric adiponectin's existence is best confirmed in a single experiment in which the redox state and oligomerization status of adiponectin are determined simultaneously. We used collision-induced dissociation nanoelectrospray ionization tandem mass spectrometry to accomplish this goal. In agreement with the results in Figure 4A, the native mass spectrum of purified bovine serum adiponectin octadecamers contains a single dominant species centered on the +48 charge state and corresponding to a molecular mass of approximately 475 kDa (Figure 5A). Independently, the molecular mass of monomeric units within purified bovine serum octadecamers was assessed after reduction by DTT and heat denaturation using MALDI-TOF mass spectrometry. The mass spectrum of denatured and reduced adiponectin octadecamers showed a single, but broad, species with a mass-to-charge ratio of 26162.4 (data not shown). Based on the monomeric adiponectin

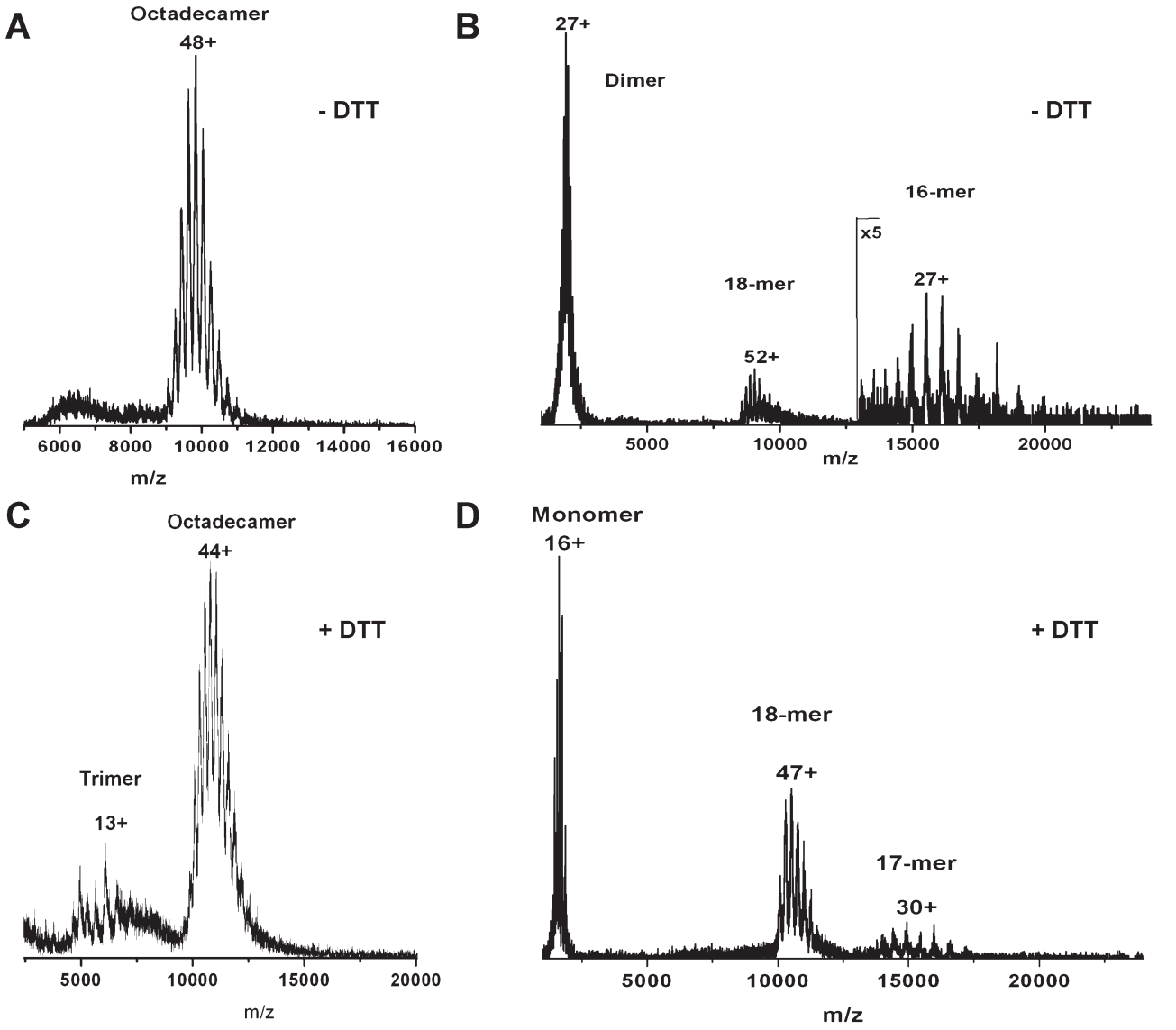

FIGURE 5: (A, C) Native and (B, D) collision-induced dissociation (CID) mass spectra of adiponectin octadecamers in the (A, B) absence or (C, D) presence of 1 mM DTT. Purified bovine serum octadecamers in 200 mM ammonium acetate at pH 7 were treated with or without 1 mM DTT at 4 °C overnight. Native and reduced adiponectin samples were ionized via nanoelectrospray and their mass-to-charge ratios determined in a quadrupole time-of-flight mass spectrometer as described in Experimental Procedures. To assess the oxidation states of octadecameric adiponectin detected in the mass spectrometer, a single charged octadecamer species was selected by the quadrupole mass analyzer and fragmented by collision with argon gas. The mass-to-charge ratios of the fragments were determined in TOF, and the charge state distributions were calculated using the MassLynx software system. The peaks corresponding to the monomers in the CID spectrum in panel D contain a predominant species with a molecular mass of 26206.7. As dimers consist of monomers with different posttranslational modifications, the peaks corresponding to the dimers in the CID spectrum in panel B are broader than those in panel D. The median molecular mass calculated from the range of dimer peaks is 52412.3.

mass determined by MALDI-TOF, the predicted mass of an octadecamer is approximately 471 kDa. This number differs from the native mass spectrometry-derived mass of 475 kDa by 0.8%, consistent with the fact that the native mass spectrometry conditions used typically lead to adducts of the oligomer with solvent and buffer salts. This confirms that the octadecameric structure of adiponectin is maintained in the gaseous environment of a nanospray mass spectrometer. The minimal covalent structure within the native octadecamer was determined by acceleration into an argon-filled collision cell to dissociate the octadecamer. The collision-induced dissociation (CID) spectrum (Figure 5B) shows a dissociation pathway in which the octadecamers predominantly fragmented into dimers and hexadecamers (16mers). This indicates that the smallest covalent subunits within an octadecamer complex are dimers linked by disulfide bonds. Similar to the results obtained in Figures 1B and 4, treating octadecamers with low concentrations of DTT led to the conversion of only small amounts of octadecamers to lower molecular mass oligomers including trimers (Figure 5C). The octadecamers that survived the DTT treatment were selected with a quadrupole mass analyzer and dissociated through collisions with argon. In contrast to native octadecamers (Figure 5B), the CID spectrum of DTT-treated octadecamers (Figure 5D) showed ejection of single monomeric subunits from octadecamers, resulting in the appearance of monomers and heptadecamers (17mers). This indicates that the minimal covalent structure within a DTT-treated octadecamer is a monomer. Using an analytical technique capable of detecting octadecamers, dimers, and monomers simultaneously, the CID of DTT-treated octadecamers into monomers reinforces the results shown in Figure 4

and Table 1 to further confirm the existence of octadecameric adiponectin lacking disulfide bonds.

Impaired Octadecamer Reoligomerization from Fully Oxidized Hexamers. The ability of adiponectin to exist as octadecamers without disulfide bonds argues against structure stabilization of octadecamers as the primary function of forming disulfides during assembly of octadecameric adiponectin. Yet data in Figures 2 and 3 indicate a requirement for disulfide bond formation during octadecamer oligomerization. The most likely conclusion that could account for both lines of experimental observations is a need for disulfide bonds to link intermediates together during octadecamer assembly. This suggests that only the intermediate oligomers that possess free thiols near their N-terminus have the ability to further oligomerize and eventually grow into octadecamers. If this hypothesis is correct, fully oxidized intermediate oligomers without free thiols should lack the ability to oligomerize to octadecamers. Previous studies have shown that HMW adiponectin could be easily converted to hexamers with a modest decrease in pH (*15*, *17*). We thus compared the rate of low pH-derived hexamers to reassemble into octadecameric adiponectin *in vitro* with that of low pH and βME treatment-derived trimers. Without the additive effects of βME or DTT, purified adiponectin octadecamers in PBS were collapsed to mostly hexamers by decreasing the pH of the solution to 5 with glycine alone (Figure 6A). Minor amounts of an oligomer intermediate between hexamers and octadecamers were also observed (Figure 6A). While the oligomerization state of this minor oligomer remains to be defined, its levels could be decreased by adding high concentrations of NaCl (data not shown). Upon dialysis against PBS at 7.6, negligible amounts of octadecameric adiponectin re-formed from hexamers by 1 h (Figure 6A). In contrast, addition of βME to otherwise identically treated octadecamers at pH 5 resulted in appearance of trimers and, following dialysis, reassembly of octadecameric adiponectin (Figure 6A). To confirm that glycine-mediated lowering of pH alone did not produce modifications or conformational changes that disrupted the potential of hexamers to reoligomerize, the fully oxidized hexamers that remained at the end of a 2 h dialysis period were treated in 50 mM βME and dialyzed again under identical conditions and duration as the first dialysis procedure (Figure 6B,C). Following reduction of disulfide bonds by βME treatment to produce trimers (Figure 6B,C) and dialysis, significant amounts of octadecameric adiponectin had reassembled from the same hexamers that had previously failed to reoligomerize under the same conditions (Figure 6B). As shown previously in Figures 2 and 3, the reassembly of octadecameric adiponectin was accompanied by formation of disulfide bonds (Figure 6C). These results indicate that fully oxidized adiponectin hexamers can reassemble into octadecamers at only a very slow rate. They support the idea that disulfide bond formation can facilitate the assembly of adiponectin octadecamers from reduced intermediates by accelerating the rate of oligomerization.

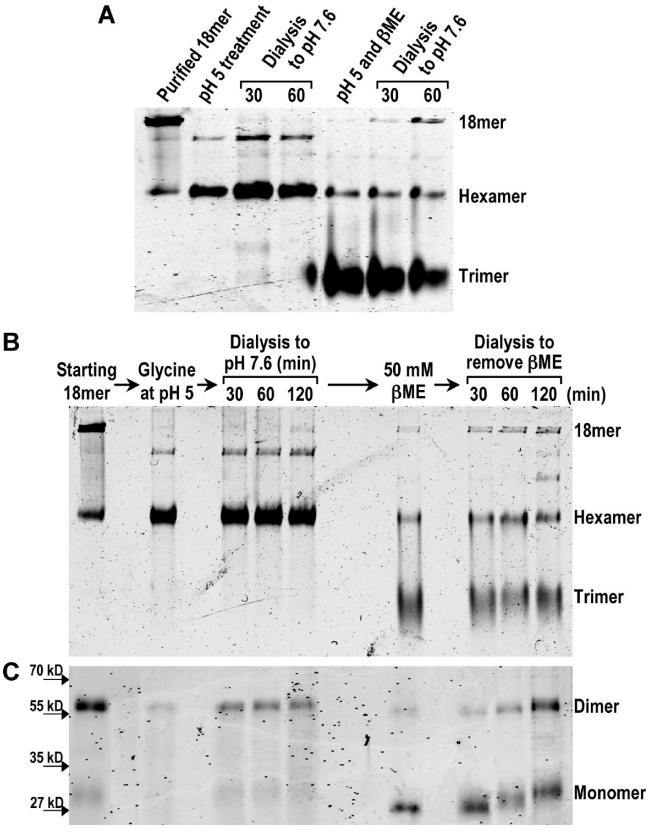

FIGURE 6: (A) Comparison of reoligomerization rates between oxidized hexamers and reduced trimers using native PAGE. Samples of adiponectin octadecamers were converted to mostly trimers by βME treatment at pH 5 while a decrease of pH to 5 alone resulted in mostly hexamers. Samples were dialyzed against PBS at pH 7.6 as described in Experimental Procedures to allow reoligomerization to occur over a 1 h period. (B) Native and (C) nonreducing denaturing PAGE analysis of the low pH-derived hexamers' potential to reoligomerize to octadecamers. Samples of hexamers generated originally by glycine treatment were dialyzed against PBS for 2 h as described in Experimental Procedures. After 2 h of dialysis, the samples were treated with βME to convert oxidized hexamers to reduced trimers, and the abilities of these trimers to reoligomerize to octadecamers and to re-form disulfide bonds over a period of 2 h were monitored in native and nonreducing denaturing gels.

DISCUSSION

The importance of decreased serum levels of HMW adiponectin in insulin resistance is well documented (*22*, *44*, *45*). As recently reviewed (*46*, *47*), mounting evidence indicates that this decrease could be the consequence of defects in adiponectin oligomerization. Currently, it is known that adiponectin oligomerization involves molecular chaperones ERp44 and DsbA-L (*35*, *36*) and ER oxidoreductase Ero1-Lα (*33*). It also requires disulfide bonds between adjacent cysteine residues near the N-terminus (*15*−*17*). However, a detailed molecular description of adiponectin oligomer assembly remains lacking. Without such knowledge, it is impossible to understand the mechanisms by which ERp44, DsbA-L, and Ero1-Lα influence adiponectin oligomerization and why levels of HMW adiponectin are reduced in insulin resistance. Results from the present study indicate that oligomerization of octadecamers, the largest and most abundant of HMW isoforms, occur spontaneously from trimers produced under specific reducing conditions (Figure 1). The only necessary condition is formation of disulfide bonds (Figures 2 and 3). While formation of disulfide bonds is required for reoligomerization of adiponectin octadecamers, we were able to demonstrate the presence of stable and non-disulfide-bonded octadecameric adiponectin using multiple analytical methods (Figures 4 and 5). In addition, fully oxidized hexamers were unable to reoligomerize into octadecamers at a rate comparable to reduced trimers (Figure 6). These *in vitro* data led us to propose that while octadecamers can be formed through spontaneous aggregation of smaller oligomers, the rate of formation is greatly accelerated

by stabilization of intermediate oligomers through disulfide bonds. This conclusion may have potential implications in disease processes. In insulin resistance associated with type 2 diabetes, reduced levels of circulating HMW adiponectin are likely due in part to an impaired oligomerization process (*46*, *47*). If the ER redox condition becomes excessively oxidizing, or if chaperones shielding the unpaired cysteines are lacking, the intermediate oligomers could be trapped in a fully oxidized state that could only oligomerize slowly. The decreased levels of octadecamers associated with insulin resistance could be the result of an abnormally oxidizing state in the ER or decreased levels of chaperones. Indeed, levels of DsbA-L, an adiponectin-interacting ER resident protein with thioredoxin-like domains, have been shown to be reduced in humans and mice exhibiting insulin resistance (*36*).

Thermodynamics of Adiponectin Oligomerization. Our findings have four implications. First, assembly of adiponectin octadecamers could proceed spontaneously through self-association of at least partially reduced trimers. No additional proteins are needed for assembly of octadecamers *in vitro*. Oxidation of cysteine residues near the N-terminus of adiponectin molecules into disulfides also appears to be spontaneous. While reducing equivalents in the form of DTT or βME are needed to break up octadecamers, no energy input in the form of ATP, reducing equivalents, or heat is necessary for reoligomerization of adiponectin. This suggests that both oligomerization of adiponectin and oxidative formation of disulfide bonds are energetically favorable processes. If oligomerization is thermodynamically favorable, needing only reduced trimers as starting substrates, the most likely role of ER chaperones and oxidoreductases is to influence the rate of adiponectin oligomerization and the relative distribution of different oligomers. If the *in vitro* findings in this study hold true *in vivo*, future studies on the regulation of adiponectin oligomerization can focus on the mechanisms by which ER chaperones and oxidoreductases can influence the distribution of hexamers versus HMW adiponectin. Regulation of ER redox state is not well understood but appears to be tightly controlled (*48*−*51*). If the reduction of HMW adiponectin associated with insulin resistance is a characteristic of impaired oxidative protein folding in the ER, understanding the causes of decreased HMW adiponectin formation will likely provide insight into the regulation of the ER redox state.

A major question surrounding the present study is whether the spontaneity of oligomerization is restricted to bovine adiponectin and not shared by mouse or human adiponectin. In a pioneering study using size-fractionated human plasma, Peake et al. observed conversion of adiponectin present in HMW plasma fractions to trimers following treatment with 100 mM DTT at 37 °C for 60 min (*18*). Upon removal of DTT by gel filtration, there was substantial re-formation of HMW adiponectin, even though the authors described the re-formed HMW adiponectin as "not fully assembled to the size of the HMW isoform present in plasma" (*18*). These data suggest that human HMW adiponectin could undergo reoligomerization similar to bovine adiponectin octadecamers. The inability of size-fractionated plasma containing HMW adiponectin to reassemble into *bona fide* HMW species perhaps reflects the presence of proteins found in the same fractions that interfered with the oligomerization process.

Role of Disulfide Bond Formation in Oligomer Assembly. Another implication of our *in vitro* findings is that disulfide bonds may be critical for the oligomerization process of adiponectin octadecamer in the ER in addition to having a structural role in the maintenance of fully assembled octadecamer. In Figures 4 and 5, treating mature adiponectin octadecamers purified from serum with low concentrations of DTT resulted in near total reduction of disulfide bonds but only partial collapse of small amounts of octadecamers to trimers. These data indicate that while disulfide bonds do have a stabilizing effect in maintaining mature octadecamers, their presence is nevertheless not an obligatory requirement for the octadecameric structure. At first glance, this finding appears to contradict the data showing the necessity of disulfide bond formation in octadecamer assembly (Figures 2 and 3). A hypothesis that is consistent with both findings is a need for disulfide bonds to stabilize intermediate oligomers during octadecamer assembly. Indeed, while fully reduced octadecamer was abundantly present in adiponectin treated with DTT, no intermediate species other than small amounts of trimers and hexamers were observed (Figures 4 and 5). This suggests the predicted intermediate nonameric, dodecameric, and pentadecameric species are unstable without any disulfide bonds. In contrast, what appears to be nonameric and docadecameric adiponectin species were clearly visible in Figures 2A and 3A along with octadecamer as disulfide-bonded adiponectin became more prevalent (Figures 2B and 3B). Interestingly, addition of hydrogen peroxide in dialysis buffer accelerated the oxidation of adiponectin (Figure 2B) and formation of octadecamers (Figure 2A). While peroxide has been known to induce cysteine oxidation in aqueous solution (*40*), it was recently shown to accelerate disulfide bond formation in bovine pancreatic trypsin inhibitor (*52*).

The ability to form stable octadecamers lacking disulfide bonds is likely not an exclusive property of bovine adiponectin. Schraw and colleagues successfully demonstrated the presence of an HMW-like complex in addition to trimers in a solution of recombinant mouse C39S adiponectin (C22 residue excluding signal sequence) lacking the disulfide-forming cysteine residue near the N-terminus (*19*). While the authors described the C39S HMW-like complex as having a gel filtration elution profile that was not identical to that of recombinant wild-type mouse HMW adiponectin, their data support the possibility of a higher ordered mouse adiponectin structure without disulfide bonds.

Assembly Pathways of Adiponectin Octadecamers and Hexamers. The third implication of our *in vitro* findings is that assembly of mature adiponectin hexamers and octadecamers is a distinct process. Mature adiponectin hexamers are fully oxidized (*16*), and fully oxidized hexamers failed to reassemble into octadecamers in the time frame during which reduced trimers reassembled into octadecamers in significant quantities (Figure 6). Fully oxidized hexamers may represent a dead end product that is secreted rather than retained inside the cell as precursors for HMW adiponectin. Using disulfide bonds to stabilize intermediates may be the reason why fully oxidized hexamers do not assemble into octadecamers to a significant extent. Fully oxidized hexamers do not have free sulfhydryls that could form additional disulfide bonds with other oligomers. In contrast, fully or partially reduced adiponectin oligomers have the potential to further oligomerize into larger species by forming disulfide bonds with other oligomers. This indicates that while partially overlapping, assembly pathways of hexamers and octadecamers are distinct. Fully oxidized hexamers cannot serve as building blocks for octadecamers. Relative flux through the distinct assembly pathways for adiponectin hexamer and octadecamer could account for differences in the ratio of HMW

to hexameric adiponectin among individuals or various physiological states (*22*−*25*, *44*).

If disulfide bonds indeed are responsible for bridging smaller oligomers and stabilizing larger intermediate structures, as our *in vitro* results indicate, these disulfide bonds must be rearranged prior to secretion of mature octadecamers. Mature octadecamers are not composed of trimers that are all connected to each other through disulfides. Octadecamers could be converted to hexamers without reduction of disulfide bonds (*15*). This indicates that the intermediate oligomers are held together only temporarily by disulfide bonds during assembly. These transient disulfide bonds are subsequently broken and rearranged in a manner that leads to the appearance of three oxidized hexamers within a mature octadecamer. The presence of both oxidized and reduced cysteine residues within a single trimer may facilitate trans-thiolation reactions within the octadecameric intermediate to achieve this maturation. The potential combination of disulfide formation and rearrangement suggests a proper redox environment in the ER is crucial for octadecamer assembly. An excessively oxidizing redox environment, characterized, for example, by high levels of reactive oxygen species, may prematurely lead to fully oxidized hexamers that are unable to oligomerize further. It may also make the predicted rearrangement of disulfide bonds in the final octadecameric intermediate difficult to achieve, leading to decreased secretion of octadecamers. On the other hand, if the redox environment is too reducing, disulfide bonds needed to bridge intermediate oligomers together may not occur at all.

The ER is more oxidizing than the surrounding cytosol (*53*). A crucial question is whether there is a need to protect the cysteine residues of adiponectin from premature oxidation. ERp44 and DsbA-L are ER chaperones that have been shown to influence the oligomerization of adiponectin and/or promote its secretion (*35*, *36*). It is possible that these proteins can shield the cysteine residues of adiponectin, in the case of ERp44 by directly forming mixed disulfides, from premature oxidation and as a result facilitate formation of HMW adiponectin.

Molecular Forces Holding Adiponectin Octadecamers Together. Lastly, the presence of octadecameric adiponectin lacking virtually all disulfide bonds *in vitro* demonstrated strong noncovalent interactions among constituent subunits within octadecamers. HMW adiponectin was previously shown to be easily converted to hexamers under mildly acidic conditions without reduction of disulfides within HMW adiponectin (*15*, *17*). In addition, HMW adiponectin has never been observed to convert to other oligomers in circulation (*15*, *19*, *54*) or *in vitro* under normal storage conditions (*21*). Combined, these two lines of evidence suggest that molecular interactions other than disulfide bonds likely contribute to the stability of HMW adiponectin. Nevertheless, there has been no solid experimental evidence showing the existence of such molecular interactions. Hydroxylation and subsequent glycosylation of lysine residues in the collagen domain of adiponectin have been shown to be important for HMW adiponectin formation (*18*, *55*). However, while glycosylation has been shown to stabilize the triple helical structure of collagen domains (*56*), it is not known to facilitate clustering of multiple collagen triple helices. Based upon electron micrographs, the region showing the most contact among trimers within the octadecamer is the collagen tail (*16*, *20*, *57*). While stability of long collagen fibrils is conferred by hydration forces and entropy associated with hydrophobic forces (*58*, *59*), it is not clear if these interactions are sufficient to cluster short collagen domains like those in adiponectin (only 22 Gly-X-Y or Gly-X-Pro repeats). Data from this study provided unambiguous proof of strong noncovalent interactions that can sustain an octadecameric structure for adiponectin molecules lacking disulfide bonds. We propose that the short collagen domains in adiponectin serve to provide the noncovalent interactions which maintain octadecamers, representing a novel mechanism of protein−protein interaction.

In summary, the present study attempted to address the mechanisms of adiponectin oligomerization using an *in vitro* approach based on octadecamers purified from serum. The results demonstrated abundant presence of stable and non-disulfide-bonded octadecameric adiponectin, implying the existence of strong noncovalent interactions that could drive the spontaneous assembly of octadecamers using disulfide bonds as intermediate-stabilizing forces. We hope these findings will provide the groundwork for developing future hypotheses that can be tested both *in vitro* and *in vivo* to understand the cause of decreased HMW adiponectin in insulin resistance and other disease states.

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