Colloids and Surfaces B: Biointerfaces 136 (2015) 674–677

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Short communication

Hierarchical multi-step organization during viral capsid assembly

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ARTICLE INFO

Article history:
Received 2 July 2015
Received in revised form 6 October 2015
Accepted 8 October 2015

Keywords:
HIV
Capsid
Self-assembly
Mechanism
Cryo-TEM

ABSTRACT

Formation of the HIV-1 core by the association of capsid proteins is a critical, not fully understood, step in the viral life cycle. Understanding the early stages of the mechanism may improve treatment opportunities. Here, spectroscopic analysis (opacity) is used to follow the kinetics of capsid protein assembly, which shows three stages: a lag phase, followed by a linear increase stage and terminated by a plateau. Adding pre-incubated capsid proteins at the start of the lag phase shortens it and increases the rate of assembly at the linear stage, demonstrating autoacceleration and cooperative assembly. Cryogenic transmission electron microscopy is used to probe structural evolution at these three stages. At the beginning of the lag phase, short tubular assemblies are found alongside micron long tubes. Their elongation continues all throughout the lag phase, at the end of which tubes start to assemble into bundles. Based on these results, we suggest a multi-step self-assembly process including fast nucleation and elongation followed by tubes packing into arrays.

1. Introduction

Protein self-assembly into ordered supramolecular structures is the underlying event of many physiological as well as pathological processes, varying from actin and tubulin filaments formation to amyloid fibril and viral particles association [1]. Understanding the initial stages of the assembly pathway is fundamental for the development of therapeutic intervention in the case of pathological ultrastructure formation [2]. While monitoring late stages in the assembly of various proteins is well-established [3], the detection of the initial conformers has been rather scarce due to resolution and rapid kinetics issues for many systems. HIV-1 capsid protein is the structural component forming the inner core of the infectious particle, encapsulating the viral genome that is bound to nucleocapsid protein [4]. Structural studies of native HIV-1 capsid have been greatly facilitated by the capacity of capsid protein to self-assemble *in vitro* into tubes and cones [5]. Electron diffraction and cryogenic transmission electron microscopy (cryo-TEM) revealed that the tubes formed by capsid protein *in vitro* and cores isolated from virions share common structural motifs, both composed of helical arrays of protein hexamers [6,7], indicating that the *in vitro* assembly of purified capsid protein is a reliable model for native capsid assembly *in vivo*. The *in vitro* assembly of capsid protein is concentration dependent and highly affected by molecular crowding agents [8], chemical chaperones [9] and by high ionic strength [10], which is required for reducing the electrostatic repulsion between capsid protein molecules and consequentially promoting molecular recognition,

The assembly process of HIV-1 capsid protein, similar to that of other viral capsids, was suggested to be a nucleation-dependent process [11,12]. According to the classical model, the assembly reaction originates with the formation of protein nuclei that slowly assemble into steady intermediates [13] which followed by an elongation step characterized by a fast kinetics [14]. Finally, at equilibrium, the elongated intermediates eventually give rise to the expected helical-cylindrical structures [12]. Correspondingly, the *in vitro* assembly of hepatitis B virus (HBV) capsid protein was also suggested to be initiated by a rate-limiting nucleus formation [13–15]. While the HBV capsid assembly nucleus is a putative trimer of dimers [13], the identity of HIV-1 capsid nucleus still needs to be clarified. Most structural studies (by X-ray crystallography, cryo-TEM and tomography analyses) of capsid protein organization mainly focused on the intermolecular interactions between protein subunits [6,7,16,17], hence, little is known on the kinetics of HIV-1 capsid protein assembly in its early stages.

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http://dx.doi.org/10.1016/j.colsurfb.2015.10.010
0927-7765/

2. Experimental

2.1. Protein expression and purification

Pet11a-based expression vector of HIV-1 capsid protein was kindly provided by W. I. Sundquist (University of Utah). HIV-1 capsid was expressed in BL21 (DE3) *Escherichia coli* Rosetta cells and purified as previously described [4]. Briefly, cells were grown to an OD600 ~0.8 at 37 °C and induced with 0.4 mM IPTG (isopropyl-b-D-thiogalactopyranoside) for 4 h. Cells were resuspended and lysed by 12.5 mg/ml of lysozyme in 50 mM Tris–HCl (pH 8.0) 5 mM β-mercaptoethanol. Lysates were centrifuged for 30 min at 4 °C and 10,000 rpm and capsid was precipitated from the supernatant with 25% saturated ammonium sulfate buffer, by centrifugation for 30 min at 4 °C and 20,000 rpm. The crude protein was dialyzed against 25 mM Tris–Cl (pH 8.1) and was purified by anion exchange chromatography (20 ml Q-Sepharose column, Amersham Pharmacia Biotech) using Akta prime system (GE Healthcare). The protein was flash frozen and stored at −80 °C.

2.2. Turbidity assay

Purified HIV-1 capsid protein was dialyzed into 50 mM Na_2HPO_4 (pH 8.0) solution and was triggered to assemble by rapid dilution into 50 mM Na_2HPO_4-4 M NaCl (pH 8.0). To examine the effect of ionic strength, assembly reactions were carried out at a final protein concentration of 50 μM either in the absence or presence of varying concentrations of NaCl (Fig. 1a). For the seeding experiment, capsid protein (50 μM) was pre-incubated with 0.5 M NaCl for 2 h at 25 °C, designated "seeds". Freshly thawed capsid protein with 0.5 M NaCl was monitored either in the absence or the presence of varying volume percentages of seeds (Fig. 1b). For all samples, final protein and salt concentrations were 50 μM and 0.5 M, respectively.

All assembly reactions were monitored by measuring the increase in optical density at 350 nm over time at 25 °C in 2 min intervals using Synergy HT Microplate Reader (BioTek Instruments, Inc.).

2.3. Stability of capsid protein assemblies

24 h after incubation with salt (0.5 M) at 25 °C, assembly reactions of HIV-1 capsid protein (50 μM) were sonicated using bath sonicator DG-1 mini supersonic cleaner (mrs) with operating frequency of 43 kHz and Power of 50 W.

2.4. Cryo-TEM

Aliquots were taken from the solution after different incubation times. Specimens for cryo-TEM were prepared using Leica EM GP cryo-preparation station operated at 100% relative humidity. A drop of 4 μl of solution was applied on a holey carbon TEM grid (Lacey substrate, 300 mesh, Ted Pella, Inc.) automatically blotted with a filter paper, and plunged into liquid ethane at its freezing point. The vitrified samples were stored under liquid nitrogen before being transferred to a TEM (Tecnai 12, FEI) using a Gatan workstation and cryo-holder for imaging at 98 K. The microscope was operated at 120 kV in low electron dose mode (to reduce radiation damage) and with a few micrometers under-focus to increase phase contrast. Images were recorded on a Gatan 794CCD camera.

3. Results and discussion

To study the early stages of assembly, we first monitored the assembly kinetics of HIV-1 capsid protein under mild conditions

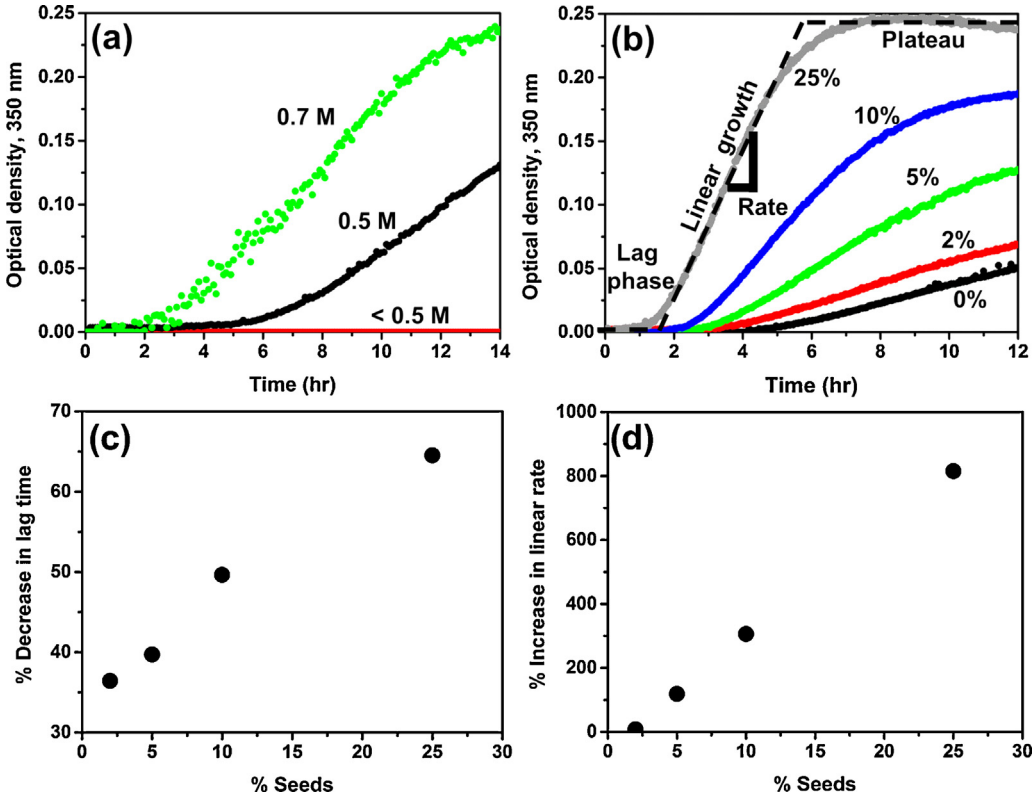

Fig. 1. Monitoring the kinetics of capsid protein assembly by turbidity assay. Capsid protein (50 μM) kinetics of assembly was monitored as a function of (a) salt concentration and (b) seeds percentage (protein pre-incubated with 0.5 M of salt for 2 h at 25 °C). The dashed lines represent the different incubation stages. The final protein and salt concentration in each sample were 50 μM and 0.5 M, respectively. The decrease in the lag time and the increase in the linear assembly rate of the reaction following the addition of seeds are shown in (c) and (d), respectively. The error bars of these measurements are smaller than the marker size.