DNA and RNA Binding by the Mitochondrial Lon Protease Is Regulated by Nucleotide and Protein Substrate*

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The ATP-dependent Lon protease belongs to a unique group of proteases that bind DNA. Eukaryotic Lon is a homo-oligomeric ring-shaped complex localized to the mitochondrial matrix. In vitro, human Lon binds specifically to a single-stranded GT-rich DNA sequence overlapping the light strand promoter of human mitochondrial DNA (mtDNA). We demonstrate that Lon binds GT-rich DNA sequences found throughout the heavy strand of mtDNA and that it also interacts specifically with GU-rich RNA. ATP inhibits the binding of Lon to DNA or RNA, whereas the presence of protein substrate increases the DNA binding affinity of Lon 3.5-fold. We show that nucleotide inhibition and protein substrate stimulation coordinately regulate DNA binding. In contrast to the wild type enzyme, a Lon mutant lacking both ATPase and protease activity binds nucleic acid; however, protein substrate fails to stimulate binding. These results suggest that conformational changes in the Lon holoenzyme induced by nucleotide and protein substrate modulation bind the affinity for single-stranded mtDNA and RNA in vivo. Co-immunoprecipitation experiments show that Lon interacts with mtDNA polymerase γ and the Twinkle helicase, which are components of mitochondrial nucleoids. Taken together, these results suggest that Lon participates directly in the metabolism of mtDNA.

The ATP-dependent Lon (La) protease is a multi-functional enzyme conserved from archaea to mammalian mitochondria (1–6). Mitochondrial Lon is a homo-oligomeric complex in which each monomer carries separate sites for the binding and hydrolysis of both ATP and protein substrate (7, 8). Lon selectively degrades aberrant polypeptides, thus serving a quality control function in protein biogenesis (9–12). The energy requirement of Lon is mechanistically similar to that described for other ATP-dependent proteases such as ClpAP and the proteasone (13–20). The binding of substrate polypeptide to Lon stimulates its ATPase activity (21). ATP hydrolysis is required for the progressive unfolding of a substrate that permits peptide bond cleavage. Conformational changes within the Lon holoenzyme likely coincide with the cycle of ATP binding and hydrolysis, ADP dissociation, and ATP re-binding, as well as with the binding and hydrolysis of protein substrate.

Lon belongs to a unique group of proteases that also bind DNA. Other proteases or protease components that bind DNA include the human adenovirus proteinase (AVP),1 the adipo-cyte-enhancer binding protein 1 (AEBP1), Gal6p/bleomycin hydrolase, and the 19 S particle of the 26 S proteasome. AVP requires two co-factors for maximal protease activity: viral DNA and a viral peptide produced by AVP proteolysis. It is proposed that AVP utilizes its nonspecific DNA binding activity to locate its viral protein substrates (22). By contrast, the AEBP1 repressor binds specifically to the adipocyte enhancer 1 element. The carboxypeptidase activity of AEBP1 is stimulated by adipocyte enhancer 1 element DNA and is required for transcriptional repression; however, the protein substrates hydrolyzed by AEBP1 remain unknown (23, 24). In yeast, Gal6p has been shown to negatively regulate galactose metabolism and also to hydrolyze bleomycin, which is widely used in cancer chemotherapy. Studies show that DNA-binding mutants of Gal6p fail to protect yeast from bleomycin toxicity (25, 26). Recent work has shown that the 19 S component of the 26 S proteasome binds specifically to the GALI–10 promoter and functions in nucleotide excision repair and transcriptional elongation in yeast (27–29). Lon shares structural and functional similarity with the ATPase subunits of the 19 S particle within their respective AAA domains. Based on these findings, it is predicted that the DNA binding activity of Lon is important for its physiological function.

In vivo studies show that Lon has a role in DNA maintenance and expression. In Saccharomyces cerevisiae, strains lacking mitochondrial Lon suffer large deletions in mtDNA and do not process and express mitochondrial RNA transcripts that contain introns (30–32). In Caulobacter crescentus, Lon is responsible for the cell cycle-dependent turnover of the CcrM DNA methyltransferase; lon mutants fail to initiate DNA replication and to complete cell division (33). In Escherichia coli, data suggest that Lon degrades the protein(s) that determine the helical density of F-type DNA mini-plasmids that replicate independently of the bacterial chromosome (34). In addition,

1 The abbreviations used are: AVP, human adenovirus proteinase; AEBP1, adipocyte-enhancer binding protein 1; BSA, bovine serum albumin; EMSA, electrophoretic mobility shift assay; FITC, fluorescein isothiocyanate; mtDNA, mitochondrial DNA; MOPS, 4-morpholinepropanesulfonic acid; NTA, nitritrocyclacetic acid; AMP-PCP, adenosine 5′-β,γ-methylenetriphosphate; POLG, polymerase γ.
Studies performed in various bacterial species suggest that Lon regulates the protein levels of transcription factors that control development, antibiotic production, or pathogenesis (35–40). In vitro studies from the 1980s examined the relationship between the DNA binding and enzymatic activities of E. coli Lon. A. Markovitz and co-workers (41, 42) purified the lon (capR9) gene product as a DNA-binding protein; independently, Chung and Goldberg demonstrated that this protein was identical to the ATP-dependent Lon/La protease (43). Both groups found that E. coli Lon bound to DNA nonspecifically, and both showed that the ATPase activity of Lon was stimulated by single- and double-stranded DNA. More recent in vitro studies have demonstrated that bacterial, mouse, and human Lon bind specifically to GT-rich DNA sequences (44–46). Although the sequence specificity of DNA binding is evolutionarily conserved, experiments show that mammalian Lon binds single-stranded DNA, whereas bacterial Lon binds double-stranded DNA (44–46). The binding site for human Lon in vitro is contained within a GT-rich DNA oligonucleotide whose sequence overlaps the light strand promoter of human mtDNA.

Little is known about the sequence specificity and regulation of nucleic acid binding by Lon and whether Lon interacts with proteins involved in mtDNA metabolism. In this study, we demonstrate that Lon binds not only to GT-rich DNA sequences found throughout the guanine-rich heavy strand of human mtDNA but also to GU-rich RNA. ATP inhibits Lon binding to DNA and RNA, whereas protein substrate increases the affinity of the protease for nucleic acid. We propose that DNA and RNA binding by the human mitochondrial Lon protease is regulated by conformational changes within the holoenzyme that are induced differentially by nucleic acid and protein substrate. In cultured cells human Lon associates with components of mitochondrial nucleoids, which are large multi-protein complexes bound to mtDNA that support the stability, expression, and inheritance of the mitochondrial genome. We show that mtDNA polymerase γ and the Twinkle helicase co-immunoprecipitate with Lon. These results are consistent with the notion that Lon participates directly in the metabolism of mtDNA.

**EXPERIMENTAL PROCEDURES**

**Expression of Recombinant Human Lon**—A cDNA encoding human Lon carrying an amino-terminal hexahistidine tag followed by a linker region (MGHHHHHHDDYIPITENLYFQGAGH) was fused to methionine 115 was expressed in the E. coli Rosetta strain (Novagen). Bacteria were grown at 37°C in 3% bactotryptone, 2% yeast extract, 1% MOPS, pH 7.2, 100 μg/ml ampicillin, 34°C, 8 g of bacteria, 1 ml 0.75 M NaCl were positive for the strain. The cells were harvested by centrifugation, and the supernatant was loaded onto a P11 column (Amersham Life Science) and eluted using Solution A. Purified human Lon was analyzed by SDS-PAGE and Coomassie Brilliant Blue staining and by immunoblotting using an anti-Lon antibody.

**In Vitro Mutagenesis of Human Lon cDNA**—The DNA encoding the Lon S855A mutant in which serine at position 855 was replaced by alanine was produced using QuikChange XL site-directed mutagenesis (Stratagene). Complementary oligonucleotide primers (coding sequence 5′-accccaagcgacgcagcagcctgacatagc-3′) were used to introduce a single point mutation that was verified by DNA sequencing. Lon S855A is a fusion protein that carries the same amino-terminal hexahistidine tag and linker as described above.

**Glutaraldehyde Cross-linking**—Cross-linking experiments were carried out as previously described (7, 8). SDS-denatured or native Lon (300 pmol of monomer) was cross-linked using 0.1% glutaraldehyde for 10 min at room temperature and then visualized on a 3% SDS-PAGE stained with Coomassie Brilliant Blue.

Δ DNA Cleavage, Protease, and ATPase Assays—Lon (5 pmol of monomer) was incubated with ADNA (0.4 μg) in a 40-μl reaction containing 2 mM NaCl, 2 mM Tris, 1 mM MgCl2, 0.5 mM CaCl2, for the time periods as indicated at 37°C; the reactions were run on a 6% agarose gel in TBE buffer. ATP-dependent degradation of casein was measured as described previously (46, 47). Briefly, wild type Lon (7.4 pmol of monomer) or LonS855A (8.4 pmol of monomer) was incubated with 160 pmol of FITC-casein in a 40-μl reaction containing 50 mM Tris–Cl, pH 8.1, 150 mM NaCl, 2 mM dithiothreitol, 10 mM Mg(OAc)2 in the presence or absence of 2 mM ATP at 37°C. At various time points, the aliquots were withdrawn, and quenched with 0.1% glutaraldehyde and centrifuged at 4°C. The level of fluorescence in the supernatant representing acid-soluble peptides derived from FITC-casein was measured spectrophotometrically. A calibration curve using known concentrations of FITC-casein was used to calculate moles of FITC-peptides generated. The ATPase activity of Lon was determined by measuring the hydrolysis of [γ-32P]ATP to [γ-32P]ADP using thin layer chromatography (48) and phosphorimaging quantification. Purified Lon (12 pmol of monomer) or LonS855A (13 pmol of monomer) was incubated in a 30-μl reaction containing 50 mM Tris–Cl, pH 8.1, 2 mM MgOAc2, 150 mM NaCl, 2 mM ATP, and 50 mM [γ-32P]ATP at 37°C. The aliquots were withdrawn at various time points and quenched with 0.5 M formic acid. The ATP-stimulated ATPase activity was assayed as above, except 440 pmol of casein was included in the reaction mixture.

**Oligonucleotides Used in Nucleic Acid Binding Assays**—The following oligonucleotides were assayed: single-stranded oligonucleotide LSPas (5′-aat aat tgt gta gtt ggg ggg tga) as previously described (45, 46) and single-stranded mtDNA sequences 1as (5′-ggc gta ggt tgg gta ggt), 2as (5′-gta gga ggg ggg tca tag ggt), 3as (5′-gta gag ggg gtg cta tag ggt), 4as (5′-gga ggg ggg tgg taa gga tgg), 5as (5′-gga ggg ggg tgg taa gga tgg), and 6as (5′-ggg gag ggg tgt tta agg ggt). The positions of oligonucleotides 1s–6s within human mtDNA (GenBank™ sequence NC_001807) are: 7114–7134, 8262–8282, 8385–8415, 10936–10956, 12387–12407, and 15258–15256, respectively. The LPSA RNA oligonucleotide was 5′-aau aac gaa gca ggg ggg uga. The respective complementary sequences were also tested. Synthetic DNA or RNA oligonucleotides were end-labeled, unincorporated radioactivity was removed, and the probes were adjusted to the same specific activity as previously described (46). DNA and RNA oligonucleotides were suspended as a stock solution in 10 mM Tris, pH 8.0. The oligonucleotides corresponding to LSPas DNA and RNA as well as to oligonucleotides G, C1, G2, and G3 form high order complexes resulting from four or more contiguous guanine residues. The uncomplexed forms of these oligonucleotides were gel-purified before end-labeling.

**Electrophoretic Mobility Shift Assay**—EMSA was performed as previously described (46) using radiolabeled probe (3.2 pmol) and purified Lon (50 pmol of monomer) incubated in a 50-μl reaction mixture. The presence or absence of unlabeled LSPas or LSPs DNA or RNA (200-fold excess), polydeoxyinosinic-deoxyctydilic acid (poly(dI-dC), 50 ng/reaction) decreased the specific DNA binding to LSPas of the LPSA RNA oligonucleotide. The respective complementary sequences were also tested. Synthetic DNA or RNA oligonucleotides were end-labeled, unincorporated radioactivity was removed, and the probes were adjusted to the same specific activity as previously described (46). DNA and RNA oligonucleotides were suspended as a stock solution in 10 mM Tris, pH 8.0. The oligonucleotides corresponding to LSPas DNA and RNA as well as to oligonucleotides G, C1, G2, and G3 form high order complexes resulting from four or more contiguous guanine residues. The uncomplexed forms of these oligonucleotides were gel-purified before end-labeling.

**Electrolytic Mobility Shift Assay**—EMSA was performed as previously described (46) using radiolabeled probe (3.2 pmol) and purified Lon (50 pmol of monomer) incubated in a 10-μl reaction mixture. The presence or absence of unlabeled LSPas or LSPs DNA or RNA (200-fold excess), polydeoxyinosinic-deoxyctydilic acid (poly(dI-dC), 50 ng/reaction), or oligonucleotide A (200-fold excess).

**Nitrocellulose Filter Binding Assay**—DNA binding reactions (30 μl) containing Binding Buffer (20 mM Hepes, pH 7.4, 2 mM MgCl2, 0.02 mM single-stranded DNA, human Lon (5 pmol) of Lon S855A (5 pmol) of isolated labeled oligonucleotide (3.2 pmol) were incubated at 37°C for 30 min. Binding Buffer (200 μl) was added to each reaction, which was then immediately nitrocellulose filter pre-equilibrated with binding buffer. The nitrocellulose filters were washed with 10 ml of Binding Buffer and analyzed by liquid scintillation counting.

**DNA binding affinity of LPSA DNA was determined using the filter assay. Total binding was determined by incubating Lon (0.8 pmol) with LPSA probe (8–16,000 fmol) in the presence and absence of casein (4 pmol) for 30 min at 37°C. Nonspecific binding was determined by adding unlabeled LPSa (800 pmol) to the reactions. Specific binding
was calculated as total binding minus nonspecific binding. Prism 3.0 software was used to calculate the Kd by nonlinear regression as well as the standard deviation (based on data obtained from three independent experiments).

**RESULTS**

**Purification of Recombinant Human Lon and Analysis of Its Enzymatic Activities**—To analyze the DNA binding and enzymatic activities of Lon in vitro, the protein was expressed and purified from E. coli. Expression of a Lon fusion protein carrying an amino-terminal hexahistidine tag was induced with isopropyl-β-D-thiogalactopyranoside (IPTG), and purified by size exclusion chromatography (Fig. 1; lane 3) and Ni2+ -NTA affinity chromatography (Fig. 1; lane 4). Complete glutaraldehyde cross-linking (30 min) of SDS-denatured and dialyzed Lon demonstrates monomers of ~100 kDa (lane 1). Complete (lane 2) and partial (1 min, lane 3) cross-linking of native Lon revealed that the complex is composed of six or seven monomers. The protein was visualized on a 3% SDS-PAGE stained with Coomassie Brilliant Blue. D, cleavage of λ DNA by Lon-containing fractions. Shown are λ DNA alone (lanes a and e); λ DNA + Lon isolated after size exclusion chromatography (lanes b and f); λ DNA + Lon eluted with either 0.2 M imidazole (lanes c and g) or 0.3 M imidazole from Ni2+ -NTA-agarose (lanes d and h); and 1-kb DNA markers (lane m; New England Biolabs).

Because we aimed to study the DNA and RNA binding activity of Lon, experiments were performed to confirm that the purified protein was nuclease-free. Fig. 1D shows that after the final purification step Lon was free of endonucleases; λ DNA remained stable even after a 24-h incubation at 37 °C with Lon (lanes g and h). By contrast, λ DNA was completely degraded after incubation under the same conditions with Lon isolated after the size exclusion step (Fig. 1D, lane f). The absence of other nucleases was shown by the stability of single- and double-stranded DNA and RNA oligonucleotides incubated with purified Lon (data not shown).

To demonstrate the protease activity of purified Lon, casein covalently labeled with fluorescein isothiocyanate (FITC-casein) was used as a substrate (46, 47, 49). The energy-dependent degradation of casein has been routinely employed to characterize bacterial, yeast, and mammalian Lon (1, 8, 46, 50). The cleavage of FITC-casein by Lon was measured spectrophotometrically as an increase in acid-soluble fluorescent casein peptides. Fig. 2A demonstrates that wild type Lon degraded FITC-casein in the presence of ATP but not in its absence; the rate of hydrolysis was 0.48 mol of casein/mol Lon/s. By contrast, LonS855A failed to degrade FITC-casein either in the presence or in the absence of ATP.

Substrate-stimulated ATPase activity of wild type Lon and the LonS855A mutant was determined using [α-32P]ATP and thin layer chromatography (48). Wild type Lon exhibited a basal ATPase activity hydrolyzing 0.81 mol of ATP/mole Lon/s. The addition of casein stimulated hydrolysis 2-fold to 1.89 mol of ATP/mole Lon/s. By contrast, LonS855A lacked detectable ATPase activity either in the presence or in the absence of casein. Taken together these results demonstrate that purified wild type Lon is an active ATP-dependent protease and that LonS855A is an enzymatic mutant that lacks both ATPase and protease activity.

**Sequence-specific DNA and RNA Binding by Human Lon in Vitro**—Previous work has demonstrated that mouse and human Lon specifically bind to a single-stranded GT-rich DNA oligonucleotide referred to as LSPas (45, 46). The sequence of LSPas overlaps with the light strand promoter anti-sense strand of human mtDNA. By contrast, the complementary LSPs sequence is not bound by Lon. Electrophoretic mobility shift assays showed that human Lon specifically bound to DNA as well as RNA oligonucleotides carrying the LSPas sequence (Fig. 3, A and B). LSPas DNA binding by Lon led to a shift in...
probe mobility that was competed by a 200-fold excess of unla-
beled LSPas DNA. No competition was observed upon the
addition of poly(dl-dc) (50 ng) or deoxyribo-oligonucleotide A
(200-fold excess) (Fig. 3A). Lon also bound specifically to LSPas
RNA and not to its complementary LSPs RNA sequence (Fig.3B).
The binding to LSPas RNA was competed by the addition of
LSPas DNA (see Fig. 5F). Lon binding to other GU-rich
RNAs was also observed; this binding was competed by both
LSPas RNA and DNA but not by spermidine (data not shown).
Lon failed to bind to the respective complementary sequences
of the GU-rich RNAs tested. These results demonstrate that
the specificity of nucleic acid binding by Lon is determined by
the sequence of purine and pyrimidine bases and not by the
sugar phosphate backbone.

Effect of Monovalent and Divalent Cations on the Interaction
of Lon with DNA—To determine the metal dependence of Lon
binding to DNA, EMSA was performed in the presence and
absence of Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$, Na$^{+}$, and K$^{+}$. The mitochondrial
matrix is a known store of Ca$^{2+}$, and several matrix enzymes
use Mg$^{2+}$ or Mn$^{2+}$ as a co-factor. Within the matrix peak
concentrations of Ca$^{2+}$ are reportedly $\approx 1$ mM (51, 52), and
concentrations of Mg$^{2+}$ and Mn$^{2+}$ are at $\approx 0.2$–1.5 mM
and $\approx 1$ mM, respectively (53–55). Fig. 3C shows that Lon bound to
LSPas DNA in the absence as well as in the presence of Mg$^{2+}$,
Mn$^{2+}$, and Ca$^{2+}$ at physiological concentrations. Inhibition
of binding was observed only at concentrations beyond those
documented to occur within the matrix. The concentrations of Na$^{+}$
and K$^{+}$ in “resting” mitochondria are both reported to be $\approx 15$
 mM (56). We found that increasing Na$^{+}$ concentrations had
little effect on the binding of LSPas DNA, and K$^{+}$ concentra-
tions affected binding only at $\approx 100$ mM.

Lon Binds to GT-rich DNA Sequences Found throughout the
Mitochondrial Genome—The human mitochondrial genome is a
16,571-base pair closed circular double-stranded DNA. The
heavy strand of mtDNA has a greater mass than the light strand
because of its high content of guanine residues. Throughout
the heavy strand there are clusters of 4–12 guanine residues every
$\approx 100$ bases, and most of these are flanked by at least one
thymidine nucleotide. We therefore tested the binding of Lon to DNA
oligonucleotides corresponding to G-rich sites within the heavy
strand as well as their respective complementary sequences.
Nitrocellulose filter binding assays demonstrated that Lon bound
preferentially to G-rich heavy strand oligonucleotides, referred to
as antisense 1–6 (1as–6as) rather than to the complementary
sense sequences (1s–6s) (Fig. 4A). Further analysis demonstrated
that Lon also bound to a DNA oligonucleotide consisting of
only guanine residues (Fig. 4B). Introduction of flanking thy-
midine residues on either side of a hexaguanine cluster increased
Lon binding to DNA. Taken together these results demonstrate
that Lon binds to G-rich DNA sequences in vitro and suggest that
Lon interacts with G-rich sites present throughout the mitochon-
drial genome.

ATP Binding Inhibits the Interaction of Lon with DNA and RNA—ATP binding and hydrolysis are essential to the cata-
ytic cycle by which Lon degrades protein substrates. The effect
of various nucleotides on the binding of LSPas DNA by Lon was
tested using the nitrocellulose filter assay. Increasing concentra-
tions of ATP inhibited DNA binding (Fig. 5A). In the presence
of 1, 2, and 5 mM ATP, Lon binding to the LSPas probe was
$\approx 80$, 50, and 30% of untreated samples, respectively. A similar
inhibitory effect was observed using increasing concentrations
of ADP, albeit to a lesser extent than ATP. The addition of
nonhydrolyzable analogs of ATP also blocked Lon binding to
DNA, demonstrating that ATP hydrolysis was not required for
inhibition (Fig. 5A). The inhibitory effects of GTP and GDP
were similar to those observed for ATP and ADP, respectively.
Little or no influence on DNA binding by Lon was observed
upon the addition of increasing amounts of AMP or GMP (Fig.5A)
or dATP, dGTP, dTTP, or dCTP (data not shown). Lon
binding to LSPas RNA was also antagonized by ATP and AMP-
PCP and was also competed by the addition of 200-fold excess
LSPas DNA (Fig. 5F).

Substrate-stimulated DNA Binding by Lon—To test whether
the binding of Lon to LSPas DNA was influenced by protein
substrate, EMSA was performed in the presence or absence of
either casein, which is degraded by Lon or native BSA, which is
not degraded (57). The addition of casein substantially in-
creased the binding of Lon to the LSPas probe, whereas BSA
had little effect (Fig. 5B). Affinity binding studies demonstrated
that the addition of casein increased the affinity of Lon
for LSPas DNA 1.6-fold. Fig. 5C shows that in the absence of
casein the approximate $K_d$ of Lon binding to LSPas DNA was
$711.2 \pm 150.6$ nM, whereas in the presence of casein the approxi-
mate $K_d$ was $201.1 \pm 46.5$ nM. The slight decrease in specific binding to LSPas at high concentrations may be caused
by factors within the experimental system that we do not
understand and thus cannot control.

Nucleotide and Protein Substrate Coordinately Regulate
DNA Binding by Lon—Once nucleotide inhibits the DNA bind-
ing activity of Lon, does protein substrate stimulate DNA bind-
ing? Conversely, once protein substrate stimulates DNA bind-
ing, does added nucleotide lead to DNA release? To ad-
dress the first question the following order-of-addition experi-
ment was performed. In Step 1, AMP-PCP was added to the
Lon-LSPas reaction for 15 min; in Step 2, casein was added,
and the reaction was incubated for an additional 15 min. AMP-
Fig. 3. Specific binding of Lon to DNA and RNA oligonucleotides. Binding of Lon to 32P-labeled LSPas DNA and RNA oligonucleotides was analyzed by EMSA. A, LSPas DNA probe was incubated either alone (lane 1); + Lon (lane 2); + Lon + 200-fold excess unlabeled LSPas (lane 3); + Lon + 50 ng poly(dI-dC) (lane 4); or + Lon + 200-fold excess DNA oligonucleotide A (lane 5). B, LSPas or LSPs RNA probe alone (lanes 1 and 4); + Lon (lanes 2 and 5); + Lon + 200-fold excess unlabeled LSPas or LSPs RNA (lanes 3 and 6). C, the effect of various cations on LSPas DNA binding was analyzed by EMSA. Lon was incubated with probe in the presence of the indicated cation concentrations.
PCP was used in these experiments because it does not support the degradation of casein (data not shown). Fig. 5D shows that AMP-PCP blocked Lon binding to LSPas DNA in a dose-dependent manner and that the subsequent addition of casein stimulated DNA binding. The level of casein stimulation depended on the concentration of AMP-PCP added in the first step; at the higher concentration of AMP-PCP, a lower stimulation of DNA binding was observed. To address whether the increase in substrate-stimulated DNA binding by Lon is nucleotide-sensitive, the following order-of-addition experiment was performed. In Step 1, casein was added to the Lon-LSPas reaction for 15 min; in Step 2, AMP-PCP was added, and the reaction was incubated for an additional 15 min. Fig. 5D shows that casein stimulated Lon binding to LSPas DNA and that the subsequent addition of AMP-PCP led to the release of LSPas. Increasing concentrations of AMP-PCP resulted in increased LSPas dissociation. These results demonstrate a functional relationship between nucleotide inhibition and protein substrate stimulation of DNA binding by Lon.

Nucleotide and protein substrate did not influence DNA binding by the Lon S855A mutant. LonS855A lacks both protease and ATPase activity (Fig. 2); however, it retains DNA binding activity (Fig. 5E). Although LonS855A bound to LSPas DNA, casein failed to stimulate DNA binding (Fig. 5E), and ATP had no effect on this binding (data not shown). Either LonS855A cannot interact with ATP and casein, or its affinity for nucleotide and protein substrate is substantially reduced. These results suggest that unlike wild type Lon, LonS855A does not undergo the conformational changes required to promote or block DNA binding in vitro.

Although ATP and protein substrate modulate DNA binding by Lon, we have not found that DNA oligonucleotides influence the ATPase or protease activities of the enzyme in vitro (data not shown). ATPase and protease assays were performed as in Fig. 2 in the presence and absence of LSPs or LSPs DNA oligonucleotides at μM final concentrations; no changes in enzyme kinetics were observed. A previous study showed that DNA stimulated ATP-dependent proteolysis by E. coli Lon (43), whereas another study showed that DNA inhibited this activity (41). It is difficult to compare our results with those of earlier experiments that used larger double- and single-stranded DNA sequences at different final concentrations.

Lon Associates with Proteins Localized to Mitochondrial Nucleoids—The binding of Lon to mtDNA sequences in vitro prompted us to investigate whether Lon associates with protein components of mitochondrial nucleoids, which are large multi-protein complexes that have been inferred to be the units of mtDNA replication, transcription, and inheritance (58–61). Recent work has shown that mtDNA POLG as well as the putative mtDNA helicase Twinkle localize to human mitochondrial nucleoids (62). 293T cells were transfected with plasmid constructs for the transient expression of either POLG or Twinkle; both proteins carried a carboxyl-terminal Myc epitope tag. Forty-eight hours post-transfection, endogenous Lon was immunoprecipitated from protein extracts of detergent solubilized cells, and the interaction between Lon and POLG or Twinkle was assayed by Western blotting. Fig. 6 demonstrates that both POLG and Twinkle co-immunoprecipitated with Lon. Overexpression of POLG or Twinkle did not alter the level of immunoprecipitated Lon (Fig. 6) nor the level of Lon in total cellular protein extracts (data not shown). These results support the involvement of Lon in regulating mtDNA metabolism in vivo.

DISCUSSION

Model for the Regulation of Nucleic Acid Binding by Lon in Vitro—The results presented here show that purified Lon binds specifically to GT-rich DNA and GU-rich RNA and that nucleotide and protein substrate modulate oligonucleotide binding by Lon. ATP binding inhibits the interaction of Lon with DNA and RNA, whereas protein substrate stimulates binding. The artificial substrate casein was used in these studies because it has been employed routinely to characterize the proteolytic activity of Lon from many different species. The stimulation of DNA binding occurs through a labile interaction between the

Fig. 4. Specific binding of Lon to GT-rich sequences found on the heavy strand of human mtDNA and to oligonucleotides consisting of only guanine residues. A, Lon binding to DNA oligonucleotides corresponding to various G-rich regions of human mtDNA (see “Experimental Procedures”) assayed by filter assay. B, Lon binding to DNA oligonucleotides consisting only of guanine or guanine and thymidine residues.
protease and substrate, because we do not observe a stable supershifted complex representing Lon, DNA, and casein (Fig. 5B). We are not able to assay the effect of casein on RNA binding, because casein contains contaminants that lead to RNA degradation. Once DNA binding has been stimulated by substrate, the addition of ATP or its nonhydrolyzable analogs leads to nucleic acid release. Conversely, once DNA binding is inhibited by nucleotide, this inhibition is overcome by the addition of protein substrate resulting in enhanced DNA binding (Fig. 5D).

We propose the following model to explain the enzymatic and DNA binding activities of Lon in vitro (Fig. 7). The Lon complexes purified in this study most likely consist of monomers in an ADP-bound form or in an ADP-depleted state (Fig. 7A). No ATP is added during the purification of Lon; thus, all or most of the bound ATP is hydrolyzed to ADP by the end of the procedure because the enzyme has a weak ATPase activity even in the absence of substrate (Fig. 2B). Nucleotide-free subunits adopt a conformation that either exposes an increased number of DNA and RNA binding sites or converts low affinity sites to high affinity sites for nucleic acid binding (Fig. 7A). The interaction of protein substrate with the Lon complex leads to ADP release, resulting in an ADP-depleted form of the enzyme (Fig. 7B). Previous studies with E. coli Lon have shown that protein substrate stimulates the release of ADP (21). Substrate-stimulated release of ADP promotes a conformation of the holoenzyme that increases further the number or affinity of DNA- and RNA-binding sites. The stoichiometry of Lon binding to nucleic acid in the presence or absence of protein substrate, ATP, or ADP is not known. In the presence of excess ATP, the majority of nucleotide-binding sites within the Lon complex are occupied by ATP, resulting in a conformation with a low affinity for DNA or RNA (Fig. 7C); thus any bound nucleic acid is released. The presence of both ATP and protein substrate leads to a cycle of peptide bond hydrolysis and ADP release. How Lon-mediated proteolysis is coordinated with DNA or RNA binding either in vivo or in vitro remains to be determined.

The stimulation of the DNA binding activity of Lon by protein substrate suggests that nucleic acid binding is regulated by specific proteins. We demonstrate that casein stimulates DNA binding by Lon, whereas BSA does not, consistent with previous work showing that Lon selectively degrades casein but not native BSA (57). Protein substrate binding and hydrolysis may regulate a cycle of mtDNA binding and release (Fig. 7). Transient association of Lon with GT-rich DNA is supported...
by the low affinity of DNA binding (Fig. 5C). However, it is also possible that some proteins interact with Lon and activate binding, even though they are not targets for degradation. Such proteins would induce a conformational change in the Lon complex promoting nucleic acid binding but not protein degradation. In so doing, another level of regulation could be imposed upon the DNA and RNA binding function of Lon.

Studies have shown that mtDNA is associated with the inner membrane and is bound by large multi-protein complexes referred to as mitochondrial nucleoids. Although the majority of mitochondrial Lon is soluble in the matrix, a fraction of Lon is associated with the inner membrane (63). Localization of Lon at the inner membrane may be explained by its interaction with mtDNA. Because Lon also interacts with mtDNA polymerase γ and the putative mtDNA helicase Twinkle (Fig. 6), Lon itself may be a component of nucleoids where it functions to degrade, process, or influence the activity of POLG, Twinkle, or other nucleoid proteins and thus regulate mtDNA metabolism.

Functional Significance of Lon Binding to GT-rich Sequences in mtDNA—The heavy strand of human mtDNA exhibits a high frequency of 4–12 contiguous guanine residues every 100 bases, many of which are flanked by at least one thymidine residue. One can envisage that those G-rich clusters that function as Lon-binding sites are bound transiently by the protease during mtDNA replication or transcription when the DNA and RNA binding function of Lon.

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DNA and RNA Binding by the Mitochondrial Lon Protease Is Regulated by Nucleotide and Protein Substrate

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