Methods xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Methods

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

Recent adaptations of fluorescence techniques for the determination of mechanistic parameters of helicases and translocases

Máté Gyimesi, Gábor M. Harami, Zsuzsa S. Kocsis, Mihály Kovács *

ELTE-MTA "Momentum" Motor Enzymology Research Group, Department of Biochemistry, Eötvös Loránd University, Pázmány Péter sétány 1/c, H-1117 Budapest, Hungary

ARTICLE INFO

Article history:
Received 3 March 2016
Received in revised form 22 April 2016
Accepted 25 April 2016
Available online xxxx

Keywords:
Kinetics
Thermodynamics
Mechanism
ATP
NTP
Hydrolysis
Translocation
Unwinding
Nucleic acid
DNA
RNA
Helicase
Translocase

ABSTRACT

Helicases and translocases are nucleic acid (NA)-based molecular motors that use the free energy liberated during the nucleoside triphosphate (NTP, usually ATP) hydrolysis cycle for unidirectional translocation along their NA (DNA, RNA or heteroduplex) substrates. Determination of the kinetic and thermodynamic parameters of their mechanoenzymatic cycle serves as a basis for the exploration of their physiological behavior and various cellular functions. Here we describe how recent adaptations of fluorescence-based solution kinetic methods can be used to determine practically all important mechanistic parameters of NA-based motor proteins. We outline practically useful analysis procedures for equilibrium, steady-state and transient kinetic data. This analysis can be used to quantitatively characterize the enzymatic steps of the NTP hydrolytic cycle, the binding site size, stoichiometry and energetics of protein-NA interactions, the rate and processivity of translocation along and unwinding of NA strands, and the mechanochemical coupling between these processes. The described methods yield insights into the functional role of the enzymes, and also greatly aid the design and interpretation of single-molecule experiments as well as the engineering of enzymatic properties for biotechnological applications.

1. Introduction and scope

Enzymatic processing of nucleic acids is fundamental for the replication of chromosomes, the transcription and translation of genes, genetic recombination and the repair of damaged genetic material. These essential processes involve protein-NA interactions and are often accompanied by the directed movement of proteins along the NA lattice. NA translocases are enzymes that use the free energy liberated during the nucleoside triphosphate (NTP; most often ATP) hydrolysis cycle to fuel unidirectional movement along DNA or RNA strands. Helicases couple this translocation activity to unwinding of base-paired NA segments and/or the displacement of other proteins bound to the tracked NA strand. The diversity of structures, mechanisms of action and cellular functions of helicases have recently been reviewed in Refs. [1–3].

Mechanistic understanding of translocase and helicase activity and, in turn, that of cellular processes involving such activities, requires the quantitative determination of the functional properties of these important enzymes. Various parameters must be determined to describe the NTP hydrolytic enzymatic cycle, the interaction of the enzyme with NA substrates of varying structure, and the coupling between each of the NTP hydrolytic, NA binding, translocation, unwinding and protein displacement activities. Theoretical frameworks for helicase action have been described in earlier studies [4–6]. In this article we will review recently introduced applications of fluorescence-based techniques that have been specifically devised for the quantitative assessment of the aforementioned enzymatic processes. We will focus on the most important aspects of experiment design and data analysis, based

Abbreviations: AU, absorbance unit (Chapter 3.1) or arbitrary unit (Figs. 2 and 5); BLM, Bloom's syndrome DNA helicase; bp, base pair; ds, double-stranded; $DxSO_4$, dextran sulfate; FRET, Förster Resonance Energy Transfer; LDH, lactate dehydrogenase; mant, methylanthraniloyl; MDCC, 7-diethylamino-3-((((2-maleimidyl)ethyl)amino)carbonyl)coumarin; NA, nucleic acid; NADH, nicotinamide adenine dinucleotide; nt, nucleotide; NATA, n-acetyl-L-tryptophanamide; NTP, nucleoside triphosphate; PEP, phosphoenolpyruvate; P_i, inorganic phosphate; PK, pyruvate kinase; ss, single-stranded; TIRF, total internal reflection fluorescence; Trp, tryptophan; WHD, winged helix domain.

* Corresponding author.
E-mail addresses: mate.gyimesi@ttk.elte.hu (M. Gyimesi), gabor.harami@gmail.com (G.M. Harami), srablue@gmail.com (Z.S. Kocsis), mihaly.kovacs@ttk.elte.hu (M. Kovács).

http://dx.doi.org/10.1016/j.ymeth.2016.04.028
1046-2023/

on equations developed specifically for the determination of functional parameters of helicases and translocases. Recent developments of applicable fluorescent labels, biosensors, and instrumental setups have been summarized in Refs. [7,8].

Functional parameters in enzymology can either characterize equilibrium conditions or describe the kinetics of transitions between discrete stable states. In equilibrium measurements, the independent variable is most often the concentration of a reaction component, and the readout is used to infer the extent of binding and/or a distribution between structural states. In kinetic measurements, the time course of a reaction signal is monitored, either in steady-state conditions or in the transient phase that occurs upon a perturbation that provokes a transit from one equilibrium or steady-state to another, e.g. via mixing of reaction components. Transients of interest often occur in sub-second time scales, necessitating the use of rapid-reaction equipment (e.g. stopped-flow, quenched-flow). Descriptions of such instrumentation, as well as those of the theoretical principles of transient kinetic analysis, are provided in Refs. [7,9,10].

Kinetic data have been routinely collected for ensembles comprising large numbers of molecules in the analyzed solution. However, technical advances in the last decades have enabled the investigation of single molecules, and the application of such techniques is spreading rapidly. In principle, all enzymatic parameters can be determined by collecting a sufficiently large amount of data on single molecules. However, technical limitations still hinder the application of single-molecule techniques for the determination of certain parameters. For some processes, no sufficiently sensitive signal is available. Other processes occur too rapidly or too slowly (infrequently) in relation to the available recording time scales. In this article we will limit our discussion to ensemble analysis, as single-molecule enzymological applications have been covered in several useful recent publications [11–20]. Ensemble methods confer the advantage that they generally require less sophisticated instrumentation. Therefore, these techniques can be readily used for the determination of the majority of important mechanistic parameters and, thus, will continue to provide a large body of mechanistic knowledge on helicases and other NA-based enzymes. The precise quantitative characterization of the enzymatic properties of these proteins in the absence and presence of interacting partners is crucial for the understanding of their complex physiological functions in essential processes such as replication, recombination and repair. Moreover, the elucidation of these mechanisms greatly aids the engineering of enzyme functions for biotechnological applications.

Importantly, the information gained using all of the steady-state, ensemble transient kinetic and single-molecule methods are to be interpreted within a common physicochemical framework of enzyme action. Thus, the models developed must be able to account for all levels of molecular behavior. Therefore, even though many processes occur *in vivo* in confined cellular environments containing a limited number of operating molecules, data recorded in each of the aforementioned conditions can provide important mechanistic information to aid the understanding of physiological and pathological phenomena. Powerful approaches will thus continue to combine ensemble and single-molecule techniques.

2. Nucleic acid binding parameters and their dependence on nucleic acid structure

NA translocases, in accordance with their *in vivo* function, interact with various NA structures ranging from simple single-stranded (ss) NAs and duplex regions (formed either by one or two strands) to complex multi-stranded structures with branches (e.g. D-loops, R-loops, Holliday junctions). Determination of the NA binding profile of these enzymes is essential to understand their function. Moreover, the determination of the binding affinity, stoichiometry of interaction and binding kinetics to various NA structures in different nucleotide states is indispensable for the construction of comprehensive mechanistic models describing the NA processing activities of these proteins. Below we will introduce some of the most versatile techniques that can be readily carried out in most labs to determine parameters of protein-NA interactions.

2.1. Equilibrium binding methods

2.1.1. Electrophoretic mobility shift assay (EMSA)

A commonly used method to determine or estimate equilibrium binding parameters (binding affinity and stoichiometry) of protein-NA interactions is the electrophoretic mobility shift assay (EMSA) [21]. EMSA is based on the separation of protein-bound and protein-free NA species via their different migration speed in non-denaturing gel electrophoresis experiments. For binding affinity determination, a fixed amount of radioactively (usually ^{32}P isotope) or fluorescently labeled NA is incubated with different amounts of the investigated protein. Radioactive labeling leaves the chemical properties of NA molecules unaffected and offers high sensitivity (detection requires 100 pM labeled molecule or less), but raises specific safety issues. Moreover, the shelf-life of labeled molecules is short due to radioactive decay (the half-life of ^{32}P is 14.3 days) (see Refs. [22,23] for labeling protocols). In contrast, fluorescently-labeled NAs are more stable and easier to handle, but fluorescent probes offer lower sensitivity (few nM or more labeled molecules required for detection) compared to radioactive labeling, and often alters the chemical features of the labeled molecule. Gel electrophoresis is carried out either in agarose or polyacrylamide gels, depending on the size of the NA and the protein-NA complex. Radioactively or fluorescently-labeled molecules are detected in the gel by using a storage phosphor screen and a phosphorimager instrument or with a fluorescence gel scanner, respectively. The optical density of bands corresponding to the bound and unbound NA is determined via densitometry by using image analysis software. Ultimately, the fraction of protein-bound NA (calculated as the ratio of the density of protein-bound NA to the summed densities of all bands) is plotted against the protein concentration to obtain a binding curve.

Despite its simple basic principle, EMSA has disadvantages. In some cases, multiple bands in the gel representing different protein-bound NA species can be detected, making the analysis more difficult. Finding experimental conditions (buffer composition, concentrations and electrophoresis conditions) that result in analyzable gels can also be labor-intensive. Protein-NA complexes can dissociate during gel electrophoresis, and thus EMSA is not a true equilibrium measurement. Due to dissociation, complexes with low lifetimes may be undetectable in EMSA assays.

2.1.2. Equilibrium methods based on fluorescence emission intensity

To overcome the limitations of EMSA, numerous fluorescence spectroscopic assays have been developed that can be used for rapid and cost-effective investigation of protein-NA interactions.

In fluorescence intensity measurements, a binding curve is obtained from the fluorescence emission change upon complex formation. Fluorophores can be intrinsic or extrinsic, the latter being fluorescent probes covalently attached either to the protein or the NA. The intrinsic Trp fluorescence emission of NA-binding proteins is often used to detect NA binding (Fig. 1A). Internally or terminally labeled NA oligonucleotides are usually obtained commercially.

Please cite this article in press as: M. Gyimesi et al., Methods (2016), http://dx.doi.org/10.1016/j.ymeth.2016.04.028