Biochimica et Biophysica Acta 1820 (2012) 1519–1525

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Helicobacter pylori hydrogenase accessory protein HypA and urease accessory protein UreG compete with each other for UreE recognition

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ARTICLE INFO

Article history:
Received 12 March 2012
Received in revised form 1 June 2012
Accepted 4 June 2012
Available online 12 June 2012

Keywords:
Metalloenzyme
Metal homeostasis
Hydrogenase
Urease
Nickel
Zinc

ABSTRACT

Background: The gastric pathogen *Helicobacter pylori* relies on nickel-containing urease and hydrogenase enzymes in order to colonize the host. Incorporation of Ni^{2+} into urease is essential for the function of the enzyme and requires the action of several accessory proteins, including the hydrogenase accessory proteins HypA and HypB and the urease accessory proteins UreE, UreF, UreG and UreH.
Methods: Optical biosensing methods (biolayer interferometry and plasmon surface resonance) were used to screen for interactions between HypA, HypB, UreE and UreG.
Results: Using both methods, affinity constants were found to be 5 nM and 13 nM for HypA–UreE and 8 μM and 14 μM for UreG-UreE. Neither Zn^{2+} nor Ni^{2+} had an effect on the kinetics or stability of the HypA–UreE complex. By contrast, addition of Zn^{2+}, but not Ni^{2+}, altered the kinetics and greatly increased the stability of the UreE–UreG complex, likely due in part to Zn^{2+}-mediated oligomerization of UreE. Finally our results unambiguously show that HypA, UreE and UreG cannot form a heterotrimeric protein complex *in vitro*; instead, HypA and UreG compete with each other for UreE recognition.
General significance: Factors influencing the pathogen's nickel budget are important to understand pathogenesis and for future drug design.

1. Introduction

Helicobacter pylori is a spiral, Gram-negative microaerophilic bacterium that has been shown to be the etiological agent of chronic atrophic gastritis and peptic ulcers [1], which can eventually develop into gastric cancers [2]. Since about 50% of the world's population is thought to be infected by the pathogenic bacterium [3], it is considered a major health threat. Despite *H. pylori* being a neutralophilic bacterium, it is able to persist for decades in the acidic habitat of the human stomach mucosa. In order to achieve this, the pathogen increases the pH in its surrounding microenvironment by using urease, an enzyme that converts urea into ammonia and carbon dioxide [4]. In addition, the gastric pathogen relies on a H_2-uptake [NiFe] hydrogenase to survive [5]. Both the urease and the hydrogenase enzymes require nickel (Ni^{2+}) in order to be catalytically active, and the Ni-maturation process involves a battery of accessory proteins.

Urease accessory proteins include UreE, UreF, UreG and UreH, the latter being homologous to UreD of other bacteria [6]. Based on extensive studies done with *Klebsiella aerogenes* genes expressed in *Escherichia coli*, UreD, UreF, UreG and UreE are expected to sequentially bind to the urease catalytic enzyme [7]. While all four proteins have been well characterized in *K. aerogenes*, most studies have focused on UreE and UreG in *H. pylori*. *Hp*UreE is a dimeric Ni^{2+} and Zn^{2+}-binding protein [8–11] thought to be the final Ni^{2+}-donor (to urease). *Hp*UreG is a GTPase enzyme [12,13] and GTP hydrolysis is needed for the activation of urease, since site-directed mutagenesis of the conserved P-loop region of UreG abolished urease activity [13]. Like *Hp*UreE, *Hp*UreG can also bind Ni^{2+} but it has a much higher affinity for Zn^{2+} [12]. While apo-*Hp*UreG has been shown to exist as a monomer in solution, interestingly Zn^{2+} binding, but not Ni^{2+} binding, appears to promote UreG dimerization [12].

Besides the four UreEFGH accessory proteins described above, a unique feature of the *H. pylori* urease maturation pathway is that two hydrogenase accessory proteins, HypA and HypB, are also required for full urease activity under nickel-limited conditions [14,15]. Indeed, *H. pylori hypA* and *hypB* mutants are deficient in urease activity, a phenotype that can be partially rescued by addition of Ni^{2+} into the growth medium [14] or by expression of additional UreE engineered to have increased Ni-binding capacity [9]. Various studies in *E. coli* have suggested that HypA would serve as a scaffold for assembly of the nickel insertion proteins along with the hydrogenase precursor protein after delivery of the iron center [16,17]. A similar role is expected for *H. pylori* HypA; previous studies have shown that HypA is found as a dimeric protein in solution, capable of binding two Ni^{2+} ions per dimer [18]. Additional work indicated that HypA contains two metal sites, an intrinsic

Abbreviations: BLI, biolayer interferometry; SPR, Surface Plasmon Resonance

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doi:10.1016/j.bbagen.2012.06.002

Zn^{2+}site and a low-affinity Ni^{2+} binding site [19–21]. Similar to *Hp*UreG, HypB is a GTPase [18] with a highly conserved P-loop motif whose inactivation by site-directed mutagenesis leads to abolishment of urease activity [13]. However, in contrast to *Hp*UreG, only Ni^{2+} induces the dimerization of *Hp*HypB [22].

In vitro and *in vivo* interactions between *H. pylori* accessory proteins specifically needed for urease maturation have been reported using an array of different methods. For instance, interactions between *H. pylori* UreE and UreG were initially reported by Rain and coworkers, using yeast two-hybrid (Y2H) technology [23]. The interaction was later confirmed by Y2H and coimmunoprecipitation [24], tandem affinity purification (TAP) [25], as well as nuclear magnetic resonance (NMR) and isothermal titration calorimetry (ITC) [8]. Results from the latter study suggested that two monomers of UreG could interact with one dimer of UreE, with a dissociation constant K_D of 4 μM; the same study also revealed a role for Zn^{2+} (but not Ni^{2+}) in stabilizing the UreE–UreG complex [8]. Similarly, interactions between *H. pylori* hydrogenase accessory proteins HypA and HypB were first suggested by Y2H studies [23]; crosslinking studies using purified HypA and HypB subsequently demonstrated that both proteins could interact, regardless of nickel or GTP provided in the reaction mixture [18]. This *in vitro* interaction was recently confirmed by Xia and coworkers who also showed that the low affinity complex (K_D of 52 μM) was present *in vivo* [26].

Finally, since hydrogenase and urease pathways share common proteins (*e.g.* HypA and HypB) in *H. pylori*, it seems that these proteins may interact at some point with urease specific chaperones, such as UreEFGH. Evidence for such interactions was provided a few years ago, when heterologous HypA–UreE and HypB–UreG complexes were observed by cross-linking of purified proteins [27]; the latter interaction was confirmed by another group via a TAP approach [25]. However cross-linking or TAP approaches permanently capture protein interactions and therefore do not provide any kinetic constants. Therefore the aims of the present study were: (i) to determine whether two complementary optical sensing methods, surface plasmon resonance (SPR) and biolayer interferometry (BLI), can be used to provide association and dissociation constants for heterodimeric complexes formed between hydrogenase and urease accessory proteins and (ii) to determine whether these proteins cooperate to form higher order structures (*e.g.* heterotrimeric or heterotetrameric structures) or compete with one another. First, we were able to confirm that the hydrogenase maturation protein HypA is able to interact *in vitro* with the urease maturation protein UreE, a critical "bridge' between both maturation pathways. Dissociation constant calculations indicated that this complex is three orders of magnitude tighter (nM range) than the K_D reported for other complexes (*e.g.* HypA–HypB or UreE–UreG). In addition, the previously described UreE–UreG complex and its dependence upon Zn^{2+} but not Ni^{2+} were further characterized. Finally, our results clearly show that HypA–UreE and UreE–UreG complexes compete with one another, and do not form heterotrimeric complexes, further demonstrating the intricate relationship between hydrogenase and urease maturation pathways.

2. Material and methods

2.1. Expression and purification of HypA, HypB, UreE and UreG

Accessory proteins HypA, HypB, UreE or UreG were expressed in *E. coli* BL21 (DE3) RIL strain (Novagen, Gibbstown, NJ, USA), from a pET21b derivative plasmid, as previously described [9,13,18]. Purification strategies were slightly modified from the ones previously published. All proteins were purified using the ÄKTA FPLC purification system (GE Healthcare, Piscataway, NJ). Briefly, HypA, HypB and UreG proteins were purified from cell-free, membrane-free supernatants, using a combination of anion exchange (5-ml Q sepharose column) followed by size exclusion chromatography (Superdex 75 16/60). The buffer used for purification of HypA, HypB and UreG was 20 mM Tris–HCl, pH 7.5 (with NaCl concentrations ranging from 25 mM to 1 M). The recombinant UreE protein was purified from cell-free, membrane-free supernatants, using a combination of cation exchange (5-ml SP Sepharose) followed by size exclusion chromatography (Superdex 75 16/60). The buffer used was 50 mM Hepes, pH 7.2, 1 mM DTT (with NaCl concentrations ranging from 25 mM to 1 M). After the last step, fractions containing the purified proteins were pooled and concentrated using centricon YM-3 devices (Millipore, Billerica, MA) and dialyzed twice against 1 L of buffer containing 50 mM Hepes, pH 7.0, 25 mM NaCl.

2.2. Protein analysis

Proteins were qualitatively analyzed on denaturing 15% SDS-polyacrylamide gels (Fig. S1). Protein concentration was determined with the BCA protein kit (Thermo Scientific Pierce, Rockford, IL) with bovine serum albumin as standard.

2.3. Inductively-coupled plasma emission spectrometry

ICP-MS was used to determine the metal content of each protein and the dialyzing buffer. All experiments were conducted on an Agilent 7500 CE octopole spectrometer. Twelve different elements (Mg, V, Mn, Fe, Co, Ni, Cu, Zn, Mo, W, Pb and U) were measured in triplicate for each protein and the dialyzing buffer. Finally the buffer-subtracted, average value for each metal was used to determine the molar equivalent per monomer.

2.4. Optical biosensing

All BLI measurements were made on a FortéBio (Menlo Park, CA) Octet QK biosensor using streptavidin sensors. Assays were performed in 96-well microplates at 25 °C. All volumes were 200 μL. Ligand proteins were biotinylated using NHS-LC-LC-biotin (succini-midyl-6-[biotinamido]-6-hexanamidohexanoate) (Thermo Scientific) at a 5:1 molar ratio of biotin to protein for 30 min at 25 °C followed by rapid exchange into HBS-P (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.01% Surfactant P-20) by passage over a rapid desalting column. Conditions were chosen according to the manufacturer so that each protein was likely randomly biotinylated at an average of 1–2 positions. After loading biotinylated ligand onto SA sensors, a baseline was established in binding buffer, HBS-P with or without addition of 1 mM $NiCl_2$ or $ZnCl_2$, prior to association at varying analyte concentrations. After the association phase, sensors were moved to buffer only to monitor dissociation. When metals were present, a subsequent dissociation phase was performed in buffer with 5 mM EDTA. Nonspecific binding to sensors without ligand was negligible (see supplemental information). Raw sensor data were analyzed using GraphPad Prism.

All SPR measurements were made on a Biacore X100 biosensor (GE Healthcare, Piscataway, NJ) using a biotin CAPture chip. Biotinylated ligand was tethered to the chip via the capture reagent prior to injection of analyte. After dissociation, regeneration was achieved in 6 M guanidium chloride and 250 mM NaOH prior to tethering of ligand for subsequent analyte injections.

3. Results

3.1. Protein analysis

HypA, HypB, UreE and UreG were each independently expressed in *E. coli* and purified to near homogeneity, as visualized on Coomassie-stained SDS-polyacrylamide gels (Fig. S1). The metal content of each protein was determined by ICP-MS. The Ni^{2+}/monomer molar ratios were 0.02, 0.002, 0.004 and 0.002 for HypA, HypB, UreE and UreG, respectively, indicating that each protein was essentially Ni^{2+}-free. Even though no Zn^{2+} was added to the growth medium, HypA was almost saturated with Zn^{2+} with a molar ratio of 0.89 ± 0.09 Zn^{2+} per monomer,