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PHYSICAL ANALYSIS OF RECOMBINANT FORMS OF THE HUMAN MITOCHONDRIAL DNA HELICASE

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Abstract

Maintenance of the mitochondrial DNA (mtDNA) genome is dependent on numerous nuclear-encoded proteins including the mtDNA helicase, which is an essential component of the replicative machinery. Human mtDNA helicase shares a high degree of sequence similarity with the bacteriophage T7 primase-helicase gene 4 protein, and catalyzes duplex unwinding in the 5'-3' direction. As purified at 300 mM NaCl, the enzyme exists as a hexamer, with a modular architecture comprising distinct N- and C-terminal domains. We present here several methods that allow the identification of the oligomeric state of the human mtDNA helicase, and probe the modular architecture of the enzyme. Despite their relatively common usage, we believe that their versatility makes these techniques particularly helpful in the characterization of oligomeric proteins.

Keywords

mitochondrial DNA replication; DNA helicase; hexamer; oligomer; human

1. Introduction

Helicases are motor proteins that utilize the energy of nucleoside triphosphate hydrolysis to perform their functions, particularly unwinding of the duplex DNA and the mechanical translocation along the nucleic acid network [1–3]. Mutations affecting helicase functions disturb basic cellular mechanisms and lead to diseases such as cancer and premature aging in humans [4]. Although some helicases can function as single polypeptides, most function as oligomeric structures containing two or more protomers that provide the enzyme with multiple active sites required in their motor protein activities [5–8]. For example, in *Escherichia coli*, the primary replicative helicase, the DnaB protein, forms a stable ring-like hexamer, specifically stabilized by the binding of multiple magnesium cations [6,9]. Other well-known replicative helicases, the bacteriophage T7 and T4 enzymes, as well as the *E. coli* transcription termination factor Rho helicase, form hexameric ring-like structures that require or are stabilized by a nucleotide cofactor and/ or DNA binding [1,8,9].

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A human protein responsible for replicative mtDNA helicase activity was identified and designated Twinkle based on its sequence similarity with the bacteriophage T7 gene 4 protein (T7 gp4) and its co-localization with mitochondrial nucleoids (T7 gp4-like protein with intramitochondrial nucleoid localization) [10,11]. Preliminary biochemical characterization of a recombinant human mtDNA helicase revealed its NTP hydrolysis and DNA unwinding properties with a 5'-3' directionality [12]. Furthermore, a minimal mtDNA replisome has been reconstituted in vitro by combining mtDNA helicase, mtDNA polymerase and mitochondrial single-strand binding protein with a suitable DNA substrate [13]. Physiological studies demonstrated that the human mtDNA helicase is essential for mtDNA maintenance and regulation of mtDNA copy number [11,14]. Because human mtDNA helicase mutations and deletions are directly linked to human disease such as autosomal dominant progressive external ophthalmoplegia, a comprehensive analysis of the enzyme is warranted.

The structure and function of the bacteriophage T7 gene 4 protein have been well studied, providing a framework for comparison with other novel helicases such as the human mtDNA enzyme. T7 gp4 is a bifunctional primase/ helicase enzyme. It contains three distinct functional domains. The N-terminal domain encompasses two subdomains, a zinc binding domain (ZBD) and an RNA polymerase domain (RPD) [15,16]. The ZBD functions in recognizing and binding DNA. The RPD synthesizes an RNA primer that is extended by the T7 DNA polymerase, T7 gene 5 protein [17,18]. The C-terminal domain is responsible for the helicase function and contains the dTTPase activity [18,19]. The N- and C-terminal domains are separated by a linker that is important for oligomerization [20,21]. Interestingly, while T7 gp4 is able to function as a primase in its monomeric form [17], multimerization into hexamers or heptamers is required for helicase activity [22].

Escherichia coli DnaB is another well-characterized hexameric helicase. DnaB is involved in both the initiation and elongation stages of chromosomal DNA replication, as well as in the replication of phage and plasmid DNA [23,24]. At the replication fork, DnaB forms a critical interaction with its corresponding primase, the DnaG protein, and this association stimulates the activities of both the helicase and the primase [25]. DnaB comprises two major domains: the N-terminal domain and the C-terminal helicase domain separated by a linker region [26,27]. It has been suggested that N-terminal domain including the linker region is essential for helicase activity in vitro, and for oligomerization under certain conditions [27,28]. Furthermore, a recent X-ray structure of the *Bacillus stearothermophilus* DnaB in complex with the DnaG shows that three molecules of the helicase binding domain of the DnaG are bound to the N-terminal domain of the DnaB hexamer, indicating that the N-terminal portion of the DnaB is crucial for the primase: helicase interaction [29]. The C-terminal portion of DnaB comprises its DNA binding, oligomerization, and ATPase activities [14,26,27].

Both T7 gp4 and the human mtDNA helicase belong to an *Escherichia coli* DnaB-like family of hexameric helicases, also known as the superfamily 4 (SF4) group of replicative DNA helicases. All members of this family contain by definition five conserved motifs (H1, H1a, H2, H3 and H4) that are located in the C-terminal region of the protein [10]. Motifs H1 and H2 are equivalent to the Walker A and B motifs found in all AAA⁺ helicases, but motifs H1a, H3, and H4 have no obvious counterparts in any other helicase family. The N-terminal region of these helicases is less well conserved. Indeed, in the T7 gp4 helicase, two domains related to the ZPD and RPD of DnaG are fused to the N-terminal portion of the T7 gp4. Several other differences exist between the members of the DnaB-like family of helicases, yet it is clear that a modular architecture is a structural characteristic of the proteins from the SF4 family. The presence of distinct structural domains within the protein is often investigated using limited proteolysis. In our recent study [30], we demonstrated using

trypsin digestion under limiting conditions that the human mtDNA helicase and bacteriophage T7 gp4 share similar structural domains. In both enzymes, the first polypeptide produced is generated by cleavage from the C-terminal portion. Digestion of T7 gp4 protein with the endoprotease Glu-C results in a loss of the ZBD domain [31], and trypsin digestion of the human mtDNA helicase follows similar pattern, with proteolytic cleavage at a homologous cleavage site in its N-terminal region [30]. Analysis of the proteolytic products of the human protein also confirmed similarities to *E. coli* DnaB. Under limited conditions, a trypsin digestion of DnaB produced N- and C-terminal structural domains generated by a single cleavage in a proteolytically sensitive region [26]. We have also observed N- and C-terminal cleavages occurring at unstructured regions of the human mtDNA helicase [30].

All helicases are involved in complex processes in which the binding and/ or hydrolysis of NTPs regulates enzyme activity and affinity toward different conformations of the DNA [23,32]. Binding of cofactors such as Mg^{2+} and NTP often induces oligomerization of the enzyme, and stimulate conformational changes that are necessary for protein function during replication. Limited proteolysis of *E. coli* DnaB protein shows a conformational change upon nucleotide binding [26]. Similarly, T7 gp4 protein undergoes transformation into numerous conformations in the presence of various nucleotides and DNA [33]. These conformations are presumed to facilitate nucleotide hydrolysis and the mechanism of single-stranded DNA (ssDNA) translocation that leads to double-stranded DNA (dsDNA) unwinding.

Although the importance of the helicase functions has been recognized, the role of the oligomeric state of the protein and the nature of interactions with cofactors remain unclear for many helicases. In this manuscript, we present several methods that have allowed us to investigate the oligomeric state of the human mtDNA helicase, and to probe its modular architecture. These include size exclusion chromatography, velocity sedimentation, glutaraldehyde cross-linking, and limited proteolysis with multiple proteases.

2. Description of Methods

2.1. Determination of the Native Molecular Mass of the Human mtDNA Helicase

2.1.1. Size Exclusion Chromatography—Size exclusion allows purification of proteins under native conditions in which macromolecular interactions are preserved. It provides information on the hydrodynamic radius of proteins and by extrapolation with molecular mass standards, can provide a molecular weight [36]. All chromatographic operations are performed at 0–4 °C. The Superdex 200 HR 10/30 column (10 mm × 300–310 mm, Amersham Biosciences) is equilibrated with at least 2 CV of running buffer (35 mM Tris-HCl pH 7.5, 330 mM NaCl, 8% glycerol, 0.5 mM EDTA, 10 mM sodium metabisulfate). A sample of mtDNA helicase (150–180 µg) in a volume approximately 10% greater than the intended volume of the injected sample is prepared in a microcentrifuge tube and centrifuged at $10,000 \times g$ for 10 min, prior to its injection onto the equilibrated column. For highest resolution, sample volumes between 0.1 and 1% of the column bed volume are recommended. The column is run at a flow rate of 0.25 ml/ min flow. Generally, separations by size exclusion chromatography are best optimized by starting with a high flow rate and a relatively small sample volume. Optimal resolution should be expected with flow rates of 0.5–1.0 ml/ min.; large molecules often require lower flow rates (0.1–0.5 ml/ min) for maximal resolution. To obtain a standard curve for Stokes' radius determination, the analysis is also performed with proteins selected as molecular mass standards: chymotrypsinogen A, hen egg ovalbumin, yeast alcohol dehydrogenase, bovine liver catalase and bovine thyroid thyroglobulin (all from Sigma-Aldrich). Amounts and volumes comparable to the amount and volume of the mtDNA helicase are injected. Fractions are

analyzed by 10% SDS-PAGE (17% for smaller molecular markers), followed by Coomassie staining. The Stokes' radius for the mtDNA helicase (8 nM) is calculated from a standard curve obtained by plotting the known values of Stokes' radii of the molecular marker proteins versus their chromatographic peaks (Fig. 1, left).

2.1.2. Velocity Sedimentation—Velocity sedimentation has a long history in the characterization of synthetic polymers, biological macromolecules, and viral particles. One of the useful properties of velocity sedimentation is that the measurement takes place with molecules free in solution, and that the size distribution is a faithful representation of the ensemble of particles in solution [37]. All sedimentation velocity steps are performed at 0–4 °C. The mtDNA helicase sample (20 µg, 0.2–0.7 ml) is layered onto preformed 12–30% glycerol gradients (10 ml) prepared in 35 mM Tris-HCl, pH 7.5, 330 mM NaCl, 1 mM EDTA in polyallomer tubes for use in a Beckman SW 41 rotor. The sample is centrifuged at 140,000 $\times g$ for 37 h at 4 °C, and the gradients are then fractionated by collecting four-drop (200 µl) fractions. To obtain a standard curve for determination of the sedimentation coefficient (*S*), the proteins selected as molecular mass standards (jack bean urease, bovine liver catalase, rabbit muscle L-lactate dehydrogenase (all from Sigma-Aldrich)) are run in parallel with the human mtDNA helicase sample. Fractions are analyzed by 10% SDS – PAGE (17% for smaller molecular markers), followed by Coomassie staining. The *S* value for the mtDNA helicase (13.6 *S*) is calculated from a standard curve obtained by plotting peak fraction numbers of the molecular marker proteins versus the known values of their sedimentation coefficients (Fig. 1, right).

2.2. Glutaraldehyde Cross-linking Analysis of the Human mtDNA Helicase

Glutaraldehyde is a wide-range, bifunctional peptide crosslinking agent. It forms covalent bonds with several functional groups in the proteins, although its cross-linking effects are dominated by reactions with the ϵ -amino group of lysine residues [38,39]. Snapshot fixation of an oligomer by cross-linking with glutaraldehyde and subsequent analysis by SDS-PAGE is a powerful tool for the analysis of oligomeric proteins [for example, see ref. 40]. Typically, phosphate and HEPES buffers at pH 7.5 to 8.0 are recommended for glutaraldehyde cross-linking versus Tris-HCl-containing buffers, due to a possible competition for glutaraldehyde binding from the 2-amino group of the Tris molecule [41]. However, we have used successfully low concentration Tris-HCl buffers in our experiments. A master reaction mixture is prepared using stock solutions in a microcentrifuge tube on ice such that each reaction (20 µl) contains 1 $\times$ cross-linking buffer (25 mM Tris-HCl pH 7.5, 80 mM NaCl, 0.1 mM EDTA, 1 mM DTT), 2.5 µg human mtDNA helicase, and 2 mM ATP and 4 mM MgCl₂ where indicated. The mixture is dispensed in 14 µl aliquots, to pre-chilled microcentrifuge tubes on ice. The helicase (2.5 µg, 6 µl) is then added to each tube, carefully avoiding bubbles, and the tubes are mixed gently by flicking. Next, the tubes are incubated for 30 min at 37 °C. A freshly prepared 0.4% glutaraldehyde solution (25% stock, Sigma-Aldrich, Grade I) is added to a final concentration of 0.02% where applicable, and the tubes are incubated for 20 min at 37 °C. The appropriate glutaraldehyde concentration should be determined empirically for each protein. Typically concentrations between 0.01–0.1% are recommended. The reactions are stopped with addition of 5 µl 5 $\times$ SDS loading buffer (50% glycerol, 2 M Tris base, 0.25 M DTT, 5% SDS, 0.1% bromophenol blue) and heated for 5 min at 100 °C. In general, NaBH₄ is utilized to terminate the glutaraldehyde cross-linking reaction via reduction of aldehyde and hydrolyzable Schiff base bonds [42]. We have determined that addition of 5 $\times$ SDS loading buffer in the amount of approximately 25% of the sample volume stops the reaction well, and represents a much safer alternative. The samples are then analyzed by 4–7% linear gradient SDS-PAGE, followed by transfer to nitrocellulose membranes at 100 mA for 10 h. In our experience, the linear gradient SDS-polyacrylamide gels are more suitable for the resolution of multiple large oligomeric

species. The appropriate polyacrylamide concentration should be determined experimentally for each protein. After protein transfer, the nitrocellulose filters are incubated for 1 h in TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20) and 5% (w/v) nonfat milk. The membranes are probed with rabbit antiserum against recombinant human mtDNA helicase (1:100 dilution in TBST buffer and 1% nonfat milk) for 2 h, and then washed in TBST buffer three times for 5 min each [35]. The membranes are then incubated for 1 h with goat-anti-rabbit IgG alkaline phosphatase conjugate (1:2000 (v/v) dilution in TBST buffer with 1% nonfat milk), and then washed in TBST buffer three times for 5 min, and once in AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) for 5 min. The membranes are developed by incubation in AP buffer containing nitro blue tetrazolium chloride (330 µg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (165 µg/ml). The developing reaction is stopped with addition of PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH of 7.5) and 20 mM EDTA. The incomplete glutaraldehyde cross-linking shown in Figure 2 most likely does not indicate partial dissociation, in terms of a hexamer: monomer equilibrium. Rather, it likely reflects either lack of, or limited accessibility to, lysine residues involved in bridging neighboring subunits [43], or to double blocking of potentially linkable interfacial lysine residues by the reagent [44]. Cross-linking of helicases with glutaraldehyde in this manner allows a versatile test of the influence of cofactors such as Mg²⁺ or nucleotides, or substrates such as DNA on oligomeric structure.

2.3. Limited Proteolysis of the Human mtDNA Helicase

Limited proteolysis represents another tool commonly used to characterize protein structure [45]. Protease-dependent digestion of a protein can help to determine the solvent accessibility of specific amino acid residues, and assess changes occurring in the absence or presence of cofactors. Buried amino acid residues are less accessible to the protease, whereas those located on the surface of a protein will be much better candidates for proteolysis. These proteolysis products can be visualized using SDS-PAGE [46].

2.3.1. Multiple Protease Digestion of the Human mtDNA Helicase—A master reaction mixture is prepared using stock solutions in a microcentrifuge tube on ice such that each reaction (30 µl) contains 1× proteolysis buffer (50 mM Tris-HCl, pH 8.0, 50% glycerol, 4 mM MgCl₂, 2 mM 2-mercaptoethanol) and 1.5 µg human mtDNA helicase. The mixture is dispensed in 24 µl aliquots to pre-chilled and numbered microcentrifuge tubes on ice. The helicase (1.5 µg, 3.6 µl) is added last to each tube avoiding bubbles, and the tubes are mixed gently by flicking. The proteases (all from Sigma-Aldrich) are then added in ratios of helicase:protease as follows: trypsin 1:1; chymotrypsin 1:0.35; thermolysin 1:1; subtilisin 1:0.005; papain 1:0.18. The applicable ratio of protein to protease and the required time of digestion need to be determined experimentally; the number of potentially proteolytically-sensitive sites can be predicted, but their accessibility to protease is not known. Next, the tubes are incubated for 10 min at 20 °C. The trypsin reaction is stopped with addition of soybean trypsin inhibitor (STI; Sigma-Aldrich, 1 µl) at a STI:trypsin ratio of 2:1. The excess of STI necessary to ensure inhibition of trypsin needs to be determined experimentally. All other protease digestions are stopped with addition of 10 mM sodium metabisulfate, 2 µg/ml leupeptin, and 1% SDS. The samples are analyzed by 10% SDS-PAGE, followed by silver staining or immunoblotting. This method can be adapted readily to test whether the proteolytic pattern of helicase changes in the presence cofactors or DNA substrates.

2.3.2. Time Course of Trypsin Digestion of the Human mtDNA Helicase—A 6× reaction mixture (180 µl) is prepared using stock solutions in a microcentrifuge tube on ice such that final concentrations are equivalent to a reaction containing 1× proteolysis buffer

(50 mM Tris-HCl, pH 8.0, 50% glycerol, 4 mM MgCl₂, 2 mM 2-mercaptoethanol) and 9 µg human mtDNA helicase. The helicase (20 µl) is added last, avoiding bubbles, and the tube is mixed gently by flicking. Trypsin (9 µg, 4.5 µl) is added such that the final helicase: trypsin ratio is 1:1. The optimal helicase: protease ratio is again determined experimentally. The master sample is incubated at 20 °C, and 30 µl aliquots are extracted at various time points as indicated in the legend to Figure 3, and the reactions are quenched by addition of STI (1 µl) at a STI: trypsin ratio of 2:1. The samples are analyzed by 10% SDS-PAGE, followed by silver staining or immunoblotting. Again, this method offers versatility to examine the influence of cofactors and substrates on the helicase digestion pattern, which is a reflection of protein conformation.

3. Concluding Remarks

Understanding the role of its oligomeric states, and elucidation of the nature of helicase: cofactor interactions, is a prerequisite for deciphering the role of the enzyme in DNA metabolism. In this report, we have presented several conventional biochemical methods that allow us to gain insight into functional forms of the human mtDNA helicase. At the same time, we expect that these protein characterization tools will offer a general approach to examine the multimeric state and modular architecture of other oligomeric proteins involved in mtDNA interactions. Each of the methods presented can be adapted to allow evaluation of the influence of myriad physiologically-relevant factors on the protein of interest.

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Figure 1. Size exclusion chromatography and velocity sedimentation of the human mtDNA helicase

Left, C-terminally His-tagged human mtDNA helicase was chromatographed on a Superdex 200 HR 10/30 column as described in the text. Proteins standards used to calibrate the column were: chymotrypsinogen A ($R_s = 28 \text{ \AA}$), hen egg ovalbumin ($R_s = 30 \text{ \AA}$), yeast alcohol dehydrogenase ($R_s = 46 \text{ \AA}$), bovine liver catalase ($R_s = 52 \text{ \AA}$) and bovine thyroid thyroglobulin ($R_s = 85 \text{ \AA}$). *Right*, N- and C-terminally His-tagged human mtDNA helicase were sedimented in 12–30 % glycerol gradients as described in the text. Protein standards run in parallel gradients were: jack bean urease (URE, 18.6 S), bovine liver catalase (CAT, 11.3 S), and rabbit muscle lactate dehydrogenase (LDH, 7.3 S).

Figure 2. Glutaraldehyde cross-linking of the human mtDNA helicase

N-terminally His-tagged human mtDNA helicase was pre-incubated under the indicated conditions for 30 min at 37° C prior to cross-linking with 0.02% glutaraldehyde for 20 min at 37° C. Samples were analyzed by 4–7% linear gradient SDS-PAGE, followed by immunoblotting. *Lane 1*, molecular weight markers; *lane 2*, absence of cofactors and glutaraldehyde; *lane 3*, presence of Mg²⁺-ATP and glutaraldehyde.

Figure 3. Time course of trypsin digestion of the human mtDNA helicase

Human mtDNA helicase was proteolyzed over a time course of 1.5 h. Samples were extracted at the indicated time points, and analyzed by 10% SDS-PAGE, followed by silver staining. *Lane 1*, undigested helicase; *lanes 2–6*, 1, 10, 30, 60, and 90 min, respectively.