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Applications of analytical ultracentrifugation to protein size-and-shape distribution and structure-and-function analyses

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ABSTRACT

The rebirth of modern analytical ultracentrifugation (AUC) began in 1990s. Since then many advanced AUC detectors have been developed that provide a vast range of versatile choices when characterizing the physical and chemical features of macromolecules. In addition, there have been remarkable advances in software that allow the analysis of AUC data using more sophisticated models, including quaternary structures, conformational changes, and biomolecular interactions. Here we report the application of AUC to protein size-and-shape distribution analysis and structure-and-function analysis in the presence of ligands or lipids. Using band-sedimentation velocity, quaternary structural changes and an enzyme's catalytic activity can be observed simultaneously. This provides direct insights into the correlation between quaternary structure and catalytic activity of the enzyme. On the other hand, also in this study, we have applied size-and-shape distribution analysis to a lipid-binding protein in either an aqueous or lipid environment. The sedimentation velocity data for the protein with or without lipid were evaluated using the $c(s,f_r)$ two-dimensional distribution model, which provides a precise and quantitative means of analyzing the protein's conformational changes.

1. Introduction

Analytical ultracentrifugation (AUC)[1] is a very precise and powerful instrument for investigating the size distribution of macromolecules in solution. However, expensive instrumentation and laborious manual data handling somehow have resulted in this powerful instrument being ignored by most scientists. This situation has been changing since 1990s when computerized data acquisition becomes mature and was complemented by the launch of many sophisticated software packages that make the AUC data very informative. Furthermore, the development of various detectors, such as Schlieren optics, UV/VIS absorption, Rayleigh interference, fluorescence, light scattering, turbidity, and multiwavelength UV/VIS optics, has made AUC more versatile that never before [1–3]. These different detection and analysis methods have already promoted AUC to a new level that is able to provide a solid strategy for examining a protein's quaternary structural state and conformational changes, as well as various heterogeneous biomolecular interactions [4].

The present study describes our application of AUC to protein size-and-shape distribution analysis and enzyme structure–function analysis. In the first section, the main protease (Mpro) from severe acute respiratory syndrome coronavirus (SARS-CoV) is chosen as an explicit example to illustrate the usefulness of AUC in enzyme research. The AUC method used here is band-sedimentation velocity, also known as active enzyme centrifugation [5,6]. The detailed theoretical background and practical aspects have been thoroughly discussed [7–12]. At that time, the complicated data analysis problems impeded the wide spread use of this technique [9]. The available literature on band-forming AUC is rather scarce. This has changed because advances in software have made handling huge datasets only a few keyboard strokes away [3,4,13,14]. Taking advantage of this, we have successfully applied the active enzyme centrifugation technique to the dimeric SARS-CoV Mpro in which dimerization is required for its normal functioning [15,16]. The enzyme was assayed by band-forming AUC at various concentrations of the substrates. The quaternary structural change and activities of the enzyme during the catalytic process are determined simultaneously. The enzyme velocities are then used in a kinetic model evaluation, which allows the derivation of the kinetic parameters K_m, k_{cat}, and the Hill coefficient for subunit cooperativity. All the results from the AUC support the existence of substrate-induced dimerization of Mpro, which is consistent with our other studies [16].

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[1] *Abbreviations used:* AEC, active enzyme centrifugation; AUC, analytical ultracentrifugation; SV, sedimentation velocity; pNA, para-nitroanilide; TQ6-pNA, Thr-Ser-Ala-Val-Leu-Gln-para-nitroanilide; SARS-CoV, severe acute respiratory syndrome coronavirus; Mpro, main protease; apoE, apolipoprotein; DHPC, dihexanoylphosphatidylcholine; PBS, phosphate-buffered saline (pH 7.6).

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The conformation of proteins after binding a specific ligand is another intriguing issue that has been studied using many other biophysical probes such as nuclear magnetic resonance, surface plasmon resonance, and fluorescence. [17–19]. Most of these methods are not able to investigate delicate structural changes at the quaternary structure level as yet. In the second part of this study, we describe an elaborate application of sedimentation velocity (SV) to the analysis of changes in protein size-and-shape distribution in either an aqueous or lipid environment. The protein chosen for this study was human apolipoprotein E3 (apoE3), a lipid-binding protein found in blood that is responsible for lipid transfer between organs [20]. The sample used in this study was apoE3-(72–166), which is presumed to have an amphipathic α-helical structure [21]. The experiments were executed with and without lipids and then analyzed by $c(s,f_r)$ two dimensional (2d) distribution model [13]. Instead of fitting the anhydrous frictional ratio (f_r) as a fix value, the 2d model is used to set a broad range for f_r during data processing. This modification helps us quantitatively characterize the size-and-shape distribution of apoE3-(72–166) in an aqueous and in a lipid environment.

As a tool that can detect in a few hours both protein quaternary structural/conformational changes in relation to either ligand binding or lipid binding, the power of AUC is not close to being fully explored. AUC can give us not only a qualitative answer, but also a quantitative measurement that is able to explain the interactions between biomolecules and how this relates to their biological functioning.

2. Description of the methods

2.1. Analysis of enzyme structure-and-function relationship in the presence of substrate

Here Mpro was used as an example to illustrate the usefulness of band-forming AUC when elucidating the role of quaternary structural changes in enzyme activity regulation. Mpro is a dimeric protein whose monomer has no catalytic activity [15,22,23]. Our previous studies have suggested that the dimerization can be induced and then further stabilized by substrate binding [16]. However, such structure-and-function investigations have always been performed in separate experiments, and it is never been possible to have identical experimental conditions. Now, by using the band-forming AUC technique, all of these technical problems have been resolved spontaneously and all of the informative data can be captured simultaneously.

A commercially available double-sector Vinograd-type [11] band-forming centerpiece (Beckman, Fullerton, CA, USA) was used for the sedimentation velocity experiments [24]. In the cell, the protein is transferred, on initiation of centrifugation, through a small channel from the sample well to the bulk sector space, which contains a substrate solution of greater density than the protein solution. The proteins will migrate as a narrow band during the sedimentation run and this created the technique's name, band-forming centrifugation. Moreover, in the presence of ligands, concentration changes in the ligands can be detected using a usual AUC detector, if the substrate-product reaction cycle involves a spectrometric or fluorometric change. This allows the measurement of enzyme function by an *in situ* kinetic assay.

2.1.1. Sample preparation

In our experiment, SARS-CoV Mpro with a 6×His tag fusion was expressed in *Escherichia coli* and purified by nickel affinity chromatography [15]. After a buffer changed using an Amicon 10K cutoff filter (Millipore, Bedford, MA, USA), the protein was stored in PBS (pH 7.3) at 4 °C. The substrate used for the assay was a synthetic hexapeptide derivative, Thr–Ser–Ala–Val–Leu–Gln–para-nitroanilide (TQ6-pNA) (purity 95–99% by HPLC), which was obtained from GL Biochem Ltd., Shanghai, China [16,25]. The enzymatic activity of Mpro was measured by a colorimetric-based peptide cleavage assay. Any increase in absorbance at 405 nm caused by para-nitroanilide (pNA) releasing was continuously monitored using a spectrophotometer. The amount of pNA released from the proteolysis is calculated using a standard curve generated by analytical grade pNA and the result is consistent with the literature ($A_{405\ nm}$ = 9.8 at 1 mM) [26].

To get a narrow protein band, the bulk substrate solution has to be denser than the protein solution. Usually, D_2O, glycerol, sucrose, or high salt (e.g., 50–100 mM excess) is chosen for this purpose [27]. We use D_2O because highly purified D_2O is commercially available; in addition, the handling of a viscous liquid is avoided, which greatly reduces the experimental lapsed time. Each sedimentation run in D_2O is completed within a couple hours rather than overnight, which is required if glycerol is used.

2.1.2. Band-forming sedimentation velocity

The AUC experiments are performed on a XL-A analytical ultracentrifuge (Beckman, Fullerton, CA, USA) with an An-50 Ti rotor [20]. In the sample well of the double-sector band-forming centerpiece, 15 μl of Mpro (1 mg/ml) was added before the cell was assembled. The other small well above the reference sector can be left empty. In total, 330 μl of substrate at different concentrations was dissolved in D_2O and then loaded into the bulk sample sector space. After equilibrating to the desired temperature, the centrifugation was spun at a rotor speed of 42,000 rpm. We found that the TQ6-pNA was cleaved and free pNA accumulated during centrifugation (detected by absorbance change at 405 nm). The absorbance spectrum of the free pNA interferes with protein absorbance at 280 nm. Therefore absorbance at 250 nm was chosen instead for detecting the protein band, while the wavelength 405 nm was used to monitor the catalytic released product pNA. The spectrum was monitored continuously using a time interval of 600 s per scan and a step size of 0.003 cm.

A typical trace of the 250 nm and 405 nm spectral results are shown in Figs. 1A and 2A, respectively. The dataset from these multiple scans at 250 nm at different time intervals were then fitted to a continuous $c(s)$ distribution model using the SEDFIT program [4,13] (<www.analyticalultracentrifugation.com>) (cited August 1, 2010). The partial-specific volume of Mpro, the solvent density, and the viscosity were calculated by SEDNTERP (<www.jphilo.mailway.com/download.htm>) (cited August 1, 2010). The first six scans at 405 nm were used to calculate the integration area in order to derive the initial velocity values.

2.1.3. Data analysis

2.1.3.1. Continuous c(s) distribution analysis for band-sedimentation velocity. SEDFIT calculates the continuous size distribution (sedimentation coefficient) using a variation based on the Lamm equation [28]. The observed continuous $c(s)$ distribution profiles can be treated as a superimposition of each subpopulation $c(s)$ of particles with sedimentation coefficients between s and $s + ds$ using the following equation,

$$a(r,t) \cong \int c(s)L(s,D(s),r,t)ds + \in \quad (1)$$

where $a(r,t)$ represents the experimentally observed signal at radius r and time t. $L(s, D(s), r, t)$ is the sedimentation profile of an ideally sedimenting monodisperse species with sedimentation coefficient s and diffusion constant D, and were calculated as the solution to the Lamm equation; $\in$ represents the noise components. In the data fitting process, the Tikhonov–Phillips method is implemented by SEDFIT [4,29] and is used at default to regularize the distribution. All