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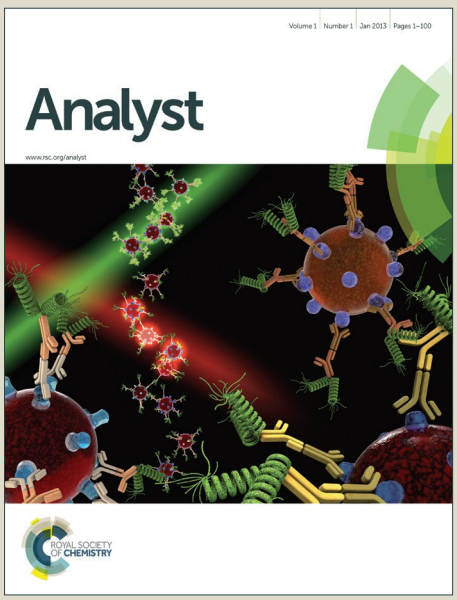

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ARTICLE

A Simple and Fast Method to Study the Hydrodynamic Size Difference of Protein Disulfide Isomerase in Oxidized and Reduced Form using Gold Nanoparticles and Dynamic Light Scattering

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The hydrodynamic dimension of a protein is a reflection of both its molecular weight and its tertiary structures. Studying the hydrodynamic dimensions of proteins in solutions can help elucidate the structural properties of proteins. Here we report a simple and fast method to measure the hydrodyamic size of a relatively small protein, protein disulfide isomerase (PDI), using gold nanoparticle probes combined with dynamic light scattering. Proteins can readily adsorb to citrate-capped gold nanoparticles to form a protein corona. By measuring the average diameter of the gold nanoparticles before and after protein corona formation, the hydrodynamic diameter of the protein can be deduced from the net particle size increase of the assay solution. This study found that when the disulfide bonds in PDI are reduced to thiols, the reduced PDI exhbits a smaller hydrodynamic diameter than the oxided PDI. This finding is in good agreement with the X-ray diffraction analysis of PDI in single crystals. In comparison with other techniques that are used for protein hydrodynamic size analysis, the current method is easy to use, requires a trace amount of protein samples, with results obtained in minutes instead of hours.

Introduction

A combination of techniques is required to study and understand the biophysical and biochemical properties of a protein. The "size" of a protein, which refers to both its absolute molecular weight and its hydrodynamic shape and dimension in solution, is often the first and most important characteristic to be analyzed.[1,2] While the molecular weight of a protein is relatively fixed, the hydrodynamic dimension of a protein is a reflection of its tertiary structure and therefore depends on the physical and functional state of the protein as well as its molecular weight. Changes in the hydrodynamic dimension of a protein can accordingly be caused by several factors, including (i) folding and unfolding of the polypeptide chain; (ii) conformational shifts under different oxidation/reduction and pH conditions; and (iii) substrate binding or dissociation. Thus, by studying changes to the hydrodynamic size of a protein, information may be obtained on the structural properties of the protein. Knowing the hydrodynamic dimension of protein monomers is particularly important for studying and detecting oligomer or aggregate formation in protein solutions, especially in biopharmaceuticals.[3]

Analytical ultracentrifugation (AUC) is a technique that has been traditionally used to study protein size.[4] However, this technique requires long hours of data acquisition and rather complicated protocols for data processing and analysis. A typical AUC experiment takes about 16 hours to complete, and only specially-trained scientists are able to use AUC for protein size analysis. Furthermore, AUC analysis requires a large amount of purified protein (≥ 10 mg). Such quantities are often beyond the reach of most biomedical research laboratories. Size exclusion chromatography (SEC), also known as gel permeation chromatography, is another technique often used for protein size analysis.[5,6] Although more readily accessible to research laboratories and less expensive than AUC, the use of SEC for protein hydrodynamic radius analysis is not straightforward. The hydrodynamic dimension of a protein is obtained indirectly, based on the elution rate of the protein from a gel column. Protein elution is affected by many variables, including the charge state of the protein, the ionic strength of the eluent, and the material, porosity and packing density of the column. SEC also requires reference standards to determine the molecular weight and hydrodynamic diameter of the protein. Other techniques such as NMR spectroscopy[7] and small angle X-ray scattering (SAXS)[8] involve expensive, sophisticated instruments that are not readily available to most research laboratories and are beyond the expertise of most researchers. Overall, there is a lack of straightforward, simple, fast, and low-cost techniques to study the hydrodynamic size of a protein.

Dynamic light scattering (DLS) is a technique that can be used to measure the hydrodynamic dimension of a protein directly in solution.[9-12] DLS is easy to use and requires protein samples in the μg rather than mg range. The hydrodynamic dimension of a protein can be obtained directly from DLS measurements without the need for sophisticated mathematical tools. DLS determines the

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hydrodynamic dimension of a protein by monitoring the scattering light intensity fluctuation of the sample solution caused by Brownian motion of the protein in solution. The diameter or radius of a protein is deduced from its diffusion coefficient using the Einstein-Stokes equation.[9] Modern DLS instruments can measure particle sizes varying from a few nanometers to a few microns. Because most proteins have hydrodynamic diameters in the nanometer range, DLS is a suitable technique to analyze the hydrodynamic dimensions of proteins.

Despite the theoretical suitability of DLS for protein size analysis, there are some significant challenges that limit its practical applications. First, most proteins have a hydrodynamic diameter in the range of a few nanometers. This dimension is very close to the lower limit of detection (LLOD) of DLS instrumentation, and the reliability of the measurement often becomes problematic near the LLOD region. Second, protein molecules - especially the smaller ones with a molecular weight around or below 50 kDa - scatter light rather weakly. The concentration of the protein therefore needs to be relatively high (often exceeding 1 mg/mL) in order to obtain sufficient light scattering intensity for DLS measurement. Yet many proteins have a tendency to form oligomers and aggregates at high concentrations. Because DLS does not perform well with samples that contain polydispersed particle sizes, it is no longer suitable for protein size measurements once protein oligomerization or aggregation occurs.

Many of the limitations for DLS protein size analysis can be readily overcome by introducing gold nanoparticle (AuNP) probes into the measurement. Metal nanoparticles, especially gold and silver nanoparticles, scatter light intensely at the region of their surface plasmon resonance wavelength. For example, a AuNP can scatter light 1000-fold stronger than a polymer bead or protein particle with similar dimensions.[13,14] Citrate ligand-capped AuNP (ctAuNP) is among one of the most extensively studied AuNPs. ctAuNPs are made by simple reduction of $HAuCl_4$ in aqueous solution with sodium citrate.[15] High quality ctAuNPs with excellent size monodispersity are available from multiple commercial vendors at low and affordable costs such as Ted Pella Inc. and Sigma-Aldrich. One of the unique properties of ctAuNPs is that proteins readily adsorb to ctAuNPs to form a "protein corona" on the surface of the nanoparticle.[16-20] By measuring the size increase of the AuNP upon protein adsorption, the hydrodynamic diameter of the protein can be indirectly deduced from the thickness of the "protein corona" as shown in Figure 1. Because of the exceptionally strong light scattering property of AuNPs, the use of DLS for protein analysis is no longer limited by the weak scattering caused by the proteins.

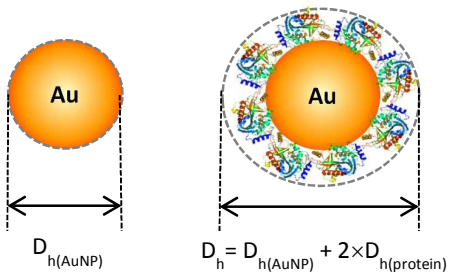

Figure 1. Illustration of AuNP-enabled DLS to analyze the hydrodynamic dimension of a protein. The protein under study is first allowed to form a monolayer of "protein corona" on the surface of a ctAuNP through physical adsorption. The hydrodynamic diameter of the AuNP before and after protein corona formation is determined using DLS. From the net increase of the AuNP average diameter, the hydrodynamic diameter of the protein, $D_{h(protein)}$, can be deduced.

Results and Discussions

In this study, we examined the hydrodynamic size of a relatively small protein, protein disulfide isomerase (PDI), using DLS alone and in combination with ctAuNPs. PDI is an enzyme that catalyzes the formation and breakage of disulfide bonds within proteins, thereby helping proteins to fold into correct conformations.[21] The oxidoreductase activity of PDI is derived from its two active thioredoxin-like domains that each contains a pair of reactive cysteine residues in a CXXC arrangement. Full-length PDI has a molecular weight of 54 kDa and can be found in both oxidized (oPDI) and reduced (rPDI) forms. In the oxidized form, the two cysteines within the CXXC motif of a thioredoxin-like domain are connected through a disulfide bond. In the reduced form, the disulfide bonds are broken into two thiols. A number of studies suggest that the redox state of PDI affects its overall tertiary structure. X-ray diffraction (XRD) analysis of both oPDI and rPDI crystals have been reported by Wang et al.[22] The unit cell of oPDI was found to have a dimension of 16.1 × 3.4 × 15.7 nm, while the unit cell of rPDI has a dimension of 3.8 × 10.1 × 12.4 nm. The XRD data suggest that both oPDI and rPDI have a disk-like overall structure, and rPDI has a smaller dimension than oPDI. On the other hand, Li et al.[23] reported a small angle X-ray scattering (SAXS) study of full-length PDI in solution that suggested PDI is a short and elliptical cylinder with an approximate dimension of 10.5 × 6.5 × 4.0 nm. The dimensions of PDI determined by different techniques in different states (solid crystal *versus* free solution) could vary significantly. To our best knowledge, no study has been reported on the direct comparison of the hydrodynamic size of oPDI and rPDI in free solution.

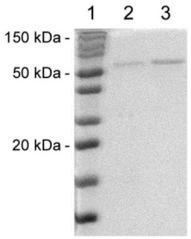

Figure 2. Purification of His-tagged PDI. Molecular mass markers (lane 1), bovine PDI from Sigma-Aldrich (lane 2), and 1 μg of our recombinant PDI (lane 3) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis with a 15% polyacrylamide gel. Samples were visualized by Coomassie stain. Select molecular mass markers are highlighted; the full range of standards included proteins of 10, 15, 20, 25, 37, 50, 75, 100, 150, and 200 kDa.

For this study, recombinant human oPDI with an N-terminal His_6 epitope tag was obtained from *Escherichia coli* expression strain BL21(DE3) and purified by Talon metal affinity chromatography. The purity of the expressed oPDI was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis as

shown by the gel image (Figure 2). rPDI was obtained by treating oPDI with DL-dithiothreitol (DTT). Further details on oPDI purification and reduction are described in the experimental section. Direct DLS measurements of pure oPDI and rPDI are shown in Figures 3A and B, respectively. Three measurements are presented for each condition. Protein concentration was 1.0 mg/mL. At the maximum laser power (4 mW, set by maximum attenuation number 11), the average light scattering intensity of pure oPDI and rPDI solution was approximately 20 kcps (kilo counts per second). Such count rates are close to the noise level of the DLS detector (~ 10-20 kcps). This low scattering intensity does not permit reliable size measurement. Even though size distribution curves and average hydrodynamic diameters were given by the instrument, the distribution curves are widely dispersed and irreproducible, as seen from the replicate measurements in Figure 3. This is a very common problem when using DLS alone to determine the hydrodynamic dimension of pure protein solutions.

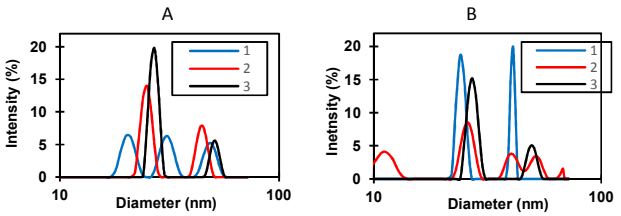

Figure 3. Intensity-averaged size distribution curves of oPDI (A) and rPDI (B) from three measurements. The size distribution curves were obtained under an attenuation of 11 at a maximum laser power provided by the instrument.

Similar measurements performed with ctAuNP probes highlighted the advantages of this system: when ctAuNP probes were introduced into the analysis, the hydrodynamic dimensions of oPDI and rPDI could be measured with excellent reproducibility. The analysis is done by simply mixing 6 µL of protein solution at different concentrations with 180 µL of ctAuNP solution. The ctAuNP used for the current study has an average diameter of 40 nm and a particle concentration of 9.0×10^{10} particles/mL. Figure 4 presents the intensity-averaged size distribution curves of pure ctAuNP (Figure 4A), ctAuNP adsorbed with oPDI (Figure 4B), and ctAuNP adsorbed with rPDI obtained from DTT reduction (Figure 4C). The concentrations of both oPDI and rPDI were 1.0 mg/mL. High-quality size distribution curves of the ctAuNP were readily obtained using an attenuation of 10, which reduces the laser power from 4 mW to 1.2 mW. Under this reduced laser power, the scattering light intensity of the ctAuNP solution is around 500-600 kcps, while the scattering intensities of the oPDI and rPDI solutions are beyond the detection limit of the instrument. It is very important to be aware of this light scattering intensity difference: the exceptionally strong light scattering intensity from the ctAuNPs versus the weak and undetectable light scattering from the proteins guarantees that the DLS only measures the size change that occurs on the AuNPs. The free proteins that are not adsorbed to the AuNPs make no contribution to the particle size measurement of the mixed protein-AuNP solution. Indeed, as seen from Figure 4B and C, the size distribution curves of the mixed protein-AuNP solutions remain monodispersed. Only the peak of AuNPs, but not proteins, is seen from the size distribution curves.

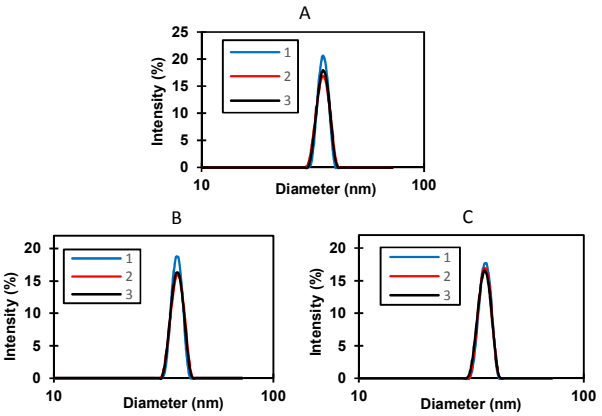

Figure 4. Intensity-averaged size distribution curves of pure ctAuNP (A), ctAuNP upon adsorption of oPDI (B) and ctAuNP upon adsorption of rPDI (C).

The average particle size of the pure ctAuNP solution increased following protein adsorption. Figure 5 presents the net increase in average particle diameter as a function of protein concentration, using oPDI or rPDI at concentrations from 0.5 to 4.0 mg/mL. The molar concentrations of measured PDI (10-80 µM) exceeded the molar concentration of AuNP (0.1 nM) by 100,000-fold. Protein adsorption on the ctAuNP surface clearly reached saturation at protein concentration between 2.0-4.0 mg/mL (Figure 5). This saturated, concentration-dependent effect is very important as a quality control protocol to determine the reliability of the protein hydrodynamic diameter measurement: appearance of a response plateau at increasing protein concentrations confirms a stable protein monolayer is formed on the AuNP surface, and the average particle size increase of the protein-adsorbed AuNP solution is due to the formation of the protein corona rather than to the formation of a small amount of AuNP aggregates induced by the binding of protein oligomers.[24] If protein oligomers or aggregates were present in the solution, the average particle size of the AuNP-protein solution would increase continuously with increasing protein concentrations. The size distribution curves as presented in Figure 4 also confirmed that the average particle size increase was not caused by aggregate formation. DLS is extremely sensitive to particle aggregate formation because the scattering intensity of particles is proportional to the sixth power of the radius of the particle. A small amount of particle aggregates can shift the particle size dramatically, broaden the size distribution curves, or lead to the formation of multiple size peaks. As shown in Figure 4, the monodispersity of the protein-adsorbed AuNPs remains almost exactly the same as pure AuNPs, narrow and highly monodispersed.

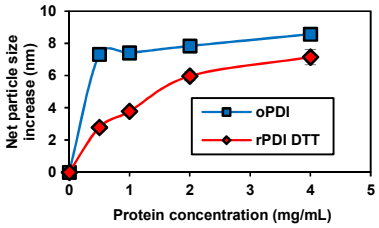

Figure 5. Concentration-dependent study of oPDI and rPDI adsorption to ctAuNPs. The net particle size increases of the assay solution upon protein adsorption compared to the original ctAuNPs are presented in the plot.

By combining the net increase in particle size measured at two protein concentrations, 2.0 and 4.0 mg/mL, it was determined that the hydrodynamic diameter of the ctAuNP increased by 8.2 and 6.6 nm upon adsorption of oPDI and rPDI, respectively. The standard deviation σ of the size measurement for pure ctAuNP was 0.8 nm. The measured increases in ctAuNP diameter upon adsorption of oPDI or rPDI exceeded largely 3σ and therefore represent statistically significant differences. From the increases in particle size, it is determined that the hydrodynamic diameter of PDI was 4.1 nm in the oxidized state and 3.3 nm in the DTT-reduced state. Our current study reached the same conclusion as drawn from other reported studies: rPDI assumes a more compact conformation than oPDI. In DLS measurement, it is assumed that the proteins are spherical particles. If we convert the volumes of oPDI and rPDI in the crystal structures as determined by XRD into the equivalent volumes of spherical particles, the diameters of oPDI and rPDI would be 5.9 and 4.8 nm, respectively. The hydrodynamic sizes of oPDI and rPDI determined by the current AuNP-DLS analysis in solution are smaller than the sizes that were determined by XRD in solid crystals. A large scale molecular dynamics simulation study recently reported by Yang et al. revealed that human PDI adopts more compact conformations in solution than in crystal structures by forming inter-domain interactions.[25] Our current study provides direct experimental evidence corroborating the SAXS study as reported by Li et al.[23] and theoretical modelling studies.

Experimental

Chemicals and materials. Citrate ligand-capped gold nanoparticles (ctAuNP) with a diameter of 40 nm (15707–1, conc. 9.0×10^{10} particles/mL) was purchased from Ted Pella, Inc. (Redding, CA). DL-dithiothreitol (DTT) ($C_4H_{10}O_2S_2$, Cat. No. D0632) was purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. A Tris buffer with 200 mM NaCl, 20 mM Tris and pH 7.6 was used to prepare protein solutions.

Dynamic light scattering (DLS) measurements. A ZetasizerNano ZS90 DLS system equipped with a green laser (532 nm, 4 mW) and an Avalanche photodiode detector (quantum efficiency >50% at 532 nm) (Malvern Instruments Ltd., England) was used for particle size analysis. The incident laser power can be adjusted by using different attenuations. DTS applications 5.10 software was applied to analyze the data. All sizes reported here were based on intensity average. The intensity average particle size was obtained using a non-negative least squares analysis method. For each sample, three DLS measurements were conducted with a fixed run time of 20 s. A detection angle of 90° was used for size measurement.

Expression and purification of protein disulfide isomerase (PDI). Dr. Lloyd Ruddock (University of Oulu) kindly provided a plasmid harboring the human coding sequence for mature PDI (amino acids 18-508) with an in-frame, N-terminal His_6 epitope tag. *Escherichia coli* strain BL21(DE3) transformed with the His_6-PDI plasmid was grown at 37°C to an $O.D._{600}$ of 0.8-.09 in 500 mL Luria-Bertani broth containing 50 μg/mL of ampicillin. PDI expression was then induced with the addition of 2 mM isopropylthiogalactoside, and the culture was grown for another 4-6 h at 25°C. The bacterial pellet generated from low-speed centrifugation was frozen at -80°C and subsequently thawed for lysis in equilibration buffer (200 mM NaCl, 20 mM $NaPO_4$ pH 7.5) containing 100 μg/mL of lysozyme and a Protease Inhibitor Cocktail from Sigma-Aldrich. The lysate was clarified by centrifugation, and the supernatant was incubated at 4°C with Talon metal affinity resin for at least 4 h under constant rotation. After the resin was washed twice with equilibration buffer, PDI was eluted from the resin using increasing concentrations of imidazole (10, 20, and 40 mM). Aliquots of 2 mL were collected from 8 mL total for each imidazole concentration, and the purity of the eluted protein was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis with Coomassie stain. Purified PDI was dialyzed against deionized sterile water with a resistivity of 18 MΩ•cm. Protein concentration was determined, and lyophilized aliquots of 200 μg PDI / tube were frozen at -80°C.

Preparation of oxidized PDI (oPDI) and reduced PDI (rPDI) solutions. A stock solution of oPDI with a concentration of 4 mg/mL was obtained by dissolving 800 μg lyophilized protein into 200 μL Tris Buffer. oPDI solutions with other concentrations (2.0, 1.0, 0.5 mg/mL) were prepared by serial dilutions of the original (4 mg/mL) oPDI in Tris buffer. 50 mM DTT and 50 mM β-me solutions were prepared in Tris Buffer. To initiate the reduction of oPDI, 1 μL DTT (50 mM) was added to 50 μL of oPDI stock solution. The final concentration of DTT was 1 mM. The solution was incubated at room temperature for one hour. Following this incubation, the two rPDI solutions were serial diluted into three different concentrations (2.0, 1.0, and 0.5 mg/mL) in Tris buffer that contained 1 mM DTT accordingly.

Adsorption study of oPDI and rPDI with ctAuNP. A 180μL sample of ctAuNP solution was placed in a sample cuvette and then supplemented with 6 μL of oPDI or rPDI solutions at four different concentrations (0.5, 1.0, 2.0, and 4.0 mg/mL). For control samples with 0 mg/mL of PDI, 6 μL Tris buffer (for oPDI study) or 6 μL Tris buffer containing 1 mM DTT (for rPDI studies) was added to the ctAuNP solution. The average particle size of the mixed protein-ctAuNP solutions was measured following a 10 min incubation at room temperature.

Conclusions

In this study, we demonstrated a very simple, fast and straightforward method to measure the hydrodynamic size of a protein using DLS combined with citrate-capped AuNP probes. PDI is a small protein. Nevertheless, we used the new method to determine not only the hydrodynamic diameter of PDI, but to also observe the size difference between oxidized and reduced forms of this small protein. Our data confirmed the long-standing surmise that rPDI has a smaller and more compact conformation compared to oPDI. Compared to the mg quantities of protein required for AUC or SEC, DLS only needs a few micrograms of protein. Furthermore, DLS analysis can be completed within minutes instead of the hours required for AUC data processing. These considerations, along with the relatively low-cost, easy-to-use DLS instrument, highlight the potential and advantages of using DLS in

combination with AuNP probes to study the hydrodynamic dimensions of proteins.

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