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Measuring the effect of ligand binding on the interface stability of multimeric proteins using dynamic light scattering

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Abstract

We have demonstrated that an approach using guanidine hydrochloride at low concentrations to progressively disrupt protein-protein interactions can be quantitated using dynamic light scattering. This approach is sensitive enough to detect ligand-induced changes of subunit-subunit interactions for homo-hexameric glutamate dehydrogenase, allowing $\Delta\Delta G$ of reversible subunit dissociation to be calculated. The use of dynamic light scattering makes this approach generally applicable to soluble proteins to monitor the relative strength of protein-protein interactions with a particular emphasis on assessing the impact of ligand binding on such interfaces.

Keywords

Multimeric proteins; dynamic light scattering; ligand-induced stability; fluorescence spectroscopy

Protein-protein interactions are integral to biological processes and may function to increase catalytic efficiency [1] or play crucial roles in the allostery mediated by subunit-subunit interactions [2]. Traditional techniques used to examine the strength/stability of protein-protein or subunit interfaces, such as size exclusion chromatography or circular dichroism, are unable to distinguish between altered quaternary or secondary structure, respectively, and loss of tertiary structure. Other methods, optical tweezers [3], atomic force microscopy [4] and fluorescence polarization [5], require modified proteins incorporating a tag or specialized, capital equipment for data acquisition. We have developed a technique that uses dynamic light scattering (DLS) to monitor changes in the translational diffusion coefficient (D_T) as a function of increasing denaturant to assess dissociation of protein-protein or subunit-subunit oligomers with little concomitant tertiary structure denaturation, monitored by protein fluorescence. This approach examines the strength of protein-protein or subunit interactions and retains the sensitivity to measure ligand-induced changes at these interfaces.

Guanidine hydrochloride (GudHCl) has been employed by us [6] and others [7-10] to perturb protein-protein interactions monitored by activity measurements, size exclusion chromatography, ultracentrifugation and light scattering. Similarly, salt- and pH or buffer-induced quaternary structure [11,12] have been studied, but except for Hanlon *et al.* [12],

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these approaches lacked quantitation. No general approach has yet unified quantitatively measuring protein-protein or subunit-subunit interaction stability/strength as a consequence of ligand-induced changes in quaternary structure.

Combining two techniques has previously been used in the context of protein folding. Circular dichroism to follow secondary structure was coupled with tryptophan fluorescence to assess tertiary structure [13]. Ligand effects on quaternary structure were also monitored by ultracentrifugation and light scattering to infer aggregation in high molecular weight complexes [14]. Here, we use DLS to measure the translational diffusion coefficient and derive the hydrodynamic radius as a function of increasing denaturant. To demonstrate that the denaturant, GuDhCl, at low concentrations disrupts subunit interfaces without affecting tertiary structure, we used protein fluorescence in combination with DLS. Furthermore, we demonstrate, through addition of ligands, that their effect on interface stability of multimeric proteins can be directly measured.

To confirm that low concentrations of GuDhCl do not disrupt tertiary structure, three well-studied, globular proteins, lysozyme (Sigma Aldrich), ovalbumin (OVA-GE Healthcare) and bovine serum albumin (BSA-Pierce), were examined. Protein solutions, 0.25mg/ml, were prepared in 0.1M phosphate, pH 7 (buffer 1) and diluted with buffer 1 and 6M GuDhCl (Sigma-Aldrich-molecular biology grade) to yield protein solutions at 0.125mg/ml at 0, 0.5, 1, 1.5 and 2M GuDhCl. Protein concentrations were verified by their absorbance at 280nm. Protein/GuDhCl samples were incubated on ice for 15 minutes and then equilibrated to room temperature before DLS or fluorescence measurements

Light scattering measurements were collected using a miniDAWN Tristar laser photometer with a Wyatt quasi-elastic light scattering (QELS) attachment (Wyatt Technology) using a 690nm laser and collection at a 90° scattering angle. Samples were continuously flowed into the sample chamber through a 0.2µm filter (Whatman) at 40cc/h. Data, >50 data points per GuDhCl concentration, were collected and processed using the AstraV ver.5.1.9.1 software package (Wyatt Technology). Briefly, using QELS analysis, the time-dependent fluctuations (using a 1 second acquisition time) in the scattered light were fit to a second order correlation function which was then fit via a non-linear least squares algorithm to the correlation function for a monodisperse sample to retrieve the correlation function decay rate, Γ . The translation diffusion coefficient, D_T , was then derived:

$$D_T = \frac{\Gamma}{q^2}$$

where q is the magnitude of the scattering vector given by:

$$q = \frac{4\pi\eta_0}{\lambda_0} \sin(\theta/2)$$

where η_0 is the solvent index of refraction, λ_0 is the vacuum wavelength of the incident light, and θ is the scattering angle. With the calculated D_T , the hydrodynamic radius (R_H) was determined using the Stokes-Einstein equation:

$$R_H = \frac{KT}{6\pi\eta D_T}$$

where k is the Boltzmann constant, T is temperature in Kelvin, and η is solvent viscosity. Refractive index and viscosity were adjusted from the buffer values, 1.3206, and $8.945 \times 10^{-3} \text{g}/(\text{cm} \cdot \text{sec})$, respectively, using published values for GudHCl effects on refractive index [15] and viscosity [16]. Fluorescence spectra (300-400nm), with excitation at 280 nm, were recorded in triplicate using an Aminco-Bowman 2 fluorometer. Buffer controls including increasing GudHCl concentrations were subtracted from appropriate protein spectra. At 2M GudHCl, the control spectrum accounted for <3% of measured intensity.

Fluorescence spectra of lysozyme, ovalbumin and BSA demonstrated that at 0.5-2M GudHCl no effect on the emission maximum occurs, Figures 1A,B, indicating that the tertiary structure is unaltered. In 6M GudHCl, the emission maximum shifts to significantly longer wavelengths indicating extensive protein unfolding, Figure 1B. The hydrodynamic radius (R_H) as a function of GudHCl is shown in Figure 1C. Lysozyme shows no change in R_H ($1.97 \pm 0.03 \text{nm}$) and agrees with the reported value of 2.05nm [17]. OVA at 0-1M GudHCl diffuses as a larger species and transitions to a smaller species at 1.5M GudHCl. OVA aggregates have been observed at concentrations greater than 0.169mg/ml [18], consistent with the larger species observed at 0-1M GudHCl. At >1M GudHCl, OVA has an R_H of $2.79 \pm 0.1 \text{nm}$ similar to the reported R_H of 2.8nm [19] suggesting we have captured the aggregate to monomer transition by this method. BSA is known to undergo monomer-dimer-aggregate transitions [20]. At 0M GudHCl the R_H value is consistent with the dimeric BSA R_H of 4.52nm compared to published value of 4.5 nm [21]. BSA transitions to the monomeric form at $\geq 0.5 \text{M}$ GudHCl. Together, the fluorescence and DLS data show that the proposed GudHCl concentrations do not disrupt tertiary structure of these model proteins, and this method is capable of measuring aggregate to monomer (OVA) and dimer to monomer transitions (BSA), respectively.

Next we tested whether the combination of DLS and protein fluorescence was sensitive enough to measure ligand-induced changes of subunit interaction stability. L-glutamic dehydrogenase (GDH-Sigma-Aldrich), a hexameric enzyme, provided a model system known to undergo a hexamer-trimer-monomer (H-T-M) protein denaturation [22] that could be assessed in the absence of substrate, with the substrate (glutamate) or an alternative substrate (norvaline) [23]. A theoretical R_H for hexamer, trimer and monomer could be calculated from GDH's three dimensional coordinates [23] by determining the R_H of a prolate ellipsoid of equivalent dimensions given by the equation: $R_H = (ab^2)^{1/3}$ where a and b are the half-lengths of the crystallographic prolate ellipsoid long and short axes, respectively. These calculations can be used as an approximation of their solution counterparts. For fluorescence and DLS measurements, GDH was diluted to a final concentration of 0.15mg/ml as described above for lysozyme, OVA and BSA. When present, ligands were at the following concentrations: 20mM L-glutamic acid (Sigma Aldrich) or 100mM L-norvaline (Sigma Aldrich). Experiments were completed as described above.

No significant effect on the emission maximum was seen over the 0-2M GudHCl range independent of ligand, Figure 2A, indicating that tertiary structure was unperturbed. In contrast, the R_H shows a downward trend as expected for an H-T-M equilibrium, Figures 2B,C. At GudHCl concentrations 0-1M, the enzyme remains a hexamer with R_H values of 6.9-4.8nm, Figure 2C, as compared to crystallographically-derived R_H , Figure 2D. At 1.5M GudHCl, apoGDH transitions to a trimer species ($R_H=4\text{nm}$). ApoGDH becomes monomeric at 2M GudHCl ($R_H=2.5\text{nm}$) while glutamate-GDH and norvaline-GDH retain trimer species. For the transition:

the equilibrium constant for the hexamer dissociation is $K_{eq} = [\text{Trimer}]^2/[\text{Hexamer}]$. We used a relationship similar to that used in protein unfolding [24], $K_{eq} = f^2/(1-f)$, where for the reversible hexamer-trimer transition, $f = (R_{H, \text{Hexamer}} - R_{H, (\text{GudHCl})}) / (R_{H, \text{Hexamer}} - R_{H, \text{Trimer}}) \times \text{Total} [\text{Hexamer}]$, assuming that at 0M GudHCl all protein is hexameric. We used the average experimental value for the hexamer R_H , 6.77nm. Although larger than the crystallographic, hexamer R_H , at 0M GudHCl the experimental R_H value should accurately represent a pure hexameric species whereas the crystallographic value is an approximation. As the trimer species cannot be isolated to directly determine its R_H , the calculated R_H value of 4.7nm was used for the calculation. Using the experimental R_H values at 0.5M GudHCl for $R_{H, (\text{GudHCl})}$, we calculated the equilibrium constant for hexamer dissociation at 0.5M GudHCl of $9.42 \times 10^{-6} \text{M}$ for enzyme in the absence of ligand compared to a value of $2.79 \times 10^{-6} \text{M}$ in the presence of norvaline and $5.34 \times 10^{-6} \text{M}$ in the presence of glutamate. By analogy with the effects of mutations on stability [25], we can calculate a $\Delta\Delta G$ ($\Delta\Delta G = -RT \ln K_1/K_2$ where K_1 and K_2 are the respective equilibrium constants in the presence and absence of ligand) produced by norvaline binding as 3.04kJ/mole at 300°K, indicating that norvaline binding stabilizes the hexamer by this amount. With glutamate a $\Delta\Delta G$ of 1.42kJ/mole is obtained indicating significantly less stabilization than with norvaline.

We have demonstrated that an approach using GudHCl at low concentrations (0-2M) to progressively disrupt protein-protein interactions can be quantitated using DLS. Furthermore, we have shown that this approach is sensitive enough to detect ligand-induced changes in the strength of subunit interactions of an oligomeric enzyme, reported as $\Delta\Delta G$ values. The use of DLS makes this approach generally applicable to soluble proteins to monitor the relative strength of protein-protein interactions with a particular emphasis on assessing the impact of ligand binding on these interactions.

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References

1. Mark JK, et al. Inhibition of mitogen-activated protein kinase phosphatase 3 activity by interdomain binding. *J Biol Chem.* 2008; 283:28574–28583. [PubMed: 18694935]
2. Schuller DJ, et al. The allosteric ligand site in the Vmax-type cooperative enzyme phosphoglycerate dehydrogenase. *Nat Struct Biol.* 1995; 2:69–75. [PubMed: 7719856]
3. Stout AL. Detection and characterization of individual intermolecular bonds using optical tweezers. *Biophys J.* 2001; 80:2976–2986. [PubMed: 11371470]
4. Zhang, X., et al., editors. Atomic force microscopy of protein-protein interactions. Springer; New York: 2009.
5. Jameson DM, Seifried SE. Quantification of protein-protein interactions using fluorescence polarization. *Methods.* 1999; 19:222–233. [PubMed: 10527728]
6. Bell ET, Bell JE. Catalytic activity of bovine glutamate dehydrogenase requires a hexamer structure. *Biochem J.* 1984; 217:327–330. [PubMed: 6696727]
7. Ásgeirsson B, et al. Dissociation and unfolding of cold-active alkaline phosphatase from Atlantic cod in the presence of guanidinium chloride. *Eur J Biochem.* 2000; 267:6403–6412. [PubMed: 11029583]
8. Fukushima K, et al. Denaturation of bovine liver glutamate dehydrogenase by guanidine hydrochloride. Correlation between enzymatic activity and molecular state. *J Biochem.* 1985; 97:1111–1117. [PubMed: 4030718]
9. Doucette PA, et al. Dissociation of human copper-zinc superoxide dismutase dimers using chaotrope and reductant. *J Biol Chem.* 2004; 279:54558–54566. [PubMed: 15485869]

10. Sanyal SC, et al. The folding of dimeric cytoplasmic malate dehydrogenase. *Eur J Biochem.* 2002; 269:3856–3866. [PubMed: 12153583]
11. Lee HS, et al. Dissociation/association properties of a dodecameric cyclomaltodextrinase. *FEBS Journal.* 2006; 273:109–121. [PubMed: 16367752]
12. Hanlon AD, et al. Free-solution, label-free protein-protein interactions characterized by dynamic light scattering. *Biophys J.* 2010; 98:297–304. [PubMed: 20338851]
13. Bowie JU, Sauer RT. Equilibrium dissociation and unfolding of the arc repressor dimer. *Biochemistry.* 1989; 28:7139–7143. [PubMed: 2819054]
14. Pal GP, et al. Dissociation and aggregation of calpain in the presence of calcium. *J Biol Chem.* 2001; 276:47233–47238. [PubMed: 11551918]
15. Wilkins DK, et al. Hydrodynamic radii of native and denatured protein measured by pulse field gradient NMR techniques. *Biochemistry.* 1999; 38:16424–16431. [PubMed: 10600103]
16. Kielley W, Harrington W. A model for the myosin molecule. *Biochim Biophys Acta.* 1960; 41:401–421. [PubMed: 14408979]
17. Kawahara K, Tanford C. Viscosity and density of aqueous solutions of urea and guanidine hydrochloride. *J Biol Chem.* 1966; 241:3228–3232. [PubMed: 5912116]
18. Sabate R, Esterlrich J. Aggregation characteristics of ovalbumin in beta-sheet conformation determined by spectroscopy. *Biopolymers.* 2002; 67:113–120. [PubMed: 12073933]
19. Nemoto N, et al. Dynamic light scattering of aqueous solutions of linear aggregates induced by thermal denaturation of ovalbumin. *Biopolymers.* 1993; 33:551–559. [PubMed: 8467064]
20. Levi V, Gonzalez FLF. Reversible fast-dimerization of bovine serum albumin detected by fluorescence resonance energy transfer. *Biochim Biophys Acta.* 2002; 1599:141–148. [PubMed: 12479415]
21. Bohme U, Scheler U. Effective charge of bovine serum albumin determined by electrophoresis NMR. *Chem Phys Lett.* 2007; 435:342–345.
22. Inoue T, et al. Light-scattering study of subunit association-dissociation equilibria of bovine liver glutamate dehydrogenase. *Biochim Biophys Acta.* 1984; 786:144–150. [PubMed: 6722167]
23. Smith TJ, et al. Structures of bovine glutamate dehydrogenase complexes elucidate the mechanism of purine regulation. *J Mol Biol.* 2001; 307:707–720. [PubMed: 11254391]
24. Klotz IM. Equilibrium constants and free energies in unfolding of proteins in urea solutions. *Proc Natl Acad Sci U S A.* 1996; 93:14411–14415. [PubMed: 8962065]
25. Zarrine-Afsar A, Davidson AR. The analysis of protein folding kinetic data produced in protein engineering experiments. *Methods.* 2004; 34:41–50. [PubMed: 15283914]

Figure 1. Monomeric proteins maintain tertiary structure at low guanidine hydrochloride concentrations

A) Emission spectra at increasing concentrations of GudHCl (0- 2M) show no shift in tryptophan emission fluorescence indicating proteins retain tertiary structure. Three replicates were completed per concentration; average is plotted with standard deviation. B) Average maximal fluorescence wavelength plotted from A; black—BSA, open-ovalbumin, gray—lysozyme. Error is ± 0.5 nm. C) R_H derived from DLS data (colored as in B) as a function of increasing GudHCl concentration.

Figure 2. Dynamic light scattering can monitor subunit stabilization by ligand binding

A) Maximal fluorescence from emission spectra of glutamate dehydrogenase (GDH) apo (black), +glutamate (20mM, open) or +norvaline (100mM, gray) at increasing concentrations of GuHCl (0- 2M) show no shift in tryptophan emission fluorescence indicating proteins retain tertiary structure. GDH at 6M GuHCl is included as a reference point for tryptophan emission of denatured enzyme. Three replicates were completed per concentration, averaged data are shown. Error is ± 0.5 nm. B) Cartoon diagram of GDH transitions from native, hexamer to trimer to monomer. Diagram based upon PDB code 1HWZ [23]. C) R_H derived from DLS data for GDH +/- ligands (colored as in A) as a function of increasing GuHCl concentration. D) R_H of GDH calculated from prolate ellipsoid of x-ray crystal structure (PDB code 1HWZ [23]).