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Zinc enhances adiponectin oligomerization to octadecamers but decreases the rate of disulfide bond formation

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Abstract Adiponectin, a hormone secreted from adipocytes, has been shown to protect against development of insulin resistance, ischemia–reperfusion injury, and inflammation. Adiponectin assembles into multiple oligomeric isoforms: trimers, hexamers and several higher molecular weight (HMW) species. Of these, the HMW species are selectively decreased during the onset of type 2 diabetes. Despite the critical role of HMW adiponectin in insulin responsiveness, its assembly process is poorly understood. In this report, we investigated the role of divalent cations in adiponectin assembly. Purified adiponectin 18mers, the largest HMW species, did not collapse to smaller oligomers after treatment with high concentrations of EDTA. However, treatment with EDTA or another chelator DTPA inhibited the oligomerization of 18mers from trimers in vitro. Zn^{2+} specifically increased the formation of 18mers when compared with Cu^{2+}, Mg^{2+}, and Ca^{2+}. Distribution of adiponectin oligomers secreted from zinc chelator TPEN-treated rat adipocytes skewed toward increased proportions of hexamers and trimers. While we observed presence of zinc in adiponectin purified from calf serum, the role of zinc in disulfide bonding between oligomers was examined because the process is critical for 18mer assembly. Surprisingly, Zn^{2+} inhibited disulfide bond formation early in the oligomerization process. We hypothesize that initial decreases in disulfide formation rates could allow adiponectin subunits to associate before becoming locked in fully oxidized conformations incapable of further oligomerization. These data demonstrate that zinc stimulates oligomerization of HMW adiponectin and possibly other disulfide-dependent protein assembly processes.

Keywords Adiponectin · Zinc · Disulfide bonds · Adiponectin oligomerization · Macromolecular protein assembly · Adipocyte

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Introduction

Adiponectin is an adipocyte-secreted hormone with insulin-sensitizing, cardioprotective, and anti-inflammatory functions (Matsuzawa et al. 2004; Whitehead et al. 2006; Ouchi et al. 2006; Goldstein et al. 2009). The primary sequence of adiponectin has four distinct domains: an N-terminal signal peptide, a region with no recognizable secondary structure but with a stretch of highly conserved residues, a short collagenous triple-helical domain with 22 G-X-Y or G-X-P repeats, and a C-terminal globular domain with a high degree of structural similarity with tumor necrosis

factor-α (Tsao et al. 2002a). The globular domains of adiponectin appear to be the region where contacts with receptors are made (Yamauchi et al. 2003).

With a mature monomeric molecular weight of approximately 25 kD (24.5 kD protein plus approximately 0.5 kD of *O*-linked glycosylation), adiponectin homo-oligomerizes into 75 kD trimers, 150 kD hexamers, and several species of higher molecular weight (HMW) oligomers (Suzuki et al. 2007; Tsao et al. 2002b, 2003), the largest of which is a 450 kD octadecamer (18mer) (Suzuki et al. 2007; Briggs et al. 2009). Of these oligomers, a decrease in HMW adiponectin is most closely associated with a variety of disease states including type 2 diabetes and obesity (Kaser et al. 2008; Basu et al. 2007; Aso et al. 2006; Hara et al. 2006; Pajvani et al. 2004), and its levels are predictive of future development of type 2 diabetes (Zhu et al. 2010). The reason for this decrease is presently unknown. Adiponectin complexes do not interconvert in serum (Schraw et al. 2008; Pajvani and Scherer 2003; Waki et al. 2003), suggesting the cause for decreased HMW adiponectin associated with insulin resistance states lies in decreased production and secretion from adipocytes. These data underlie the importance of studying how adiponectin oligomerizes into the HMW species.

Relatively little is known regarding adiponectin oligomerization mechanisms and the molecular interactions responsible for maintaining the structure of the HMW oligomers. Adiponectin monomers spontaneously trimerize into a globular head stabilized by strong hydrophobic interactions (Shapiro and Scherer 1998) with a triple helical collagenous tail (Tsao et al. 2002a). To generate oligomers larger than trimers, formation of disulfide bonds between conserved cysteine residues located in the highly conserved region near the N-terminus of adjacent adiponectin molecules are necessary (Tsao et al. 2003; Pajvani and Scherer 2003; Waki et al. 2003). We showed recently that while disulfide bonds do contribute to the structural integrity of octadecameric adiponectin, the 18mers are stable in physiological buffer in the absence of disulfide bonds (Briggs et al. 2009). These results indicate that disulfide bonds are necessary for the oligomerization process, with their presumed role being stabilization of intermediate assembly oligomers.

HMW adiponectin collapses to hexamers when pH is lowered to 5 or below but is stable under alkaline conditions (Briggs et al. 2009; Pajvani and Scherer 2003; Schraw et al. 2008; Hada et al. 2007). Although pKa perturbation within proteins' local environment can lead to exceptions, a titration event near pH 5–6 suggests protonation of histidine residues' imidazole groups is responsible for disruption of HMW adiponectin structure. Electron microscopy based structural analysis suggests extensive interactions near the N-terminus of adiponectin 18mers (Radjainia et al. 2008). There are two conserved histidines located near the N-terminal end of the collagenous domain of each adiponectin monomer for a total of six histidine residues per trimer. These two histidines are spaced to form an HXXH motif, a sequence pattern associated with metal-binding (Chakrabarti 1990). This observation led us to hypothesize that acidic pH could result in protonation of metal-chelating histidine residues in adiponectin, thereby releasing the putative coordinated metal and collapsing the HMW complex.

In this study, we examined the requirement for metals in adiponectin oligomerization. We found that metal chelators ethylenediaminetetraacetic acid (EDTA) and diethylene triamine pentaacetic acid (DTPA) inhibited the assembly of 18mers from trimers while Zn^{2+}, but not Cu^{2+}, Mg^{2+}, or Ca^{2+}, specifically enhanced formation of 18mers. One mechanism by which zinc facilitates adiponectin oligomerization is decreased rate of disulfide formation which likely resulted in less trimers becoming oxidized and trapped in a conformation incapable of further oligomerization. Taken together, our data demonstrates that zinc plays an important role in the oxidative assembly of HMW adiponectin.

Materials and methods

Purification of HMW adiponectin

HMW adiponectin was purified from calf serum (Invitrogen, Carlsbad, CA) or fetal bovine serum (Atlanta Biologicals, Atlanta, GA) as described previously(Briggs et al. 2009). Purified bovine adiponectin consists primarily of 18mers with small amounts of hexamers as determined by gel filtration chromatography (Tsao et al. 2002b) and native nano-electrospray ionization mass spectrometry (Briggs et al. 2009). Bovine adiponectin is 87% identical to human adiponectin with 92% similarity over the entire length of the

mature region of the protein (lacking signal peptide) with no gaps. Bovine and human adiponectin have nearly identical molecular mass, pI, and hydrophobicity.

Isolation and preparation of rat primary adipocytes

Visceral fat pads from the ovarian, mesenteric, and perirenal regions were surgically removed from euthanized 6- to 7-week old female Zucker rats, weighed, and placed in Krebs–Ringer Hepes bicarbonate buffer (KRHB, 120 mM NaCl, 4 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM $NaCO_3$, 200 mM adenosine, and 30 mM Hepes, pH 7.4) with 1% Fraction-V BSA and 3.3 mg/ml type II collagenase ($\geq$125 U/mg, Sigma-Aldrich, St. Louis, MO) at a ratio of 1 ml buffer to 1 g tissue. The tissue was minced, transferred to 50 ml flasks, and kept at 37°C in orbital shaking incubator or waterbath rotating at 120 rpm until no tissue chunks were visible. Upon complete digestion and adipocyte dissociation (usually 30 min), the cell suspension was poured through sterile cotton gauze (wetted with KRHB buffer). KRHB buffer was added to raise the total volume of cell suspension to 7 ml and centrifuged for 5 min at 550 rpm. The media below the floating adipocyte layer was removed and replenished with fresh KRHB buffer. This process was repeated for three more times with KRHB and then twice with RPMI media (Invitrogen) supplemented with 1% BSA. After the last wash in RPMI/1% BSA, volume of floating adipocytes was estimated in 15-ml conicals and RPMI/1% BSA was added at 1:1 volume ratio. Aliquots of adipocyte suspensions were transferred to tissue culture plates and maintained at 37°C/5% CO_2 in tissue culture incubator for 16 h in *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) at indicated concentrations. Dimethyl sulfoxide at 0.2% volume was added to both control and TPEN-treated adipocytes. At end of incubation, conditioned media were separated from adipocytes by centrifugation and amounts of secreted adiponectin oligomers were determined by immunoblot analysis detailed below. The adipocytes were lysed in Tris-buffer saline (TBS) supplemented with Complete protease inhibitor cocktail (Roche) by sonication. The Institutional Animal Care and Use Committee of University of Arizona in accordance with the Public Health Service Animal Welfare Policy approved all animal experiment protocols.

Native PAGE and non-reducing SDS-PAGE analysis of adiponectin oligomerization and oxidation states

Native and denaturing gel electrophoresis are used for, respectively, determination of adiponectin oligomerization and oxidation states. In denaturing, non-reducing, gel electrophoresis, adiponectin with reduced thiols migrate as monomers while adiponectin oxidized in disulfide bond forms migrate as dimers. This technique is not capable of differentiating between reduced adiponectin with free thiols from adiponectin with oxidized sulfenic and sulfinic acids and sulfoxides. Samples for native PAGE were prepared and separated in 7% Tris–acetate gels as previously documented (Briggs et al. 2009). Samples for non-reducing SDS-PAGE were prepared and separated in 11% Tris–glycine SDS-PAGE gels as described previously (Briggs et al. 2009). Prior to heat denaturation, molar excess *N*-ethylmaleimide (NEM) was added at the described concentrations to protect adiponectin cysteine residues from becoming oxidized and the disulfides from the reducing agents present in assembly reactions. Native gels were stained using GelCode Blue Stain Reagent (Thermo Fisher Scientific, Rockford, IL) per manufacturer's protocol. SDS-PAGE gels were stained by Coomassie Brilliant Blue G-250 and destained in 25% methanol/10% acetic acid. Stained gels were scanned and intensity of protein bands quantitated using the LI-COR Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE). Due to diffuse trimeric adiponectin bands on native gels, we calculated the amount of the various oligomers of adiponectin by dividing the integrated intensity by the area of the region of interest. The intensities of the dimer and monomer bands on denaturing gels were used to calculate mass ratios of adiponectin dimers to monomers and in turn the mass ratios were multiplied by 0.5 to obtain the mole ratios of dimers to monomers. Oligomeric states of adiponectin in native gels were determined by comparing their migration distances to those of defined standards whose oligomer states were independently verified by gel filtration chromatography or native nano-electrospray ionization mass spectrometry (Tsao et al. 2002b; Briggs et al. 2009). Native and denaturing immunoblot analyses were performed by transferring adiponectin oligomers or adipocyte-conditioned media fractionated under native conditions in 7% Tris–acetate gels or denaturing

conditions in 10% Tris–glycine-SDS gels to nitrocellulose filters at 1 mA per cm^2 gel area for 90 min in a semi-dry transfer apparatus (Hoefer, Inc., Holliston, MA) in 25 mM Tris pH 8.3, 192 mM glycine, and 15% methanol. Filters were blocked in TBS supplemented with 0.1% Tween-20 and 10% non-fat dried milk, pH 7.5 and probed overnight with a rabbit antiserum raised against recombinant globular adiponectin [expressed and purified as previously described (Fruebis et al. 2001)] or purified immunoglobulins to BiP (Cell Signaling) diluted at 1:2000 in the same blocking buffer followed by washing and incubation with horseradish peroxidase—conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). After extensive washing, the membranes were developed using chemiluminescent reagent (Thermo Fisher Scientific) and exposed to blue X-ray films or scanned in ChemiDoc XRS (Bio-rad). The scanned images of the chemiluminescent-exposed membranes were exported to tiff format and ImageJ was used to perform densitometry.

Adiponectin oligomerization assays based on gradual removal of reducing agent

Purified HMW adiponectin at 10 μM monomer concentration was collapsed to trimers in 200 mM β-mercaptoethanol (βME) for 1 h at 37°C. Following the particular treatments in different experiments (described in detail in the Results section), individual reactions were transferred to MINI-Dialysis units (Thermo Fisher Scientific) and reassembly of adiponectin oligomers was initiated by dialysis against PBS to remove βME and allow disulfide bond formation. Concentration of adiponectin was adjusted to 5 μM monomers and that of βME to 100 mM at the start of the reassembly reaction. Aliquots of samples undergoing dialysis were removed at specific time points and degrees of adiponectin oligomerization and oxidation were determined by native and non-reducing SDS-PAGE, respectively, as described above. For native analysis of adiponectin oligomerization states, samples were removed from dialysis and 4× sample loading buffer (400 mM Tris–HCl pH 8.6, 40% glycerol, and 0.01% bromophenol blue) was added to give a final concentration of 1×. Samples were placed on ice until the completion of the time course, then all of the samples were analyzed on native gels. For non-reducing denaturing analysis of adiponectin disulfide-bonding states, aliquots were removed from dialysis units and NEM was immediately added at a 2:1 molar excess of βME concentration before dialysis and incubated at 25°C for 20 min to alkylate thiols present in adiponectin and remaining βME to fix redox states.

In oligomerization experiments requiring faster rates of adiponectin oxidation, purified HMW adiponectin in PBS (10 μM monomer) was first treated with lower concentrations of βME (50 mM) for at least 45 min at 37°C, then concentrated glycine was added to adjust pH to 4 (final glycine concentration approximately 50 mM) and incubated for an additional 30 min at 37°C. After equal volumes of PBS at pH 7.6 were added to re-neutralize the samples to pH 7, various chelators and divalent cations were added and allowed to incubate for a minimum of 10 min at 25°C before start of dialysis against PBS. To accelerate disulfide bond formation, H_2O_2 was added to the PBS dialysis buffer at a final concentration of 1 mM. Concentration of adiponectin was adjusted to 5 μM monomers and that of βME to 25 mM at the start of the reassembly reaction. Samples were collected at 1 and 3 h after the start of dialysis and treated in 50 mM NEM before frozen at −80°C until native and non-reducing SDS-PAGE analyses.

Adiponectin oligomerization assay in redox buffer with defined reduction potential

Purified HMW adiponectin (10 μM monomer) was treated with 10 mM DTT for a minimum of 1 h at 37°C or overnight at 4°C. Afterwards, the DTT was removed by diluting the samples to 0.5 ml with PBS containing a minimum of 1.67 mM reduced glutathione (GSH) and with pH adjusted to 6 by addition of glycine to 25 mM, and centrifuged down to 50 μl in Amicon Ultra-0.5 centrifugal filter units with 30 kDa molecular weight cut-off (Millipore, Billerica, MA). This procedure was repeated six times. Following DTT removal, the concentration of glycine was increased to 50 mM to further reduce the pH to 4 and incubated for 15 min at 25°C to collapse to trimers. The pH of the reactions was then returned to 7 by tenfold dilution in PBS. Subsequently zinc chloride (final concentration 10 μM) or deionized water was added and incubated for another 10 min at 25°C. Oligomerization of adiponectin was initiated by addition of oxidized glutathione (GSSG) to a final

concentration of 1.32 mM. With dilution of GSH concentration to 0.34 mM, the reduction potential of the reactions was -120 mV. Following incubation for the indicated amount of time at 25°C, aliquots of reactions were removed and redox states fixed by addition of NEM to 10 mM. Oligomerization and oxidation states of adiponectin were determined using native and non-reducing SDS-PAGE analyses described above.

Adiponectin reoligomerization assays with hexamers as starting substrate

Purified HMW adiponectin (10 μM monomer) was collapsed to hexamers by decreasing the pH to 4 in 75 mM glycine and incubating for 30 min at 37°C. The samples were divided and allowed to incubate for a minimum of 15 min at 37°C in the absence or presence of 10 μM $ZnCl_2$. The samples were then neutralized by adding 1.5-fold volume of PBS at pH 7.6 (final monomer concentration of adiponectin was 4 μM). To initiate reoligomerization, βME was added to the hexamers at a final concentration of 2 mM and incubated at 37°C. Samples were removed at 5, 15, 60 and 120 min after βME addition. NEM (20 mM final concentration) was added to fix the redox state of adiponectin and the samples were immediately frozen at −80°C. Oligomerization and oxidation states of adiponectin were determined using native and non-reducing SDS-PAGE analyses as described above.

Zinc content in adiponectin

Concentrations of zinc in adiponectin purified from calf serum were determined in an Elan DRC-II inductively coupled plasma-mass spectrometer (ICP-MS) system housed in Arizona Laboratory for Emerging Contaminants. Purified calf serum adiponectin (predominantly 18mers) dissolved in PBS were diluted 50-fold prior to instrument loading. Separate batches of purified calf serum adiponectin were treated first with 25 mM EDTA followed by six rounds of dialysis against 18.2 MΩ deionized water overnight and loading into spectrometer without dilution. No significant differences in average zinc content were observed between samples treated with EDTA and those not treated with EDTA.

Analytical ultracentrifugation

Purified HMW adiponectin (30 μM monomer) was treated with 100 mM βME at 37°C for 1 h followed by adjustment of pH to 4 in 150 mM glycine to generate trimers. The samples were treated with 100 μM TPEN for 15 min at 37°C prior to re-neutralization by dilution in equal volume PBS. The reactions were then dialyzed three times against excess PBS with 10 mM βME in 10,000 MWCO Slide-A-Lyzer dialysis cassettes (Thermo Scientific) for 30–45 min each time to reduce TPEN concentrations. Half of the samples were treated with 100 μM $ZnCl_2$ and oxidative oligomerization was initiated by dialysis in PBS with 1 mM H_2O_2 in the absence or presence of 100 μM $ZnCl_2$. Higher concentrations of $ZnCl_2$ were used in these experiments to saturate the remaining TPEN. Reactions with and without zinc were kept separate. After 2 h at 25°C, the oligomerization states of the reactions were assessed in a Beckman Coulter XL-I analytical ultracentrifuge as previously described (Park et al. 2010) except absorbance scans were taken at 280 nm. The buffers loaded into the solvent compartment of the two-sector sedimentation velocity cells were aliquots of the dialysis buffer that were in contact with oligomerization reactions last. The sedimentation coefficients for adiponectin oligomers were calculated using direct boundary modeling of the sedimentation velocity data using SEDFIT software package (Schuck and Rossmanith 2000). Sedimentation coefficients under standard conditions ($s_{20,w}$) are reported. The partial specific volume of mature bovine adiponectin lacking the signal peptide (0.7236 ml/g at 20°C) and the density and viscosity of the buffer were calculated using the SEDNTERP program (Laue et al. 1992).

Statistical analysis

Data are presented as mean ± SE. Statistical comparisons between one treatment group and the corresponding control group were performed using Student's paired, two-tailed, *t*-test while those between the two groups over several time points were determined by two-way ANOVA with replication. In experiments with more than one treatment group the differences between one particular treatment group and the controls and those between different treatment groups were assessed using one-way repeated-measures

ANOVA with Tukey HSD post hoc test. The particular statistical analyses applied to the results in specific experiments are described in legends to figures and table.

Results

EDTA does not collapse purified HMW adiponectin in absence of reducing agents at neutral pH

Prior studies showed that HMW adiponectin collapses to hexamers under mildly acidic conditions (Briggs et al. 2009; Pajvani and Scherer 2003; Schraw et al. 2008; Hada et al. 2007). As detailed in the Introduction, protonation of functional groups capable of coordinating divalent cations could underlie pH sensitivity of HMW adiponectin. To determine if divalent cation coordination contributes to the structural stability of HMW adiponectin, we treated purified bovine HMW adiponectin at 0.1 μM monomer concentration with 5, 10, or 100 mM of the divalent cation chelator EDTA for 1 h at 37°C and subsequently assessed adiponectin oligomer states by native immunoblot analysis as described in "Materials and methods" section. We observed that EDTA at concentrations as high as 100 mM was not able to collapse the octadecameric isoform of adiponectin (Fig. 1), but lowering the pH of the samples to 4 did convert 18mers to hexamers. We also observed that treating mouse or human serum with 100 mM EDTA at 37°C for 30 min had no effect on the distribution of adiponectin complexes (data not shown).

Divalent cation chelators inhibit the oligomerization of octadecameric adiponectin

It is possible that EDTA was unable to collapse purified adiponectin 18mers in a manner similar to low pH treatment (Fig. 1) because of tighter binding between zinc and 18mers or inaccessibility to zinc binding sites. Adiponectin trimers will be less likely to have high affinity for zinc or buried zinc-binding sites than 18mers, therefore we asked if metals could play a role in the assembly of HMW adiponectin from trimers. To answer this question we collapsed purified HMW adiponectin to trimers by treatment with 200 mM βME then added 20 mM EDTA and assayed

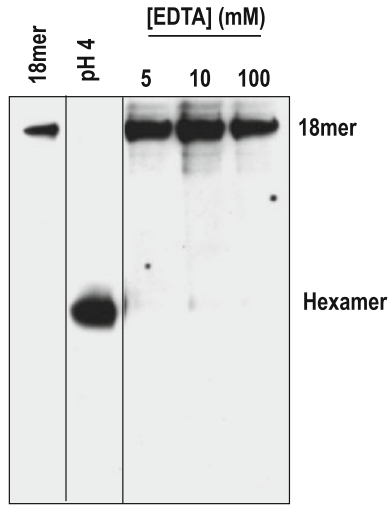

Fig. 1 Stability of adiponectin in EDTA. Purified bovine adiponectin (0.1 μM monomer equivalent) was incubated in the indicated concentrations of EDTA at pH 7 or in 50 mM glycine at pH 4 for 1 h at 37°C. Adiponectin oligomers were separated by native PAGE and their amounts determined by immunoblot analysis as described in "Materials and methods" section. The figure is composed of three different portions of the same exposure (separated by dividing *lines*) and is representative of three independent experiments

oligomerization of adiponectin by dialysis to remove βME over a period of 3 h. Treatment with 20 mM EDTA led to a decrease in the oligomerization of adiponectin 18mers (47.1 ± 2.2% vs. 34.9 ± 1.4% of total oligomers by mass at 180 min for the control and EDTA-treated samples, respectively, Fig. 2a, c). While EDTA treatment had no effect on hexamer levels (Fig. 2a, d), the decrease in 18mer formed was accompanied by higher concentrations of trimers in the EDTA-treated samples (17.2 ± 1.7% vs. 30.7 ± 4.2% at 180 min for the control and EDTA-treated samples, respectively, Fig. 2a, e).

Previously we showed that oligomerization of adiponectin was accelerated when disulfide bond formation was enhanced by H_2O_2 and abrogated when thiols were alkylated (Briggs et al. 2009). To determine if EDTA-induced inhibition of adiponectin oligomerization was caused by decreased disulfide formation, we examined the levels of oxidized dimers and reduced monomers in reassembly reactions over a 3-h period. Despite its inhibitory effect on adiponectin oligomerization, presence of 20 mM EDTA led to increased molar ratios of dimers to monomers (3.49 ± 0.04 vs. 2.80 ± 0.26 at 180 min, Fig. 2b, f) consistent with increased disulfide bond formation.

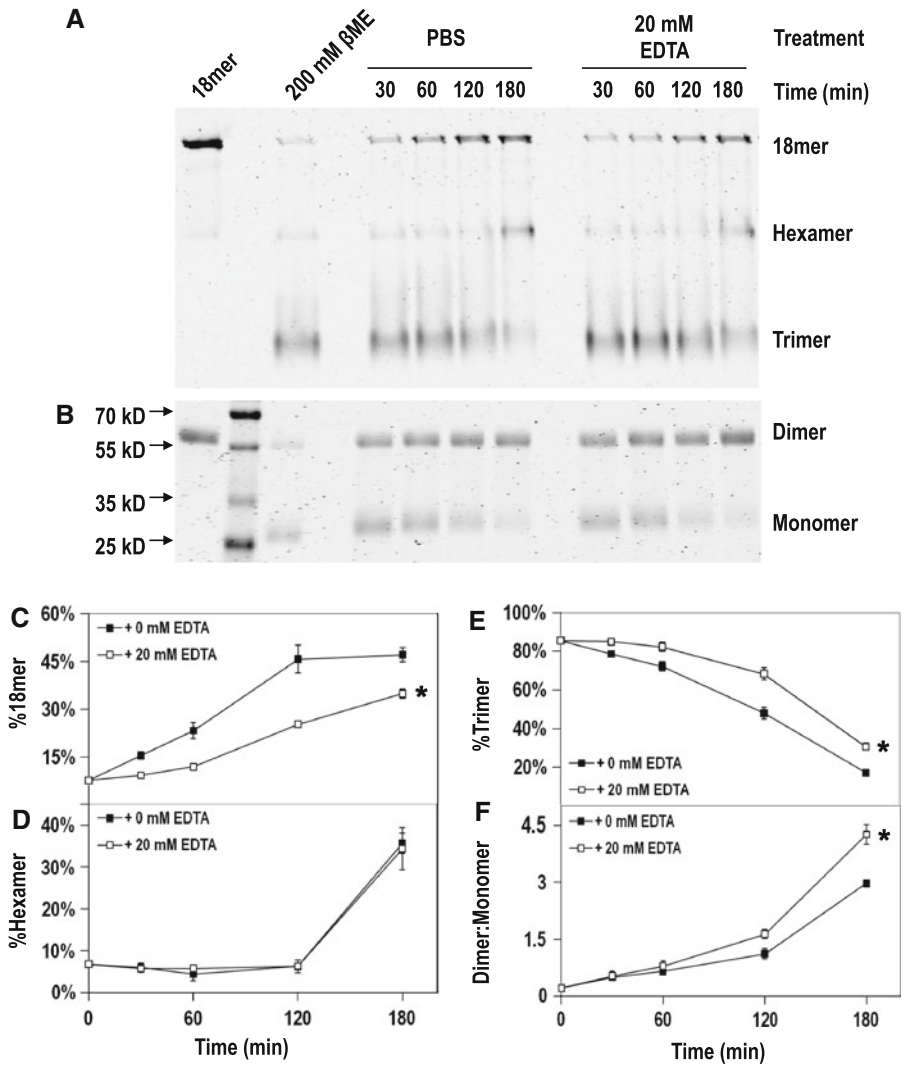

Fig. 2 Reassembly of adiponectin oligomers after treatment with 20 mM EDTA. Purified HMW adiponectin was collapsed to trimers by incubation in 200 mM βME for 1 h at 37°C. Following collapse, EDTA (20 mM final concentration) or PBS was added and incubated for 30 min at 37°C. The individual oligomerization reactions were then started by dialysis against PBS. At 30, 60, 120, and 180 min into dialysis, samples were removed for **a** native PAGE analysis of oligomer distribution and **b** non-reducing SDS-PAGE analysis of adiponectin redox states as oxidized dimers or reduced monomers. Average abundance of **c** 18mers, **d** hexamers, and **e** trimers as percentages of total oligomers by mass was plotted against time in dialysis. The extent of adiponectin oxidation during oligomerization reactions is depicted as **f** mole ratios of dimers to monomers. Data in **c**–**f** were averaged from three independent experiments and presented as mean ± SE. *$P < 0.001$ between treatment groups in two-way ANOVA with replications

Taken together, these data suggest that one or more divalent cations that are chelated by EDTA may facilitate adiponectin assembly despite the decreased rate of disulfide formation.

To ensure that high concentrations of EDTA did not inhibit adiponectin oligomerization by directly binding to adiponectin and physically blocking the association between its subunits, we performed the oligomerization assay in the presence of lower concentrations of EDTA (5 mM) and even lower concentrations (100 μM) of DTPA and neocuproine. DTPA and neocuproine chelate specific metals with higher stability constants than EDTA (Piu et al. 1997; Hart 2000; Smith and McCurdy 1952). The concentration

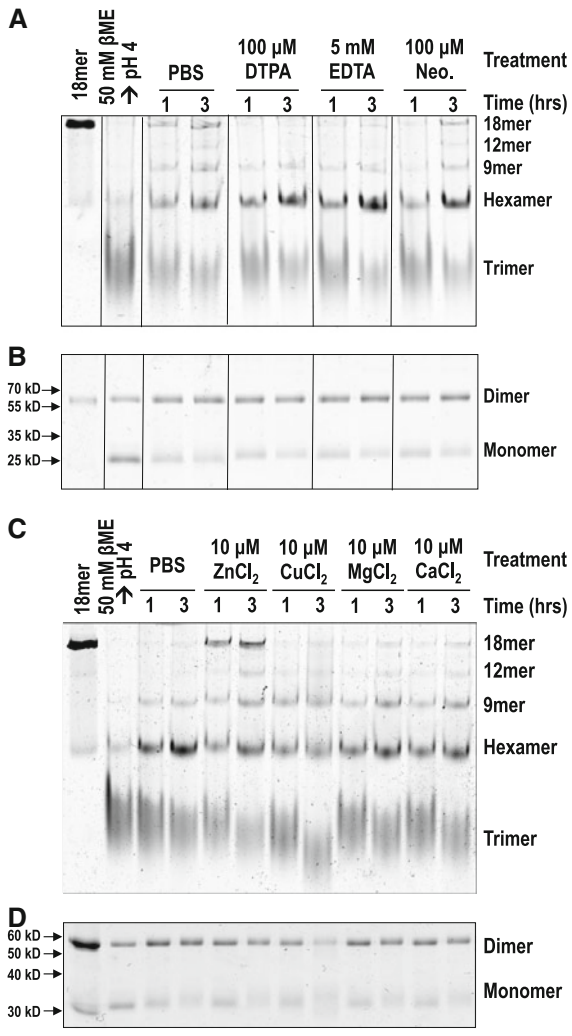

Fig. 3 Adiponectin **a** oligomer distribution and **b** redox state during oxidative reassembly from trimers after treatment with three different chelating agents, DTPA, EDTA, and neocuproine. Purified HMW adiponectin was collapsed to trimers by sequential incubations in 50 mM βME at pH 7 and in 50 mM glycine at pH 4 for 30 min at 37°C each. Upon neutralization with PBS, the samples were treated with 100 μM DTPA, 5 mM EDTA, or 100 μM neocuproine (Neo.) for at least 15 min at 25°C. Samples were then dialyzed against PBS containing 1 mM H_2O_2 for 3 h. At 1 and 3 h, aliquots of reactions were quenched with NEM and assessed for oligomer distribution using native PAGE and redox states using non-reducing SDS-PAGE. Average abundances of the oligomers from four independent experiments are listed in Table 1. Adiponectin **c** oligomer distribution and **d** redox state during reassembly from trimers after treatment with zinc, copper, magnesium, or calcium. HMW adiponectin was collapsed to trimers and allowed to re-oligomerize as described in "Materials and methods" section after the trimers were treated with 10 μM $ZnCl_2$, $CuCl_2$, $MgCl_2$, or $CaCl_2$ prior to dialysis. At 1 and 3 h into dialysis, samples were removed, quenched with NEM, and analyzed using native PAGE for oligomer distribution and non-reducing SDS-PAGE for redox states as oxidized dimers or reduced monomers. At the end of 3 h, 18mers and 12mers accounted for, respectively, 4.6 ± 0.3 and 6.0 ± 0.9% of total oligomers by mass in control reactions. In contrast, reactions in presence of $ZnCl_2$ had 21.6 ± 2.7% 18mers and 9.7 ± 0.6% 12mers after 3 h. Differences in 18mers and 12mers between PBS control and zinc-treated reactions were significant at $P < 0.05$ in paired, two-tailed, Student's *t*-test. There was a tendency for trimers and hexamers to be decreased in zinc-treated groups compared with controls (16.1 ± 0.3% vs. 23.2 ± 2.2% for trimers and 32.6 ± 2.0% vs. 44.8 ± 4.5% for hexamers). There were no statistically significant differences in any of the other oligomers between PBS control reactions and reactions with any of the other divalent cations at the end of 3 h

of βME used to collapse HMW adiponectin was correspondingly decreased and pH was lowered to 4 to complete the collapse to trimers. Upon collapse to trimers, the samples were re-neutralized to pH 7 prior to addition of EDTA, DTPA, or neocuproine and assembly reactions were started by dialysis to remove βME as described in "Materials and methods" section. As disulfide bond formation is necessary for adiponectin oligomerization (Briggs et al. 2009), 1 mM hydrogen peroxide was included in dialysis buffer to accelerate oxidation of adiponectin, but no additional chelators were added to dialysis buffer. We observed that oligomerization of trimers in the presence of 100 μM DTPA led to decreased 18mer formation compared with control reactions without DTPA after 3 h (Fig. 3a; Table 1). There was a strong tendency for oligomerization reactions with 5 mM EDTA to exhibit decreased concentrations of 18mers ($P = 0.08$) after 3 h (Fig. 3a; Table 1). Neocuproine, a 1,10-phenanthroline derivative with methyl groups at the 2 and 9 positions originally identified as a cuprous copper chelator (Smith and McCurdy 1952), was not as effective as DTPA or EDTA in decreasing formation of 18mers (Fig. 3a; Table 1), but presence of each of the three chelators in oligomerization reactions resulted in decreased proportions of 9mers and increased proportions of hexamers generated (Fig. 3a; Table 1). Interestingly, the dimer to monomer ratios of adiponectin following addition of EDTA (2.3 ± 0.2), neocuproine (2.0 ± 0.2), or DTPA (1.9 ± 0.2) were increased ($P < 0.05$ in one-way repeated-measures ANOVA with Tukey HSD post hoc test) when compared with control (1.3 ± 0.1) (Fig. 3b). Collectively, the chelator-induced alterations in the distribution toward increased hexamers

Table 1 Average abundance of adiponectin oligomers as percentages of total oligomers by mass at the end of 3-h reassembly reactions after treatment with listed chelating agents

	PBS (%)	DTPA (%)	EDTA (%)	Neocuproine (%)
18mer	11.9 ± 3.9	2.1 ± 0.4*	3.8 ± 0.8	6.2 ± 1.6
12mer	5.6 ± 0.7	2.6 ± 0.6*	2.7 ± 0.5*	3.7 ± 0.7*
9mer	23.7 ± 1.5	11.7 ± 2.0**	11.0 ± 1.2**	12.5 ± 1.4**
6mer	30.3 ± 1.4	51.1 ± 2.4**	54.1 ± 3.7**	48.2 ± 2.4**
3mer	28.5 ± 3.7	32.5 ± 3.3	28.4 ± 3.9	29.4 ± 3.9

Results are mean ± SE averaged from four independent experiments

* and ** denote, respectively, $P < 0.05$ and 0.01 between control and specific chelator-treated groups using one-way repeated-measures ANOVA with Tukey HSD post hoc test

and decreased higher oligomers during reassembly reactions suggest a role of divalent cations in the enhancement of octadecameric adiponectin oligomerization.

Zinc selectively enhances the oligomerization of octadecameric adiponectin

EDTA and DTPA bind to Zn^{2+}, Cu^{2+}, Ca^{2+}, and Mg^{2+} with varying affinities (Hart 2000), therefore, we assessed oligomerization of adiponectin after treatment with these divalent cations. The counter ion for all reactions was chloride. After collapsing HMW adiponectin to trimers by incubation in βME followed by lowering of pH to 4 as described in "Materials and methods" section, 10 μM of the various metal ions or 5 mM EDTA, as a negative control, were added to individual reactions and allowed to incubate for at least 10 min at 25°C. Reassembly reactions were then initiated by dialysis against PBS, pH 7.6, containing 1 mM H_2O_2. After 3 h in dialysis, samples with prior Zn^{2+} treatment showed on average a 370 and a 62% increase in the proportion of 18mers and 12mers, respectively, compared with controls (Fig. 3c). Notably, no differences in the concentrations of adiponectin dimers and monomers were observed (Fig. 3d), indicating that adiponectin redox status did not change. The other divalent cations tested did not affect the oligomerization or oxidation of adiponectin during reassembly (Fig. 3c, d). These data indicate a prominent and specific role for zinc in enhancing adiponectin oligomerization in vitro.

To determine the effect of Zn^{2+} on adiponectin oligomerization from reduced trimers using a technique independent of native gel electrophoresis, we performed analytical ultracentrifugation to distinguish adiponectin oligomers using sedimentation velocity. Figure 4 shows absorbance scans and the fitted c(s) distribution from selected sedimentation velocity experiments in the absence or presence of 100 μM $ZnCl_2$. Compared with reassembly reactions in absence of zinc (Fig. 4a, b), those performed in the presence of 100 μM zinc (Fig. 4c, d) showed a 290% increase and a 66% decrease, respectively, in the amount of 18mers and 6mers as percentages of all oligomers (Fig. 4b, d).

Oligomerization and oxidation of adiponectin in the reassembly experiments described above proceeded gradually as reducing agents were removed and oxidizing agents introduced by dialysis. We next asked if zinc ions can enhance adiponectin 18mer formation when reducing agents were first removed before reoligomerization was started by direct incubation in defined redox buffers. After treatment with DTT to reduce disulfide bonds and before lowering of pH to 4 to fully collapse purified HMW adiponectin, DTT was removed by successive rounds of dilution in PBS and concentration in centrifugal filter units as described in "Materials and methods" section. Upon neutralization by dilution in PBS, collapsed adiponectin was allowed to re-oligomerize in the presence or absence of 10 μM zinc chloride in the physiologically relevant glutathione redox buffer maintained at a relatively oxidizing −120 mV reduction potential, which is conducive to disulfide bond formation. As shown in Fig. 5a, based on averages of two independent experiments, the addition of Zn^{2+} increased the abundance of adiponectin 18mers and at the 2-h time point by 145.5%. The increased assembly of 18mers was accompanied by a decrease in hexamer and trimer concentrations 2 h into incubation with the glutathione

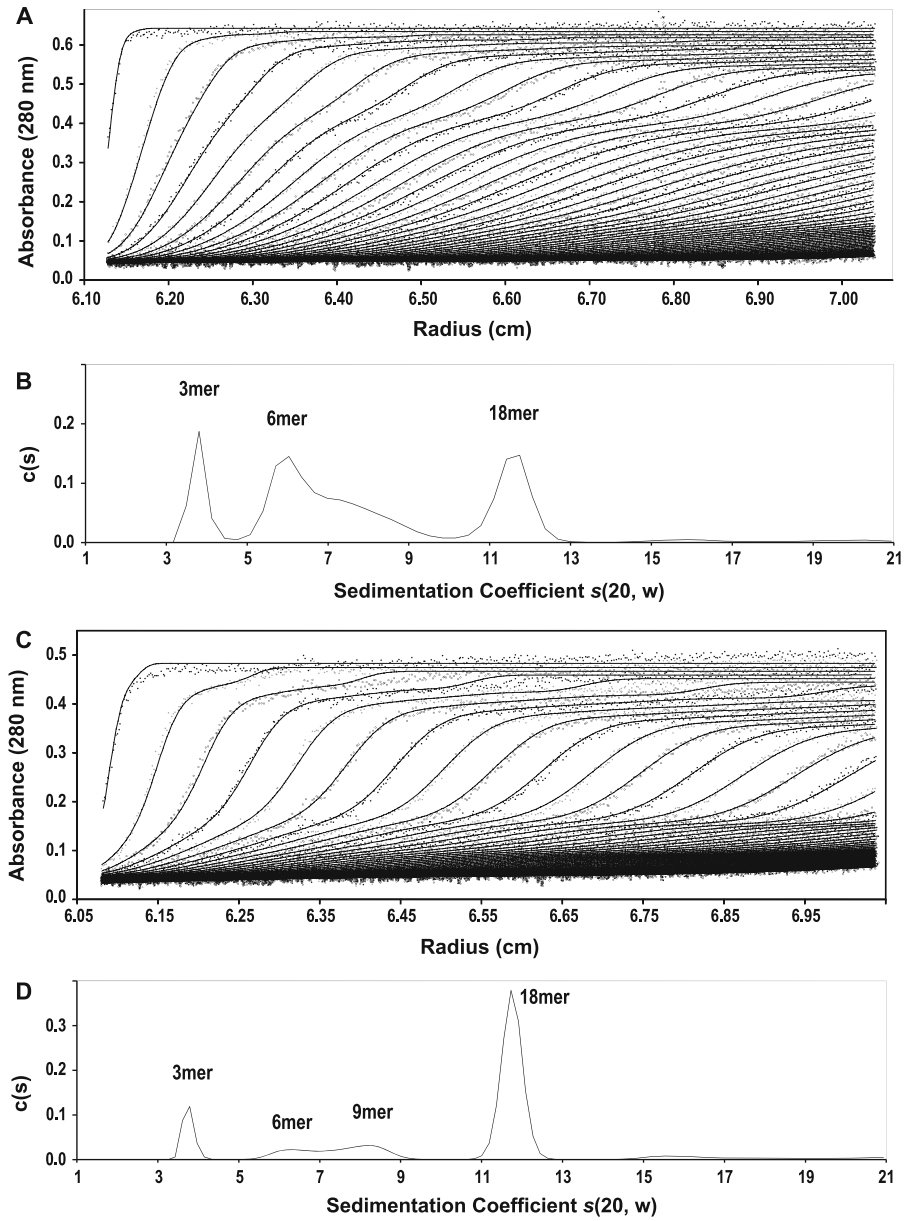

Fig. 4 Sedimentation velocity analyses of adiponectin oligomers during oxidative reassembly reactions in **a**, **b** absence or **c**, **d** presence of 100 μM $ZnCl_2$. Shown in the figure are **a**, **c** absorbance scans (*circles*) and the fit curves (*solid curves*) and **b**, **d** sedimentation velocity c(s) distributions from selected oxidative adiponectin reassembly reactions as described in detail in "Materials and methods" section in presence or absence of zinc. Of the three independent reassembly reactions in absence of zinc, the one with the highest proportion of 18mers is shown. Of the four independent reassembly reactions in presence of zinc, the one with the lowest proportion of 18mers is shown. Individual absorbance readings from consecutive scans are separated by use of *solid*, *shaded*, and *open circles* for better visibility. On average the oligomer distributions in assembly reactions conducted with zinc were 69.4 ± 1.4% 18mers, 17.6 ± 1.5% combined 6mers and 9mers, and 13.0 ± 0.3 trimers. Average oligomer distributions in reactions without zinc were 17.7 ± 5.0% 18mers, 51.2 ± 1.4% combined 6mers and 9mers, and 31.1 ± 6.4% trimers. 6mers and 9mers could not be clearly distinguished in some of the reactions and are combined in calculations of percentages of oligomers. Differences in 18mers and combined 6mers and 9mers between samples with and without zinc were significant at, respectively, $P < 0.01$ and $P < 0.001$ in unpaired, two-tailed, Student's *t*-test

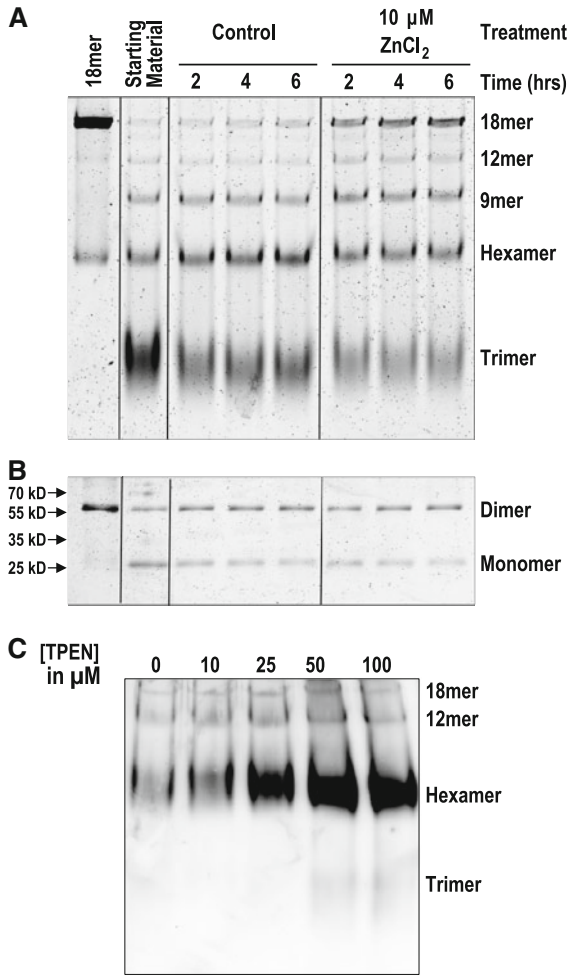

Fig. 5 Adiponectin **a** oligomerization and **b** redox states during reassembly of oligomers in glutathione-based redox buffer maintained at −120 mV reduction potential after treatment with zinc chloride. Purified HMW adiponectin was reduced by incubation in 10 mM DTT, which was subsequently removed by repeated buffer exchange in centrifugal filtration units as described in "Materials and methods" section. Upon collapse in pH 4 and re-neutralization, reassembly of adiponectin oligomers was started by addition of oxidized and reduced glutathione at final concentrations of 1.32 and 0.34 mM, respectively. After 2, 4, and 6 h in glutathione-based redox buffers, samples were removed and analyzed for adiponectin oligomerization states using native PAGE and redox states using non-reducing SDS-PAGE. **c** Secretion of adiponectin oligomers from isolated rat primary adipocytes following 16 h of treatment in 0, 10, 25, 50, and 100 μM TPEN. Adipocytes from visceral adipose depots were prepared as described in "Materials and methods" section. The amounts of adiponectin oligomers secreted into conditioned media at the end of incubation period were determined using native immunoblot analysis. Multiple exposures of chemiluminescent-treated blots represented in **c** were taken and densitometry was performed on those with comparable intensities among adipocyte-conditioned media from different rats using ImageJ. Results were averaged from adipocyte-conditioned media prepared from three different rats. While there were no differences in the amounts of 18mers and 12mers secreted with or without TPEN, secretion of hexamers increased in a TPEN dose-dependent manner. At 50 and 100 μM TPEN, average amount of hexamers in adipocyte-conditioned media was approximately twice that of non-treated control groups (5859 ± 1077 arbitrary densitometric units for control groups vs. 12093 ± 1282 and 11105 ± 1073 for, respectively, 50 and 100 μM TPEN-treated groups, $P < 0.05$ and $P < 0.06$ in paired Student's *t*-test). Although not statistically significant, there was a trend for increased secretion of trimers upon TPEN treatment

redox couple (12.1 and 24.5%, respectively, compared with control reactions lacking zinc, Fig. 5a). We were unable to completely prevent disulfide bond formation during removal of DTT by repeated dilution and centrifugation (Fig. 5b). Nevertheless, incubation in glutathione redox buffer did lead to increased disulfide bond formation by 2 h and no differences in dimer and monomer levels were observed between zinc-treated and control groups at all time points (Fig. 5b). These results indicate that zinc can enhance adiponectin oligomerization under defined redox conditions.

TPEN-treated rat adipocytes secreted higher levels of adiponectin hexamers and trimers

Because zinc enhanced oligomerization of adiponectin 18mers and 12mers in vitro (Figs. 3, 4, 5), we investigated whether treating isolated rat primary adipocytes with the zinc-selective and cell-permeable chelator TPEN would inhibit HMW adiponectin formation and secretion ex vivo. TPEN-treated primary adipocytes secreted increasing amounts of hexamers in a dosage-dependent manner (Fig. 5c). In addition, we observed consistent secretion of trimeric adiponectin from adipocytes treated with 50 μM and 100 μM TPEN while none of the mock-treated adipocyte samples exhibited any secretion of trimers (Fig. 5c). In contrast, TPEN did not produce significant changes in the amount of secreted HMW adiponectin between treated and control adipocytes (Fig. 5c), resulting in a pattern of adiponectin oligomer distribution in which secreted hexamers predominated over 12mers and 18mers upon TPEN treatment. TPEN is known to induce ER stress (López et al. 2009). To determine if adipocytes treated with TPEN displayed ER stress, we assessed the levels of the ER stress marker binding immunoglobulin protein (BiP)

in cell lysates using immunoblot analysis. Of the three different rats from which adipocytes were prepared, we detected BiP in adipocyte lysates from only one rat, and no differences in the levels of BiP were observed between cells treated with varying concentrations of TPEN (data not shown, lysates from 3T3-L1 adipocytes treated with thapsigargin used as positive control). The relatively high hydrophobicity of TPEN likely led to significant distribution into the large lipid droplet that occupies greater 90% of an adipocyte's volume and limiting its ability to induce ER stress.

Zinc inhibits disulfide bond formation in adiponectin

To determine potential mechanisms by which zinc enhances adiponectin 18mer oligomerization, we performed ICP-MS analyses for zinc on 6 different batches of purified calf HMW adiponectin. Zinc was present at an average ratio of 1.14 ± 0.37 trimers per zinc ion. Data in Fig. 5c showed that TPEN-treated adipocytes secreted significantly higher amounts of hexamers without producing more HMW adiponectin, suggesting zinc may inhibit disulfide formation between trimers to form hexamers. In addition, zinc and other metals are known to coordinate with reduced cysteines in proteins and protect them from oxidation (Morgan et al. 2009; Maret 2006; Paget and Buttner 2003; Wilcox et al. 2001; Krezel et al. 2007; Li and Maret 2009). We therefore postulated that zinc could be keeping the cysteines in adiponectin reduced during the oligomerization process. We previously showed that fully oxidized hexamers undergo additional oligomerization to 18mers poorly because additional disulfide bonding needed to stabilize intermediate 9mers and 12mers could not occur (Briggs et al. 2009). As shown in Fig. 5b, the oxidation states of adiponectin are similar between zinc-treated and control groups from 2 to 6 h, indicating the assembly reaction had reached completion before the first time point. It is possible that effects of zinc on adiponectin redox state could be occurring at earlier time points when more cysteines are available to coordinate Zn^{2+}. To investigate assembly and oxidation at earlier time points, we examined adiponectin oligomerization and redox states at 10, 20, 30, 45, 60 and 120 min after initiating the reaction by incubating in glutathione redox buffer kept at −120 mV. The addition of zinc to the reaction led to a lower oxidized-dimer-to-reduced-monomer-ratio at time points from 10 to 45 min (Fig. 6b, d). These results are indicative of reduced disulfide bond formation rate. The mole ratios of dimers to monomers in reactions containing Zn^{2+} were 43, 57, and 65% of those in reactions without Zn^{2+} at, respectively, 20, 30, and 45 min time points ($P < 0.05$ in Student's paired, two-tailed, *t*-tests). At 60 and 120 min, the dimer-to-monomer mole ratios were similar between reactions with or without Zn^{2+} (Fig. 6b, d). Interestingly, oligomerization of adiponectin 18mers accelerated during the 0 to 60 min time span ($P < 0.05$ in two-way ANOVA with replications, Fig. 6a, c). Similar to the results in Fig. 5a, higher proportions of adiponectin 18mers were observed at 120 min following treatment with zinc (24.5 ± 4.6% vs. 8.7 ± 3.6% for controls, Fig. 6a, c). These data suggest that zinc enhances oligomerization of adiponectin by decreasing the rate of disulfide bond formation.

Zinc-mediated enhancement of adiponectin oligomerization is diminished under thiol-limited conditions

Results in Fig. 6 showed that disulfide bond formation was no longer inhibited by Zn^{2+} 60–120 min after initiation of reoligomerization, when concentrations of reduced monomers became decreased relative to oxidized dimers. We reasoned this could be due to decreased availability of thiols to coordinate Zn^{2+} leading to the loss of inhibition to form disulfide bonds. To determine whether or not the effect of zinc on adiponectin oligomerization depends upon high concentrations of free thiols, we performed reassembly experiments under conditions in which dimers predominated over monomers at the beginning of reoligomerization. Purified HMW adiponectin was collapsed to hexamers by addition of glycine to reach pH of 4. Reoligomerization to 18mers was initiated by adding βME to a final concentration of 2 mM in the absence or presence of 10 μM $ZnCl_2$. βME caused a portion of adiponectin to become reduced by 15 min and amounts of oxidized dimers and reduced monomers reached a steady state mole ratio of 0.6–0.7 to 1 by 1 h (Fig. 7b, f). These experiments were performed in the presence of βME where reassembly of adiponectin 18mers occurred by rearrangement of

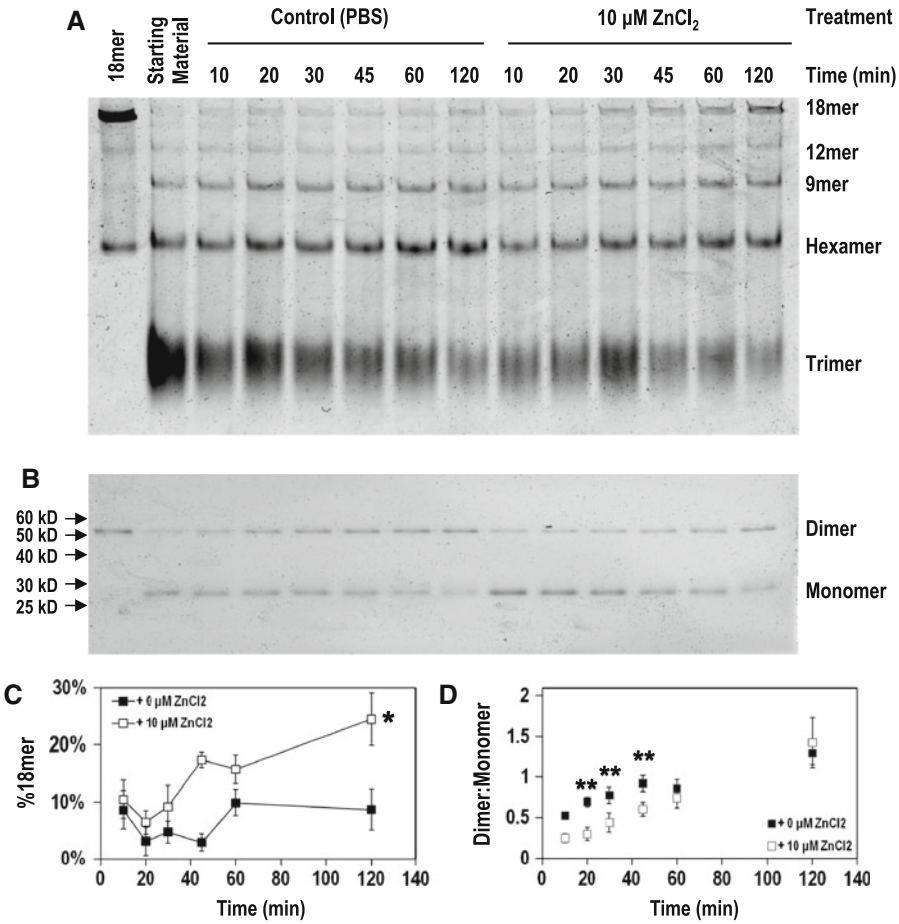

Fig. 6 Initial rates of adiponectin reoligomerization and oxidation in glutathione redox buffer. Collapse and reassembly of HMW adiponectin in presence and absence of Zn^{2+} were performed as detailed in "Materials and methods" section and in legends to Fig. 5. Samples were removed at 10, 20, 30, 45, 60, and 120 min after start of reoligomerization in glutathione redox couple, quenched in 10 mM NEM, and assayed for **a** oligomer distribution using native PAGE and **b** redox states using non-reducing SDS-PAGE. Average abundance of **c** 18mers as percentages of total oligomers by mass during reoligomerization reactions in the absence (*closed squares*) or the presence (*open squares*) of zinc was plotted against time. The extent of adiponectin oxidation during reoligomerization reactions is represented as **d** mole ratios of dimers to monomers. Data in **c**, **d** were averaged from three independent experiments and presented as mean ± SE. $*P < 0.001$ between zinc-treated and control groups in two-way ANOVA with replications. $**P < 0.05$ between zinc-treated and control groups in paired Student's *t*-test

hexamers into higher oligomers and trimers. In contrast to reoligomerization reactions in which there were more monomers than dimers as substrates (Figs. 5, 6), zinc only had marginal effects on the formation of 18mers (Fig. 7a, c) when starting conditions contained higher proportions of oxidized dimers (Fig. 7b, f). These results are consistent with the hypothesis that the ability of zinc to promote adiponectin oligomerization is due to inhibition of disulfide formation when concentrations of trimers in a reduced state are high.

Discussion

Numerous studies have found that serum levels of HMW adiponectin are lower in people with type 2 diabetes and obesity when compared to normal individuals (Kaser et al. 2008; Basu et al. 2007; Aso et al. 2006), underscoring the importance of studying the mechanism of adiponectin assembly. Here we report the divalent cation zinc specifically promotes oligomerization of adiponectin 18mers in vitro. Our data point to inhibition of unproductive disulfide bond

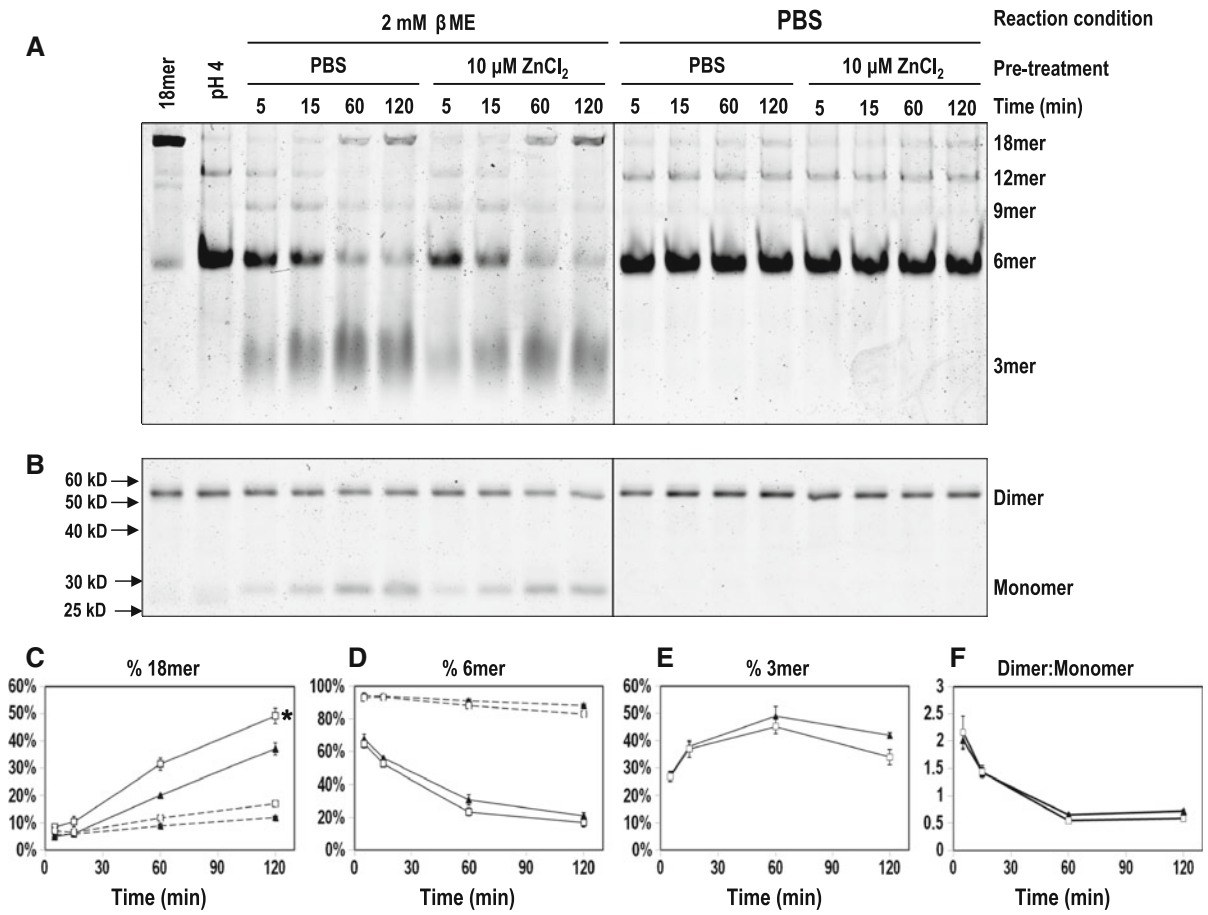

Fig. 7 Rearrangement of adiponectin oligomers in presence of zinc. HMW adiponectin was collapsed to hexamers by incubation in 75 mM glycine, pH 4, for 30 min at 37°C. Subsequently, the samples were treated with either PBS or 10 μM $ZnCl_2$ for 10 min at 37°C. The reactions were divided and incubated with 2 mM *β*ME or PBS for an additional 5, 15, 60, and 120 min. At each time point samples were quenched in 20 mM NEM and subjected to **a** native PAGE analysis to assess adiponectin oligomer distribution and **b** non-reducing SDS-PAGE analysis to determine levels of oxidized dimer and reduced monomer forms of adiponectin. Levels of **c** 18mers, **d** hexamers, and **e** trimers as percentages of total adiponectin by mass averaged from three independent experiments are plotted against time after addition of *β*ME. Extent of adiponectin reduction by *β*ME over time is shown as **f** mole ratios of dimer to monomer. *Solid lines* represent samples treated in 2 mM *β*ME. *Dashed lines* represent controls mocked treated with PBS. *Open squares* represent samples treated with $ZnCl_2$ while *closed triangles* represent samples mocked treated with PBS. $^*P < 0.05$ between zinc- and PBS-treated groups matched for *β*ME treatment using two-way ANOVA with replications

formation as a likely mechanism for enhanced adiponectin oligomerization.

While disulfide bonds are critical in the formation of HMW adiponectin, fully oxidized hexamers are unable to oligomerize further (Briggs et al. 2009). Results in Fig. 6 of the present study suggest that other oligomers including trimers also oligomerize poorly after becoming oxidized. Under the highly oxidizing conditions of −120 mV, oligomerization from trimers occurred at a fast rate but plateaued quickly (Fig. 6). Overall, in contrast to assays in which oligomerization proceeded slowly via dialysis of reducing agents (Figs. 2, 3, 4), those occurring under defined and oxidizing conditions exhibited poor 18mer formation unless zinc was added (Figs. 5, 6). These data indicate that although disulfide bonds are necessary for adiponectin oligomerization, excessively rapid disulfide formation impairs 18mer assembly. The data suggest two types of disulfide bonding in adiponectin oligomerization—those that are productive toward formation of 18mers (between subunits) and those that are unproductive (within subunits). As unproductive

disulfide bond formation has pseudo first order kinetics and productive disulfide bond formation has second order kinetics, rapid disulfide bonding under oxidizing conditions without increased interactions between trimers will favor unproductive disulfide bonds and lead to oxidized intermediates with fixed oligomerization state.

This line of reasoning led to the hypothesis that the ability of zinc to enhance oligomerization of 18mers may be related to decreased rate of disulfide bond formation. Indeed, as shown in Fig. 6, presence of zinc decreased the rate of disulfide bond formation during the initial time points of the adiponectin reoligomerization assay. It is significant that the effect of zinc on disulfide formation is limited to the early stages of adiponectin reoligomerization. The concentrations of monomers with free thiols relative to dimers with disulfides were higher at early time points (Fig. 6b, d), suggesting Zn^{2+} interacted with thiols in adiponectin. Stabilization of thiolate ions by Zn^{2+} could decrease their reactivity and thereby reduce the rate of disulfide bond formation. As reduced adiponectin became disulfide-bonded, the concentration of free thiols dropped correspondingly and resulting in decreased availability to interact with Zn^{2+}. This could explain why zinc had no effect on the rate of disulfide bond formation at later time points when concentrations of disulfide-bonded dimers exceeded those of reduced monomers (Fig. 6b, d). Consistent with this interpretation, zinc-mediated enhancement of adiponectin oligomerization is less apparent in reoligomerization reactions that had lower starting concentrations of free thiols (Fig. 7). Taken together, the data in Figs. 6 and 7 suggest that the mechanism by which zinc decreases disulfide bond formation rate in adiponectin is stabilization of the reduced form of adiponectin.

The idea that zinc can stabilize the reduced form of a redox-active protein and modulate the reduction potential of coordinating thiols has precedence. Oxidation of the CX_3C motif of the mitochondrial small Tim proteins prevents import into mitochondria and binding of zinc greatly stabilizes the reduced form of the CX_3C motif (Morgan et al. 2009). Zinc-binding also decreases the formation of disulfide bonds that inhibit activity of certain voltage-gated potassium channels (Wang et al. 2007). In enzymes and signaling proteins found in both prokaryotes and eukaryotes, zinc-binding cysteine-rich regions often serve as sensors of oxidative stress with zinc functioning to inhibit oxidation of key cysteine residues (Maret 2006; Paget and Buttner 2003; Wilcox et al. 2001). As is the case with adiponectin in which zinc could slow down disulfide formation but could not prevent it, the thiols coordinating zinc in these zinc-containing redox sensors undergo oxidation resulting in the release of zinc ions (Bonham and Vacratsis 2009; Maret 2006; Ortiz de Orué Lucana et al. 2012; Wilcox et al. 2001).

The positive effects of zinc on adiponectin oligomerization were apparent in both native gel electrophoresis (Figs. 3, 6) and sedimentation velocity (Fig. 4) techniques. Higher proportions of 18mers were observed in sedimentation velocity experiments compared with those analyzed using native gel electrophoresis in both absence and presence of zinc (Figs. 3c, 4, 5a, 6a). This is likely due to the increased concentrations of adiponectin used in reassembly reactions analyzed by sedimentation velocity (30 μM) versus native gel electrophoresis (10 μM).

The original impetus for the present study was the observation that lowering of pH below 5 resulted in collapse of HMW adiponectin to hexamers (Briggs et al. 2009; Pajvani and Scherer 2003; Schraw et al. 2008; Hada et al. 2007). This observation prompted us to hypothesize that coordination of metal ions by histidine residues could stabilize HMW adiponectin. EDTA treatment did not collapse mature adiponectin 18mer (Fig. 1), but interactions between metals and proteins often have high affinity (Maret 2006; Sellin and Mannervik 1984; Jackson et al. 2001) and are thus difficult to disrupt by chelators. It remains possible that metal coordination is necessary for structural stability of adiponectin oligomers or their assembly. This possibility is supported by results from ICP-MS analyses showing presence of zinc in purified calf serum adiponectin. Zinc has been documented as influencing protein complex assembly reactions through effects on both equilibria and assembly kinetics. Hexameric insulin forms a stable complex with two zinc ions in which each subunit contributes a single histidine residue to coordinate zinc (Dodson and Steiner 1998; Dunn 2005). The stoichiometry of insulin hexamer to zinc is similar to the observed ratio of one adiponectin trimer per zinc ion. Zinc has been shown to contribute to the stability of other multimeric complexes, potentially through formation of inter-subunit binding sites (Green et al. 1991; Hernández-Santoyo et al. 2011; Lee et al. 1997; Rosenbaum et al. 2011; Jahng et al. 2002). Additionally, it is able to

influence the kinetics of amyloid formation (Bush et al. 1994; Esler et al. 1996).

The observations that treatment with EDTA or DTPA decreased formation of adiponectin 18mers in reoligomerization reactions (Figs. 2, 3) are consistent with divalent cations having a structural role in stabilization of adiponectin. They are also consistent with zinc-mediated decrease in disulfide bond formation rate as mechanism for enhanced oligomerization. Decreased formation of adiponectin 18mers following EDTA and DTPA treatments were associated with increased ratios of dimers to monomers indicating increased oxidation (Figs. 2, 3). DTPA, a chelator similar in structure to EDTA but with 100 times stronger affinity to some divalent cations including zinc and copper (Hart 2000), inhibited adiponectin oligomerization and led to increased oxidation at concentration as low as 100 μM (Fig. 3).

While amounts of total zinc are relatively high in most cells, the intracellular concentration of free zinc is thought to be extremely low with most zinc ions bound to proteins (Maret and Li 2009). Studies using fluorescent probes with 0.7 nM dissociation constant for zinc have shown that ER, Golgi, and mitochondria likely have higher concentrations of free zinc (Tomat et al. 2008; Stork and Li 2010; Burdette et al. 2001). Thus it is plausible for zinc to modulate adiponectin oligomerization in vivo. Indeed, rat primary adipocytes treated with TPEN secreted significantly higher proportions of hexamers and trimers relative to HMW adiponectin than those secreted from mock-treated cells (Fig. 5c), a pattern consistent with enhanced oxidation but impaired adiponectin oligomerization.

In summary, we demonstrated that zinc enhances the oligomerization of HMW adiponectin in vitro. One mechanism by which this occurs is decreased rate of disulfide bond formation. These results illustrate a need to study the potential role of zinc as a chaperone for HMW adiponectin formation in vivo.

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References

Aso Y, Yamamoto R, Wakabayashi S, Uchida T, Takayanagi K, Takebayashi K, Okuno T, Inoue T, Node K, Tobe T, Inukai T, Nakano Y (2006) Comparison of serum high-molecular weight (HMW) adiponectin with total adiponectin concentrations in type 2 diabetic patients with coronary artery disease using a novel enzyme-linked immunosorbent assay to detect HMW adiponectin. Diabetes 55:1954–1960

Basu R, Pajvani UB, Rizza RA, Scherer PE (2007) Selective downregulation of the high molecular weight form of adiponectin in hyperinsulinemia and in type 2 diabetes: differential regulation from nondiabetic subjects. Diabetes 56:2174–2177. doi:10.2337/db07-0185

Bonham CA, Vacratsis PO (2009) Redox regulation of the human dual specificity phosphatase YVH1 through disulfide bond formation. J Biol Chem 284:22853–22864

Briggs DB, Jones CM, Mashalidis EH, Nuñez M, Hausrath AC, Wysocki VH, Tsao TS (2009) Disulfide-dependent self-assembly of adiponectin octadecamers from trimers and presence of stable octadecameric adiponectin lacking disulfide bonds in vitro. Biochemistry 48:12345–12357. doi:10.1021/bi9015555

Burdette SC, Walkup GK, Spingler B, Tsien RY, Lippard SJ (2001) Fluorescent sensors for Zn(2+) based on a fluorescein platform: synthesis, properties and intracellular distribution. J Am Chem Soc 123:7831–7841

Bush AI, Pettingell WH, Multhaup G, d Paradis M, Vonsattel JP, Gusella JF, Beyreuther K, Masters CL, Tanzi RE (1994) Rapid induction of Alzheimer A beta amyloid formation by zinc. Science 265:1464–1467

Chakrabarti P (1990) Geometry of interaction of metal ions with histidine residues in protein structures. Protein Eng 4:57–63

de Orué Ortiz, Lucana D, Wedderhoff I, Groves MR (2012) ROS-mediated signalling in bacteria: zinc-containing Cys-X-X-Cys redox centres and iron-based oxidative stress. J Signal Transduct 2012:605905. doi:10.1155/2012/605905

Dodson G, Steiner D (1998) The role of assembly in insulin's biosynthesis. Curr Opin Struct Biol 8:189–194. doi:10.1016/S0959-440X(98)80037-7

Dunn MF (2005) Zinc-ligand interactions modulate assembly and stability of the insulin hexamer—a review. Biometals 18:295–303. doi:10.1007/s10534-005-3685-y

Esler WP, Stimson ER, Jennings JM, Ghilardi JR, Mantyh PW, Maggio JE (1996) Zinc-induced aggregation of human and rat beta-amyloid peptides in vitro. J Neurochem 66:723–732

Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE, Lodish HF (2001) Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. Proc Natl Acad Sci USA 98:2005–2010. doi:10.1073/pnas.041591798

Goldstein BJ, Scalia RG, Ma XL (2009) Protective vascular and myocardial effects of adiponectin. Nat Clin Pract Cardiovasc Med 6:27–35. doi:10.1038/ncpcardio1398

Green SM, Ginsburg A, Lewis MS, Hensley P (1991) Roles of metal ions in the maintenance of the tertiary and quaternary structure of arginase from Saccharomyces cerevisiae. J Biol Chem 266:21474–21481

Hada Y, Yamauchi T, Waki H, Tsuchida A, Hara K, Yago H, Miyazaki O, Ebinuma H, Kadowaki T (2007) Selective purification and characterization of adiponectin multimer species from human plasma. Biochem Biophys Res Commun 356:487–493

Hara K, Horikoshi M, Yamauchi T, Yago H, Miyazaki O, Ebinuma H, Imai Y, Nagai R, Kadowaki T (2006) Measurement of the high-molecular weight form of adiponectin in plasma is useful for the prediction of insulin resistance and metabolic syndrome. Diabetes Care 29:1357–1362. doi:10.2337/dc05-1801

Hart JR (2000) Ethylenediaminetetraacetic acid and related chelating agents. In: Ullmann's encyclopedia of industrial chemistry. Wiley-VCH, Weinheim. doi:10.1002/14356007.a10_095

Hernández-Santoyo A, Landa A, González-Mondragón E, Pedraza-Escalona M, Parra-Unda R, Rodríguez-Romero A (2011) Crystal structure of Cu/Zn superoxide dismutase from *Taenia solium* reveals metal-mediated self-assembly. FEBS J 278:3308–3318. doi:10.1111/j.1742-4658.2011.08247.x

Jackson GS, Murray I, Hosszu LL, Gibbs N, Waltho JP, Clarke AR, Collinge J (2001) Location and properties of metal-binding sites on the human prion protein. Proc Natl Acad Sci USA 98:8531–8535. doi:10.1073/pnas.151038498

Jahng AW, Strang C, Kaiser D, Pollard T, Pfaffinger P, Choe S (2002) Zinc mediates assembly of the T1 domain of the voltage-gated K channel 4.2. J Biol Chem 277:47885–47890. doi:10.1074/jbc.M208416200

Kaser S, Tatarczyk T, Stadlmayr A, Ciardi C, Ress C, Tschoner A, Sandhofer A, Paulweber B, Ebenbichler CF, Patsch JR (2008) Effect of obesity and insulin sensitivity on adiponectin isoform distribution. Eur J Clin Invest 38:827–834. doi:10.1111/j.1365-2362.2008.02028.x

Krezel A, Hao Q, Maret W (2007) The zinc/thiolate redox biochemistry of metallothionein and the control of zinc ion fluctuations in cell signaling. Arch Biochem Biophys 463:188–200. doi:10.1016/j.abb.2007.02.017

Laue TM, Shah BD, Ridgeway TM, Pelletier SL (1992) Computer-aided interpretation of analytical sedimentation data for proteins. In: Harding SE, Rowe AJ, Horton JC (eds) Analytical ultracentrifugation in biochemistry and polymer science. Royal Society of Chemistry, Cambridge, pp 90–125

Lee SP, Xiao J, Knutson JR, Lewis MS, Han MK (1997) Zn^{2+} promotes the self-association of human immunodeficiency virus type-1 integrase in vitro. Biochemistry 36:173–180. doi:10.1021/bi961849o

Li Y, Maret W (2009) Transient fluctuations of intracellular zinc ions in cell proliferation. Exp Cell Res 315:2463–2470. doi:10.1016/j.yexcr.2009.05.016

López JJ, Redondo PC, Salido GM, Pariente JA, Rosado JA (2009) *N*,*N*,*N*',*N*'-tetrakis(2-pyridylmethyl)ethylenediamine induces apoptosis through the activation of caspases-3 and -8 in human platelets. A role for endoplasmic reticulum stress. J Thromb Haemost 7:992–999. doi:10.1111/j.1538-7836.2009.03431.x

Maret W (2006) Zinc coordination environments in proteins as redox sensors and signal transducers. Antioxid Redox Signal 8:1419–1441. doi:10.1089/ars.2006.8.1419

Maret W, Li Y (2009) Coordination dynamics of zinc in proteins. Chem Rev 109:4682–4707. doi:10.1021/cr800556u

Matsuzawa Y, Funahashi T, Kihara S, Shimomura I (2004) Adiponectin and metabolic syndrome. Arterioscler Thromb Vasc Biol 24:29–33. doi:10.1161/01.ATV.0000099786.99623.EF

Morgan B, Ang SK, Yan G, Lu H (2009) Zinc can play chaperone-like and inhibitor roles during import of mitochondrial small Tim proteins. J Biol Chem 284:6818–6825. doi:10.1074/jbc.M808691200

Ouchi N, Shibata R, Walsh K (2006) Cardioprotection by adiponectin. Trends Cardiovasc Med 16:141–146. doi:10.1016/j.tcm.2006.03.001

Paget MS, Buttner MJ (2003) Thiol-based regulatory switches. Annu Rev Genet 37:91–121. doi:10.1146/annurev.genet.37.110801.142538

Pajvani UB, Scherer PE (2003) Adiponectin: systemic contributor to insulin sensitivity. Curr Diabetes Rep 3:207–213

Pajvani UB, Hawkins M, Combs TP, Rajala MW, Doebber T, Berger JP, Wagner JA, Wu M, Knopps A, Xiang AH, Utzschneider KM, Kahn SE, Olefsky JM, Buchanan TA, Scherer PE (2004) Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. J Biol Chem 279:12152–12162. doi:10.1074/jbc.M311113200

Park CK, Stiteler AP, Shah S, Ghare MI, Bitinaite J, Horton NC (2010) Activation of DNA cleavage by oligomerization of DNA-bound SgrAI. Biochemistry 49:8818–8830. doi:10.1021/bi100557v

Piu P, Sanna G, Masia A, Zoroddu MA, Seeber R (1997) Potentiometric and spectroscopic study of ternary complexes ofcopper(II), substituted 1,10-phenanthrolines and oxidisedglutathione. J Chem Soc Dalton Trans 2369–2372. doi:10.1039/A608272F

Radjainia M, Wang Y, Mitra AK (2008) Structural polymorphism of oligomeric adiponectin visualized by electron microscopy. J Mol Biol 381:419–430. doi:10.1016/j.jmb.2008.06.015

Rosenbaum E, Ferruit M, Durá MA, Franzetti B (2011) Studies on the parameters controlling the stability of the TET peptidase superstructure from *Pyrococcus horikoshii* revealed a crucial role of pH and catalytic metals in the oligomerization process. Biochim Biophys Acta 1814:1289–1294. doi:10.1016/j.bbapap.2010.11.008

Schraw T, Wang ZV, Halberg N, Hawkins M, Scherer PE (2008) Plasma adiponectin complexes have distinct biochemical characteristics. Endocrinology 149:2270–2282. doi:10.1210/en.2007-1561

Schuck P, Rossmanith P (2000) Determination of the sedimentation coefficient distribution by least-squares boundary modeling. Biopolymers 54:328–341. doi:10.1002/1097-0282(20001015)54:5<328:AID-BIP40>3.0.CO;2-P

Sellin S, Mannervik B (1984) Metal dissociation constants for glyoxalase I reconstituted with Zn^{2+}, Co^{2+}, Mn^{2+}, and Mg^{2+}. J Biol Chem 259:11426–11429

Shapiro L, Scherer PE (1998) The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor. Curr Biol 8:335–338

Smith GF, McCurdy WH (1952) 2,9-Dimethyl-1,10-phenanthroline. Anal Chem 24:371–373. doi:10.1021/ac60062a029

Stork CJ, Li YV (2010) Zinc release from thapsigargin/IP3-sensitive stores in cultured cortical neurons. J Mol Signal 5:5. doi:10.1186/1750-2187-5-5

Suzuki S, Wilson-Kubalek EM, Wert D, Tsao TS, Lee DH (2007) The oligomeric structure of high molecular weight adiponectin. FEBS Lett 581:809–814. doi:10.1016/j.febslet.2007.01.046

Tomat E, Nolan EM, Jaworski J, Lippard SJ (2008) Organelle-specific zinc detection using zinpyr-labeled fusion proteins in live cells. J Am Chem Soc 130:15776–15777. doi:10.1021/ja806634e

Tsao TS, Lodish HF, Fruebis J (2002a) ACRP30, a new hormone controlling fat and glucose metabolism. Eur J Pharmacol 440:213–221

Tsao TS, Murrey HE, Hug C, Lee DH, Lodish HF (2002b) Oligomerization state-dependent activation of NF-kappa B signaling pathway by adipocyte complement-related protein of 30 kDa (Acrp30). J Biol Chem 277:29359–29362. doi:10.1074/jbc.C200312200

Tsao TS, Tomas E, Murrey HE, Hug C, Lee DH, Ruderman NB, Heuser JE, Lodish HF (2003) Role of disulfide bonds in Acrp30/adiponectin structure and signaling specificity. Different oligomers activate different signal transduction pathways. J Biol Chem 278:50810–50817. doi:10.1074/jbc.M309469200

Waki H, Yamauchi T, Kamon J, Ito Y, Uchida S, Kita S, Hara K, Hada Y, Vasseur F, Froguel P, Kimura S, Nagai R, Kadowaki T (2003) Impaired multimerization of human adiponectin mutants associated with diabetes. Molecular structure and multimer formation of adiponectin. J Biol Chem 278:40352–40363. doi:10.1074/jbc.M300365200

Wang G, Strang C, Pfaffinger PJ, Covarrubias M (2007) Zn^{2+}-dependent redox switch in the intracellular T1–T1 interface of a Kv channel. J Biol Chem 282:13637–13647

Whitehead JP, Richards AA, Hickman IJ, Macdonald GA, Prins JB (2006) Adiponectin—a key adipokine in the metabolic syndrome. Diabetes Obes Metab 8:264–280. doi:10.1111/j.1463-1326.2005.00510.x

Wilcox DE, Schenk AD, Feldman BM, Xu Y (2001) Oxidation of zinc-binding cysteine residues in transcription factor proteins. Antioxid Redox Signal 3:549–564. doi:10.1089/15230860152542925

Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R, Kadowaki T (2003) Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 423:762–769. doi:10.1038/nature01705

Zhu N, Pankow JS, Ballantyne CM, Couper D, Hoogeveen RC, Pereira M, Duncan BB, Schmidt MI (2010) High-molecular-weight adiponectin and the risk of type 2 diabetes in the ARIC study. J Clin Endocrinol Metab 95:5097–5104. doi:10.1210/jc.2010-0716