Biochimie 94 (2012) 1647–1659

Contents lists available at SciVerse ScienceDirect

Biochimie

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

Research paper

The ribosome modulates the structural dynamics of the conserved GTPase HflX and triggers tight nucleotide binding

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ARTICLE INFO

Article history:
Received 3 March 2012
Accepted 12 April 2012
Available online 25 April 2012

Keywords:
HflX
Nucleotide binding
Ribosome
Limited trypsinolysis
GTPase-activated state
Stopped-flow

ABSTRACT

The universally conserved GTPase HflX is a putative translation factor whose GTPase activity is stimulated by the 70S ribosome as well as the 50S but not the 30S ribosomal subunit. However, the details and mechanisms governing this interaction are only poorly understood. In an effort to further elucidate the functional mechanism of HflX, we examined its interaction with the 70S ribosome, the two ribosomal subunits (50S and 30S), as well as its ability to interact with guanine nucleotides in the respective ribosomal complexes using a highly purified *in vitro* system. Binding studies reported here demonstrate that HflX not only interacts with 50S and 70S particles, but also with the 30S subunit, independent of the nucleotide-bound state. A detailed pre-steady-state kinetic analysis of HflX interacting with a non-hydrolyzable analog of mant-GTP, coupled with an enzymatic probing assay utilizing limited trypsinolysis, reveal that HflX·GTP exists in a structurally distinct 50S- and 70S-bound form that stabilizes GTP binding up to 70 000-fold and that may represent the "GTPase-activated" state. This activation is likely required for efficient GTP-hydrolysis, and may be similar to that observed in elongation factor G. Results reported here address the surprising low affinity of free HflX for GTP and suggest that cellular HflX will mainly exist in the HflX·GTP·ribosome-bound form. A minimal model for the functional cycle of HflX is proposed.

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1. Introduction

Members of the P-loop family of GTPases are ubiquitous throughout all domains of life. These essential proteins function as nucleotide-dependent molecular switches, and modulate processes such as signal transduction, DNA replication, and protein synthesis [1,2]. The functional cycle of GTPases is regulated by the phosphorylation state of the bound nucleotide; the *apo* and GDP-bound states represent inactive forms, while upon binding to GTP, the protein adopts a functionally active state. GTP-hydrolysis triggers a conformational change in the enzyme, reverting the protein back to its inactive, GDP-bound form. This GTPase cycle can be modulated by a variety of factors within the cell: guanine nucleotide exchange factors (GEFs) catalyze the release of bound GDP, thus facilitating binding of GTP; GTPase activating proteins or factors (GAPs) stimulate the intrinsic GTPase activity of the enzyme, and guanine nucleotide dissociation inhibitors (GDIs) regulate the release of GDP, providing an additional temporal regulation of the enzyme.

Only eight members of the vast family of P-loop GTPases are conserved in all domains of life [1]. These universally conserved GTPases can be subdivided into two groups: (i) the well characterized GTPases translation elongation factor (EF) Tu and G, initiation factor (IF) 2, and the protein secretion factors Ffh and FtsY [1]; (ii) GTPases of largely unknown function, consisting of the proteins YihA, YchF, and HflX [1]. Mounting evidence suggests that the latter GTPases are also involved in protein synthesis, because YchF associates with ribosomal subunits and polysomes in *Trypanosoma cruzi* [3], YihA (YsxC) interacts with the 50S ribosomal subunit in *Bacillus subtilis* [4], and HflX associates with 50S ribosomal subunits in both *Chlamydophila pneumoniae* and *Escherichia coli* [5,6]. Interestingly

Abbreviations: P-loop, phosphate-binding loop; GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein; GDI, guanine nucleotide dissociation inhibitor; EF, elongation factor; IF, initiation factor; mant, 2′(3′)-*O*-(*N*-methylanthraniloyl); X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; IPTG, isopropyl-β-D-galactopyranoside; PMSF, phenylmethylsulfonylfluoride; MWCO, molecular weight cutoff; PK, pyruvate kinase; PEP, phosphoenolpyruvate; K_D, equilibrium dissociation constant; FRET, fluorescence resonance energy transfer; IC_{50}, 50% inhibitory concentration; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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doi:10.1016/j.biochi.2012.04.016

the intrinsic GTPase activity of HflX is significantly stimulated by the 70S ribosome as well as the 50S ribosomal subunit, but partially inhibited by the antibiotic chloramphenicol [7].

Previous work based on co-fractionation experiments using HflX from *E. coli* and *C. pneumoniae* revealed that HflX interacts with the 50S subunit in either the GDP- or GDPNP-bound state [5,6]. Furthermore, HflX was found to co-purify with an RNA species similar in size to 16S rRNA [5]. Recent work on HflX from *Sulfolobus solfataricus* indicates that association with the 50S subunit does not require the presence of a nucleotide, though it may be stabilized by guanine nucleotides [8]. In our current work we clarify, using microfiltration and ultracentrifugation experiments based on our detailed knowledge of the nucleotide binding properties of free HflX [7], that *E. coli* HflX does interact with the 30S and 50S ribosomal subunits as well as the 70S ribosome, and that this interaction does not require the presence of GDP or GTP. Based on this information we wanted to answer the question of how these interactions affect the structural dynamics of HflX as well as its nucleotide binding properties. To this end we used limited trypsinolysis to probe the structural dynamics of HflX in various nucleotide- and ribosome-bound states, and performed a detailed fluorescence resonance energy transfer (FRET)-based pre-steady-state kinetic analysis of HflX interacting with mant-guanine nucleotides in the presence of 70S ribosomes or the respective ribosomal subunit (30S and 50S).

Our findings provide a critical first step toward mapping the functional cycle of HflX on the ribosome and suggest that *in vivo* the ribosome efficiently modulates the structure and the GTP binding properties of HflX to overcome the surprisingly low GTP binding affinity previously observed for the free protein [7].

2. Materials and methods

2.1. Materials

Chemicals were obtained from VWR, Sigma, or Invitrogen, unless otherwise specified. Nucleotides and mant-nucleotide derivatives were purchased from Invitrogen or Medicorp (mant-nucleotides $\geq$94% purity based on manufacturers specifications). Recombinant 6X His-tagged HflX was purified as previously reported [7]. Vacant ribosomes and ribosomal subunits were purified from *E. coli* MRE600 cells essentially as described in [9], but using a Ti 45 rotor rather than a Ti 50.2 rotor. As HflX rapidly hydrolyzes GTP in the presence of 70S ribosomes or 50S ribosomal subunits, the non-hydrolyzable GTP analog GDPNP (or mant derivatives) was utilized in binding studies where 70S ribosomes and ribosomal subunits were present. Protein mass spectrometry was performed at the Institute for Biomolecular Design at the University of Alberta.

2.2. Microfiltration

HflX·ribosome complexes were formed by incubating HflX (5 μM) with 70S ribosomes or the 30S and 50S ribosomal subunits (1 μM respectively) in the presence of nucleotides (1 mM), and when indicated with chloramphenicol (1 mM), in 20 μL $TAKM_7$ buffer (50 mM Tris-Cl pH 7.5 at 4 °C, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$) at 37 °C for 15 min. Following incubation on ice for 5 min, 480 μL $TAKM_7$ was added. Samples were centrifuged in Vivaspin-500 (100 kDa MWCO, GE) columns at 10 000× *g* to 20 μL final volume, diluted to 500 μL, refiltered, mixed with SDS-loading buffer (50 mM Tris-Cl pH 6.8 at 20 °C, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and subsequently analyzed using a 12% SDS-PAGE run at 200 V for 1 h and stained with Coomassie blue. Gels were subsequently scanned and the ratio of HflX to the respective ribosomal proteins was determined using the gel analysis function of ImageJ [10] to determine the intensity of each band, followed by a comparison of the respective intensities of 6X His-tagged HflX (50.5 kDa) to that of ribosomal proteins S1, S2, L1, and L2 (61.2, 26.8, 26.7, and 30.0 kDa respectively), taking into account the size and amino acid composition of each protein.

2.3. Ultracentrifugation

HflX·ribosome complexes were formed by mixing HflX (1 μM), ribosomes or ribosomal subunits (0.1 μM), and nucleotides (1 mM) in 400 μL $TAKM_7$ buffer at 37 °C for 10 min. Complexes were loaded on a 1700 μL sucrose cushion (20 mM Tris-Cl pH 7.5 at 4 °C, 60 mM NH_4Cl, 5.25 mM $Mg(OAc)_2$, 0.25 mM EDTA, 10% (w/v) sucrose; nucleotide present in appropriate experiments) and centrifuged at 65 000× *g* for 24 h in a Beckman Coulter TLX ultracentrifuge using a TLA-100.3 rotor. The resulting pellet was dissolved in 30 μL $TAKM_7$ and analyzed by SDS-PAGE as above.

2.4. Pre-steady-state kinetics

Rate constants for HflX interacting with mant-GDPNP/mant-GDP were determined via FRET between the intrinsic tryptophan residues in HflX and the mant group as previously reported [7] using a KinTek SF-2004 stopped-flow apparatus. Tryptophan residues were excited at 280 nm and the fluorescence emission from the mant group was monitored after passing through LG-400-F (400 nm long-pass) cutoff filters (Newport Corp.). The resulting fluorescence traces were initially fit with a one- or two-exponential function (equations (1) and (2)), where k_{app} is the characteristic apparent rate constant, A is the signal amplitude, Fl is the fluorescence at time t, and Fl_∞ is the final fluorescence signal. Fluorescence data were normalized with respect to the initial fit, averaged (5–10 traces typically), and refit with the appropriate equation. Kinetic constants are expressed as the final fit, ±95% confidence interval. For association experiments, the obtained concentration dependence of k_{app} was fit with a linear function; the slope represents the bimolecular association rate constant. All experiments were performed in $TAKM_7$ at 20 °C.

$$Fl = Fl_\infty + A\exp(-k_{app}t) \quad (1)$$

$$Fl = Fl_\infty + A_1\exp(-k_{app1}t) + A_2\exp(-k_{app2}t) \quad (2)$$

The association of mant-nucleotides to HflX·ribosome complexes were determined by pre-forming complexes (2 μM HflX and 70S ribosomes or the respective subunits) and incubation at 20 °C for 15 min prior to use. Complexes were then rapidly mixed with 25 μL of various concentrations (5–25 μM final concentration) of mant-nucleotides at 20 °C in $TAKM_7$. The resulting fluorescence traces were then analyzed according to either equation (1) or (2), depending on the quality of the obtained fit.

To determine the rates of dissociation of mant-nucleotides from HflX·mant-nucleotide·ribosome complexes, complexes were formed by incubating 30 μM mant-nucleotide with 2 μM each HflX and 70S ribosomes (or the 30S and 50S ribosomal subunits). Experiments were then performed by rapidly mixing 25 μL of HflX·mant-nucleotide·ribosome complex with 25 μL of 300 μM unlabeled nucleotide at 20 °C in $TAKM_7$. Fluorescence traces were fit according to equations (1) or (2). When monitoring nucleotide dissociation from HflX·mant-GDPNP·50S and HflX·mant-GDPNP·70S complexes, these samples were rapidly mixed with similar complexes containing 300 μM unlabeled GDPNP.

As an additional evaluation method, fluorescence traces from each nucleotide association experiment were also analyzed by

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