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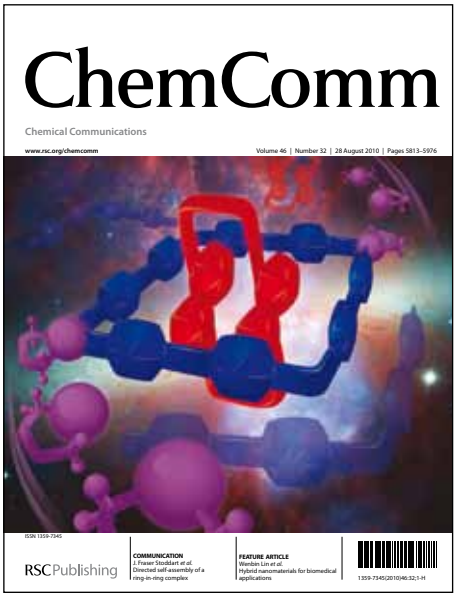

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Communication

Functional disruption of HypB, a GTPase of *Helicobacter pylori* by bismuth

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Bismuth (Bi^{3+}) binds equal molar HypB from *Helicobacter pylori* at the conserved metal site with a dissociation constant of $0.94(\pm0.25)\times10^{-17}$ μM, concomitantly induces the protein dimerization similarly to Ni^{2+}. Excess Bi^{3+} causes HypB further oligomerization, leading to HypB GTPase dysfunction. The results extend our understanding on the inhibitory mechanism of bismuth drugs against the pathogen.

The *Helicobacter pylori* (*H. pylori*) is one of the most common bacterial pathogens of humans. It infects human gastric mucosa and causes a variety of stomach diseases, such as peptic ulcer, gastritis and even gastric cancer.[1] The bacterium expresses a membrane-bound hydrogen (H_2) uptake [Ni-Fe] hydrogenase, which catalyzes reversible oxidation of molecular hydrogen (H_2) and is coupled with whole cell respiration.[2, 3] The enzyme contains one heterodimeric catalytic core and has two distinct subunits, with the small one housing Fe-S clusters involved in electron transfer and the large one comprising the Ni-Fe metallocenter as the catalytic site.[4, 5] The assembly of the metallocenter of [Ni-Fe] hydrogenase heavily relies on a battery of metallochaperones which are encoded by the *hyp* operon.[6] Two chaperones, HypA and HypB, are particularly important for the proper incorporation of Ni^{2+} to the enzyme.[7, 8] Gene knockout of either *hypA* or *hypB* of *H. pylori* led to complete loss of [Ni-Fe] hydrogenase activity, and much less efficiency in colonization of human stomach was also noted in these strains.[9, 10]

Enormous efforts have been made towards understanding the functions of HypA and HypB proteins in view of their importance. *Hp*HypA contains two distinct metal-binding sites. A Zn^{2+} site stabilizes whole protein structure whereas a N-terminal Ni^{2+} site exerts the metal delivery function.[11] HypB belongs to the SIMIBI class of GTP-binding proteins and possesses a highly conserved metal-binding site on its G-domain consisting of two cysteines and one histidine.[12-14] Disruption of the metal binding site of *E. coli* HypB abrogated the hydrogenase activity in *E. coli* cells.[15] Recent studies also demonstrated that *Hp*HypB GTPase activity could be regulated by metal binding to the site, indicating the pivotal role of the conserved metal site for proper [Ni-Fe] hydrogenase function *in vivo*.[16, 17]

Bismuth compounds such as colloidal bismuth subcitrate and ranitidine bismuth citrate have been widely used in combination with antibiotics to eradicate *H. pylori* infection,[18-20] and exhibit very low toxicity if administrated properly. Bismuth is able to bind human serum albumin and transferrin, and the binding to thiolate-containing proteins (metallothionein) and peptides may allow bismuth drugs to be detoxified.[21] The mechanisms of bismuth compounds against *H. pylori* are complex and probably involve formation of protective coating on the ulcer creator[22] and disruption of the functions of bacterial proteins or enzymes.[21, 23-26] Previously we have identified and characterized several bismuth binding proteins in *H. pylori*, which served as potential bismuth drug targets.[27-32] Due to its strong thiophilic feature,[21, 25] bismuth is commonly found to bind cysteine residues in these proteins. *Hp*HypB possesses a highly conserved CHX_nC motif (C106, H107 and C142) which is capable of binding both Zn^{2+} and Ni^{2+}.[16, 17] However, it is not clear whether the *Hp*HypB binds bismuth. Here, we investigate the binding of bismuth to *Hp*HypB, particularly the effect of such a binding on the protein aggregation and GTPase activity.

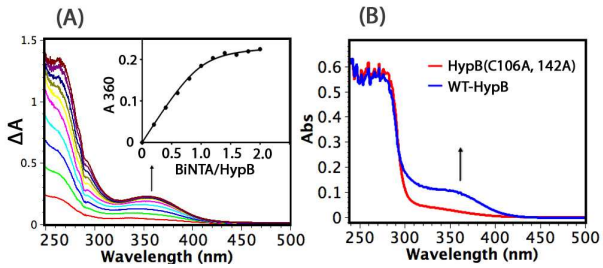

Fig. 1 (A) UV-vis difference spectra of the wild-type HypB (50 μM) upon addition of Bi-NTA (2 molar equivalents). The appearance of the absorbance at 360 nm is characteristic for Bi^{3+} binding to cysteines of the protein. The insert is titration curve plotted at 360 nm against molar ratios of [Bi-NTA]/[HypB], indicative of binding of one Bi^{3+} per HypB monomer. (B) UV-vis spectra of WT and double-cysteine mutant (C106A and C142A) of HypB in the presence of Bi-NTA. The absorbance at 360 nm can only be observed for WT-HypB, indicative of Bi^{3+} binding to the two cysteines.

To examine the interaction of *Hp*HypB with Bi^{3+}, the protein was expressed and purified as an apo-form as described previously.[17] Titration of apo-*Hp*HypB with bismuth nitrilotriacetate (Bi-NTA) was carried and monitored by UV-vis spectroscopy (ESI†). A broad absorption band at ~360 nm appeared in the difference UV-vis spectra and increased with the addition of Bi-NTA (Fig. 1A), which is characteristic for bismuth binding to cysteines.[33, 34] The absorption band levelled off at a molar ratio of 1:1 ([Bi-NTA]/[HypB]), indicative of binding one Bi^{3+} per HypB monomer. The dissociation constant between HypB and Bi^{3+} was calculated to be $0.94(\pm0.25)\times10^{-17}$ μM based on UV titration curve fitting and known Bi-NTA binding affinity (Fig. S1, ESI†). Consistently, no such an absorption band could be observed upon addition of Bi^{3+} to a solution of HypB mutant, i.e. HypB(C106A/C142A) (Table S1, ESI†), in which the two cysteines of the conserved metal-binding residues were mutated to alanines (Fig. 1B), confirming the involvement of the two cysteines in Bi^{3+} binding.

It has been demonstrated previously that the binding of Ni^{2+} to the conserved metal-binding site of *Hp*HypB could enhance

the GTPase activity of *Hp*HypB by synergistically inducing protein dimerization and reducing affinity of GDP binding to the protein. It is believed that Ni^{2+}-binding plays an important role in regulating physiological function of *Hp*HypB.[16, 17] Intrinsically, addition of Bi-NTA into Ni^{2+}-*Hp*HypB led to an increase in intensity of the Bi^{3+}-S(Cys) absorption band at 360 nm at the expense of two original absorption bands at 290 and 330 nm, assignable to S(Cys)→Ni ligand-to-metal charge transfer (LMCT) bands,[16, 28] (Fig. 2A). The kinetics of metal dissociation from both Ni-HypB and Bi-HypB were also compared. Large excess of EDTA were added to 50 μM of metal-saturated HypB solutions to a final concentration of 10 mM in the titration buffer, the absorbance at 330 and 360 nm was monitored for Ni^{2+} and Bi^{3+} respectively (Fig. 2B). The absorbance was normalized to the percentages of bound metal for comparison. Both Ni^{2+} and Bi^{3+} were released from HypB gradually with biphasic kinetics. Ni^{2+} release was fast and completed within 5 mins, with half-lives of 0.24 and 2.7 min for the fast and slow steps respectively. In contrast, Bi^{3+} release was around 5-fold slower, with half-lives of 1.2 and 15 min for the fast and slow steps respectively. The results demonstrated that the coordinated Ni^{2+} could be readily displaced by Bi^{3+}. Owing to a much lower rate of release, Bi^{3+} may interfere with the appropriate function of HypB once it binds to the protein. The changes of secondary structure of HypB upon metal binding were also examined by circular dichroism (CD) spectroscopy (Fig. S1, ESI†). There appear no evident changes on the CD spectrum of apo-HypB upon binding of 1 molar equivalent of Ni^{2+} to the protein, indicative of no obvious secondary structure change occurred. In contrast, binding of 1 molar equivalent of Bi^{3+} leads to clear changes of the CD spectrum, yielding a slight decrease in α-helix contents from 24% to 19% (Table S2, ESI†).

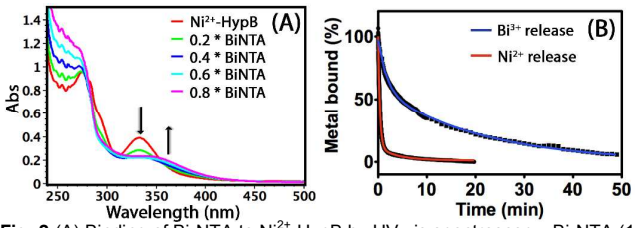

Fig. 2 (A) Binding of Bi-NTA to Ni^{2+}-HypB by UV-vis spectroscopy. Bi-NTA (1 mM) is added stepwise into a Ni^{2+}-HypB solution (50 μM). The decreases in intensity at 330 nm and increases at 360 nm indicate the displacement of the bound Ni^{2+} by Bi^{3+}. (B) Kinetics of metal release from HypB. Time-dependent absorbance at 330 and 360 nm is used to monitor the metal release from Ni^{2+}-HypB and Bi^{3+}-HypB respectively. The absorbance is normalized to the bound metal percentages.

The displacement of physiologically appropriate metal ion by other metal ions, especially at the activity sites, usually results in dysfunction of the enzymes.[35, 36] Thus, we further examined the effect of Bi^{3+} on the GTPase activity of HypB. The rates of GTP hydrolysis were measured and found to be linear within 80 mins (Fig. S2, ESI†). In agreement with previous reports, HypB supplemented with 1 molar equivalent of Ni^{2+} exhibited significantly higher GTPase activity compared with apo-HypB. Surprisingly, the effects of Bi^{3+}on HypB GTPase activity were dose-dependent. Incubation of the protein with 1 molar equivalent of Bi^{3+} led to a 4-fold increase in GTPase activity of HypB compared with the apo-HypB. Unexpectedly, its GTPase activity was completely abrogated in the presence of higher dose (2 and 3 molar equivalents) of Bi^{3+} (Fig. 3). Previously it was demonstrated that Ni^{2+}-dependent dimerization of HypB resulted in the formation of intact GTP hydrolysis sites, enhancing the GTPase activity.[17] Therefore, the dose-dependent effects of Bi^{3+} on HypB GTPase activity inspired us to further investigate the oligomerization states of HypB supplemented with different molar equivalents of Bi^{3+}. Around 200 μM apo-HypB was incubated with different equivalents of Bi^{3+} for 1 h at 4 °C. As shown in Fig. 4, HypB was eluted as a single and broad peak with an apparent molecular weight of 57.6 kDa upon incubation with 1 equivalent of Bi^{3+}, corresponding to a dimeric form of HypB (Fig. S3, ESI†). Whereas incubation of the protein with higher dose of Bi^{3+} (2 and 3 equivalents) led to decreases in the fraction of dimeric form and instead more HypB protein was eluted at lower elution volumes, indicative of the formation of a higher oligomerization state of the protein.

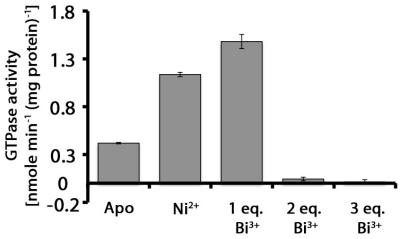

Fig. 3 GTPase activities of HypB in the absence and presence of different metal ions. The binding of Ni^{2+} or 1 molar equivalent of Bi^{3+} (10 μM) significantly enhanced apo-HypB GTPase activity (by 3-fold and 4-fold respectively). In contrast, addition of 2 and 3 molar equivalent of Bi^{3+} to the protein totally abolished HypB GTPase activity.

To further validate that the protein aggregation was induced by Bi^{3+} binding to the cysteines of the metal site of HypB, we investigated the oligomerization state of the HypB variants with metal-site mutated in the absence and presence of 1 molar equivalent of Bi^{3+}, Fig. 5.

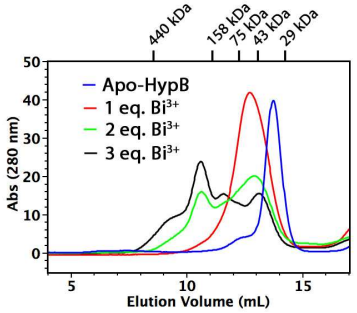

Fig. 4 Size-exclusion chromatography (analytical Tricorn Superdex 200 10/300 column) of HypB pre-incubated with Bi^{3+}. Apo-HypB was incubated with different molar equivalents of Bi-NTA prior to gel-filtration. The elution volumes of the standards were indicated based on the calibration with gel filtration High Molecular Weight (HMW) calibration kit (GE healthcare).

Notably, in the absence of Bi^{3+}, all HypB variants including a double mutant of the two conserved cysteines, HypB(C106A/C142A), single cysteine mutants, HypB(C106A) and HypB(C142A) as well as single histidine mutant HypB(H107A) were eluted as single peaks at ~10 mL , corresponding to the monomeric form. Upon incubation with 1 eq. of Bi^{3+} for 1 h at 4 °C, HypB(C106A/C142A) was eluted at exactly the same volume, indicative of no occurrence of oligomerization of the protein (Fig. 5A); whereas single cysteine mutants, HypB(C106A) and HypB(C142A) were eluted at volumes between the monomeric and dimeric forms, implying an equilibrium between the two states (Fig. 5B, C). Besides the two cysteines, a highly conserved histidine residue H107 is also involved in Ni^{2+}-binding in HypB. In contrast to the cysteine mutants, majority of HypB(H107A) was eluted as a dimeric form with a small monomeric shoulder after incubation with 1 molar equivalent of Bi^{3+} (Fig. 5D), which was similar to wild-type

HypB (Fig. S4, ESI†).

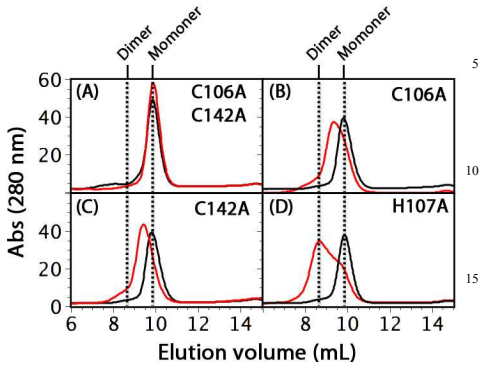

Fig. 5 Size-exclusion chromatography of HypB mutants with (red) or without (black) pre-incubation with 1 molar equivalent of Bi^{3+}. Protein samples were analyzed by Tricron Superdex 75 10/300 column. The double mutant HypB(C106A/C142A) exhibited no changes on oligomerization state upon incubation with Bi^{3+} (A). Single cysteine mutants HypB(C106A) and HypB(C142A) were eluted between the monomeric and dimeric forms (B, C). Histidine mutant HypB(H107A) was mainly eluted as a dimer (D).

As a Ni^{2+} chaperone for [Ni-Fe] hydrogenase, HypB was found to be critical for the survival and colonization of *H. pylori*.[37] Binding of an appropriate metal ion at the conserved metal site of HypB was important for its GTPase activity regulation. Our combined data demonstrated that Bi^{3+} not only binds apo-HypB, but also is able to displace Ni^{2+} from Ni-HypB, indicative of a higher affinity of Bi^{3+} than Ni^{2+} towards the protein. In comparison with Ni^{2+}, the release of Bi^{3+} from HypB was much slower, which was properly due to the tighter binding. Taken together, it is likely that Bi^{3+} competes with Ni^{2+} for HypB in *vivo* and interferes with the proper function of HypB. Unexpectedly, a dose-dependent effect of Bi^{3+} on HypB GTPase activity was observed. Similar to Ni^{2+},[17] 1 molar equivalent of Bi^{3+} induced the dimerization of HypB, which is an active form of the enzyme, thus enhancing HypB GTPase activity. However, excess of Bi^{3+} induced HypB further oligomerization, totally abolished its GTPase activity. Previously bismuth was found to disrupt the functions of certain proteins or enzymes simply *via* alternation of their quaternary structures.[27, 38] Perturbation of GTPase activity of HypB by Bi^{3+} would be harmful for *H. pylori* since it was not physiologically regulated. The present work expands our understanding on the inhibitory effects of bismuth-based drugs against *H. pylori*.

In summary, Bi^{3+} binds to the conserved metal site of HypB with high affinity and readily displaces the bound Ni^{2+}. Bi^{3+} binding perturbs physiological GTPase activity of HypB by inducing protein dimerization or oligomerization. As an essential trace element, Ni^{2+} is critical for the survival and pathogenesis of *H. pylori*. Understanding how bismuth-based drugs interfere with Ni^{2+} homeostasis in *H. pylori* merits further investigation.

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Notes and references

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TOC

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