FEBS Letters 581 (2007) 4109–4114

Eukaryotic initiation factor 2α kinase is a nitric oxide-responsive mercury sensor enzyme: Potent inhibition of catalysis by the mercury cation and reversal by nitric oxide

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Received 26 May 2007; revised 16 July 2007; accepted 16 July 2007

Available online 1 August 2007

Edited by Lev Kisselev

Abstract **The activity of one of the eukaryotic initiation factor 2α kinases, heme-regulated inhibitor (HRI), is modulated by heme binding. Here, we demonstrate for the first time that Hg^{2+} strongly inhibits the function of HRI (IC_{50} = 0.6 μM), and nitric oxide fully reverses this inhibition. Other divalent metal cations, such as Fe^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+}, also significantly inhibit kinase activity with IC_{50} values of 1.9–8.5 μM. Notably, inhibition by cations other than Hg^{2+} is not reversed by nitric oxide. Our present data support dual roles of Hg^{2+} and nitric oxide in the regulation of protein synthesis during cell emergency states.**

Keywords: Protein synthesis; Translation; eIF2α kinase; Nitric oxide; Mercury; Metal cations

1. Introduction

There are four known types of eukaryotic initiation factor 2α (eIF2α) kinases, designated GCN2, PERK, PKR, and heme-regulated inhibitor (HRI)[1] [1–4]. These kinases have a common substrate, and phosphorylate the Ser51 residue of the α subunit of eukaryotic initiation factor 2 (eIF2), leading to the termination of protein synthesis under emergency conditions. However, the sensing systems of these eIF2α kinases in response to cell stress or emergency are different. For example, GCN2 mediates the termination of translation under conditions of amino acid shortage [1] and UV exposure [2], PERK is activated during accumulation of denatured proteins [2], PKR activity is stimulated upon viral infection [3], and HRI acts when cells face a shortage of heme [4–6].

HRI senses the heme concentration in erythroid or red blood cells. Under normal conditions, heme blocks the kinase active site, whereas at reduced heme concentrations, the active site is exposed, allowing phosphorylation of eIF2α [4–6]. While HRI reportedly acts specifically in erythroid or red blood cells, the protein is expressed in almost all tissues, including brain, lung, heart, liver, spleen, kidney, thymus, and stomach [7–9]. It is proposed that HRI additionally regulates protein synthesis in response to factors other than heme. It is thus important to identify the metal cations that effectively inhibit HRI activity, similar to the heme iron.

Nitric oxide (NO) protects cells under conditions of stress and impairs biological systems [10]. Earlier studies using the purified enzyme in a reconstituted system in vivo disclose that NO stimulates HRI activity subsequent to suppression by heme [6].

Here, we examine the effects of metal cations and NO on HRI activity. Our results demonstrate that HRI is significantly inhibited by metal cations, in particular, Hg^{2+} with an IC_{50} value of 0.6 μM. Interestingly, this marked inhibition of HRI catalytic activity by Hg^{2+}, but not other metal ions, is effectively reversed by NO. Additionally, the N-terminal domain of HRI is essential for restoration of activity by NO. Based on these results, we suggest that HRI is a novel NO-responsive mercury sensor enzyme.

2. Materials and methods

2.1. Materials

Restriction and modification enzymes were acquired from Takara Bio (Otsu, Japan), Toyobo (Osaka, Japan), New England Biolabs (Beverly, MA, USA), and Nippon Roche (Tokyo, Japan). Other chemicals of the highest guaranteed grade were purchased from Wako Pure Chemicals (Osaka, Japan), and used without further purification.

2.2. Plasmid construction

A plasmid encoding $(His)_6$-tagged wild-type HRI comprising residues 1–619 (His_6 HRI) from mouse liver cDNA was originally constructed using the pET28 vector (Novagen, Madison, WI, USA), as described previously [6,11]. We introduced a PreScission™ protease (Amersham Biosciences, Piscataway, NJ, USA) recognition site in the expression vector to remove the $(His)_6$-tag. The resulting protein contains extra Gly-Pro-His residues upstream of the Met residue.

2.3. Protein expression and purification

$(His)_6$-tagged HRI was expressed in *Escherichia coli* BL21 (DE3) Codon Plus RIL (Stratagene, La Jolla, CA, USA) harboring pET28a-PreScission/HRI, and purified using a previous protocol [6,11]. The $(His)_6$ tag was cut with PreScission™ protease, according to the manufacturer's protocol. Purified $(His)_6$-free HRI proteins were

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Abbreviations: HRI, heme-regulated eIF2α kinase or heme-regulated inhibitor; eIF2α, α-subunit of eukaryotic initiation factor 2; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Fe^{3+}-hemin, Fe^{3+}-protoporphyrin IX complex; Fe^{2+}-heme, Fe^{2+}-protoporphyrin IX complex; GCN2, general control non-derepressible 2; PKR, pancreatic eIF2α kinase; PERK, PKR-like ER kinase; TBST, 20 mM Tris–HCl, pH 7.7, 137 mM NaCl containing 1% Tween 20

0014-5793/$32.00
doi:10.1016/j.febslet.2007.07.055

more than 95% homogenous, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE; 7.5% gel) followed by staining with Coomassie Brilliant Blue R250. The final yields were 0.21 and 0.51 mg/L of culture for full-length and N-terminal truncated (amino acids 146-619) HRI enzymes, respectively.

2.4. Optical absorption spectra

Optical absorption spectra were collected under both anaerobic and aerobic conditions using Shimadzu UV-2500 and Multi Spec 1500 spectrophotometers (Kyoto, Japan) maintained at 25 °C, respectively. The reaction mixture was incubated for 10 min prior to spectroscopic measurements to ensure stable solution temperature. Experiments were performed at least three times for each complex.

2.5. In vitro protein kinase assay

The in vitro protein kinase assay was conducted as described previously [6,11], with some modifications. Briefly, the kinase reaction mixture (20 μl) containing 20 mM Tris–HCl, pH 7.7, 2 mM magnesium acetate, 60 mM KCl, 2 μg of $(His)_6$-tagged eIF2α, 0.35 μM tag-free HRI (full-length and N-terminal truncated proteins), and 50 μM ATP was incubated at 15 °C for 10 or 15 min under aerobic conditions. However, experiments with NO were performed under anaerobic conditions. The reaction was terminated by adding Laemmli sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 0.002% bromophenol blue), heated for 10 min at 95 °C, and subjected to 10% SDS–PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). Phosphorylated proteins were detected by immunoblotting with an anti-phosphorylated eIF2α antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) [11].

Primary mouse anti-$(His)_6$ monoclonal IgM (H-3), goat anti-HRI IgG (S-16), rabbit anti-eIF2α IgG (FL-315), and goat anti-phosphorylated eIF2α IgG (Ser52) antibodies were purchased from Santa Cruz Biotechnology, Inc. For immunoblotting, the membrane was blocked for 1 h with 5% bovine serum albumin in Tris-buffered saline (20 mM Tris–HCl, pH 7.7, 137 mM NaCl) containing 1% Tween 20 (TBST), and incubated overnight at room temperature with primary antibody diluted in TBST. After washing with TBST, the membrane was treated with horseradish peroxidase-conjugated sheep anti-mouse IgG, donkey anti-rabbit IgG (Amersham Biosciences) or donkey anti-goat IgG (Santa Cruz Biotechnology) for 1 h. Immunoreactive protein bands were visualized with ECL reagents (Amersham Biosciences), and detected using a Fuji Film Chemiluminescence Reader LAS-3000 IDX6 (Tokyo, Japan). Band intensities were calculated using Image J 1.32j (National Institutes of Health, Bethesda, MD, USA) software.

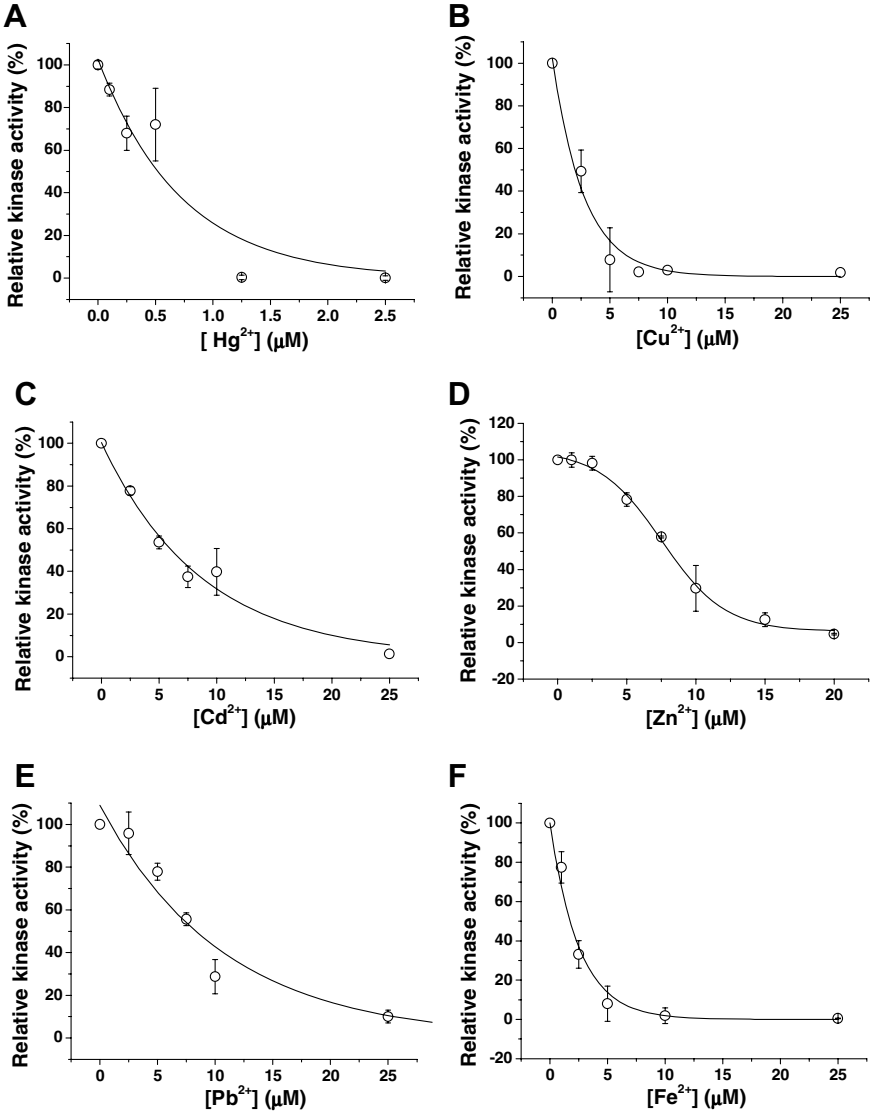

Fig. 1. Effects of Hg^{2+} (A), Cu^{2+} (B), Cd^{2+} (C), Zn^{2+} (D), Pb^{2+} (E), and Fe^{2+} (F) on the activity of full-length wild-type HRI. Inhibitory effects of these metal cations on the N-terminal truncated (first 145 amino acids) mutant were similar to those observed for the full-length enzyme (not shown).

3. Results

3.1. Effects of metal cations on HRI kinase activity

The majority of metal cations studied effectively inhibited HRI activity (Fig. 1; Table 1). Hg^{2+} inhibited HRI activity most potently with an IC_{50} value of 0.6 μM (Fig. 1A; Table 1). Other metal cations, such as Cu^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+}, significantly inhibited HRI, but with less potency than Hg^{2+}, as disclosed by their IC_{50} values of 2–9 μM (Table 1; Fig. 1). Fe^{3+}, Ni^{2+} and Co^{2+} were very weak inhibitors with IC_{50} values of 48–130 μM (Table 1). For comparison, the IC_{50} values of the Fe^{2+}-heme and Fe^{3+}-hemin complexes, which are native inhibitors in erythroid or red blood cells, were calculated as 4.1 and 9.5 μM, respectively (Table 1). Therefore, it is likely that metal cations with IC_{50} values less than 10 μM regulate HRI in vivo by competing with Fe^{2+}-heme or Fe^{3+}-hemin complexes in erythroid or red blood cells. Arsenite, AsO_3^-, which influences HRI activity in vivo [12], had a marginal effect on catalytic activity in our assays (Table 1). Essentially similar results with the different cations were obtained for the N-terminal truncated mutant.

3.2. Effects of NO on metal cation-induced inhibition

NO re-stimulates HRI activity following suppression by heme in a reconstituted system [6]. We examined the effects of NO on metal cation-induced inhibition of HRI. Interestingly, Hg^{2+}-inhibited activity was restored upon the addition of NO. Specifically, HRI activity in the presence of Hg^{2+} was enhanced up to 80-fold by NO (Fig. 2A). This marked reversal in Hg^{2+}-mediated suppression of activity is comparable to the 70-fold increase in heme-inhibited HRI activity by NO (Fig. 2B). Optical absorption and EPR spectroscopy data suggest that the formation of a 5-coordinate NO–Fe^{2+}-heme complex (Table 1S; Fig. 1S) contributes to the enhancement of heme-suppressed catalysis [6,11]. Similarly, since Hg^{2+} binds to thiols [13], it is likely that NO forms a thiol adduct in the active site, such as S–NO, that reverses catalytic suppression. The nitrite anion affects various physiological functions [14]. However, neither nitrite nor nitrate anions influenced HRI catalysis in our experiments.

Table 1
IC_{50} values (μM) of heavy metal cations for the inhibition of full-length wild-type HRI

Metal cation	
Hg^{2+}	0.6
Fe^{2+}	1.9
Cu^{2+}	2.1
Fe^{2+}-heme	4.1
Cd^{2+}	6.2
Zn^{2+}	8.2
Pb^{2+}	8.5
Fe^{3+}-hemin	9.5
Fe^{3+}	48
Ni^{2+}	57
Co^{2+}	130
AsO_3^-	110

Experiments were conducted under anaerobic conditions at least four times. Experimental errors were within 20%. The IC_{50} values of the N-terminally truncated mutant were comparable to those observed for full-length wild-type enzyme.

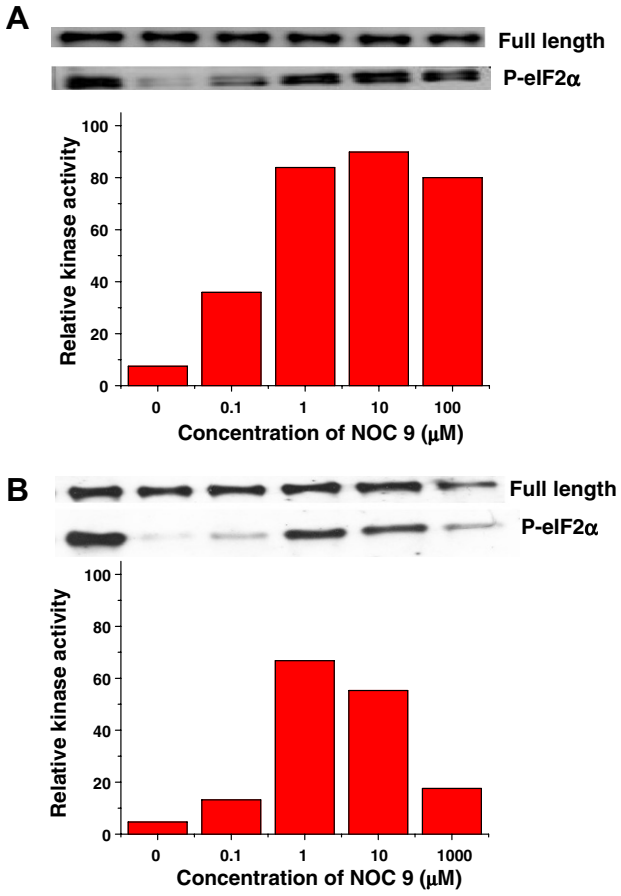

Fig. 2. (A) Effects of NO on Hg^{2+}-inhibited activity of full-length wild-type HRI. The upper bands show that all reactions contain the same amount of enzyme, while the lower bands indicate the level of phosphorylated eIF2α. The first reaction does not contain Hg^{2+}. All the other reactions contain 0.6 μM Hg^{2+} and different concentrations of NOC 9. Experiments were conducted under anaerobic conditions, and repeated at least four times. Results are presented as mean values. Experimental errors were within 20% of the mean. (B) Effect of NO on Fe^{2+}-heme-induced inhibition of full-length wild-type HRI. The first reaction does not contain Fe^{2+}-heme, while the other reactions contain 6 μM Fe^{2+}-heme and different concentrations of NOC 9. Addition of NO enhanced activity approximately 70-fold. Experiments were conducted under anaerobic conditions at least four times. NO did not affect the activity of the N-terminal truncated mutant.

3.3. Effect of the N-terminal domain on NO-induced catalytic recovery

Since the N-terminal domain of HRI is important for sensing the heme concentration [11], we determined whether it plays a role in the reversal of Hg^{2+}-mediated inhibition by NO using a truncated protein lacking the first 145 amino acids. Hg^{2+} inhibited the activity of truncated and full-length HRI to a similar extent. However, NO did not reverse the effect of Hg^{2+} on the truncated enzyme, suggesting that the N-terminal domain of HRI is essential for NO stimulation of catalytic activity.

3.4. Cross-talk between metal and heme binding

It is proposed that the same Cys residue(s) is involved in inhibition by heme, Hg^{2+}, and other cations. Accordingly, we examined the effects of metal ions on heme-bound full-length

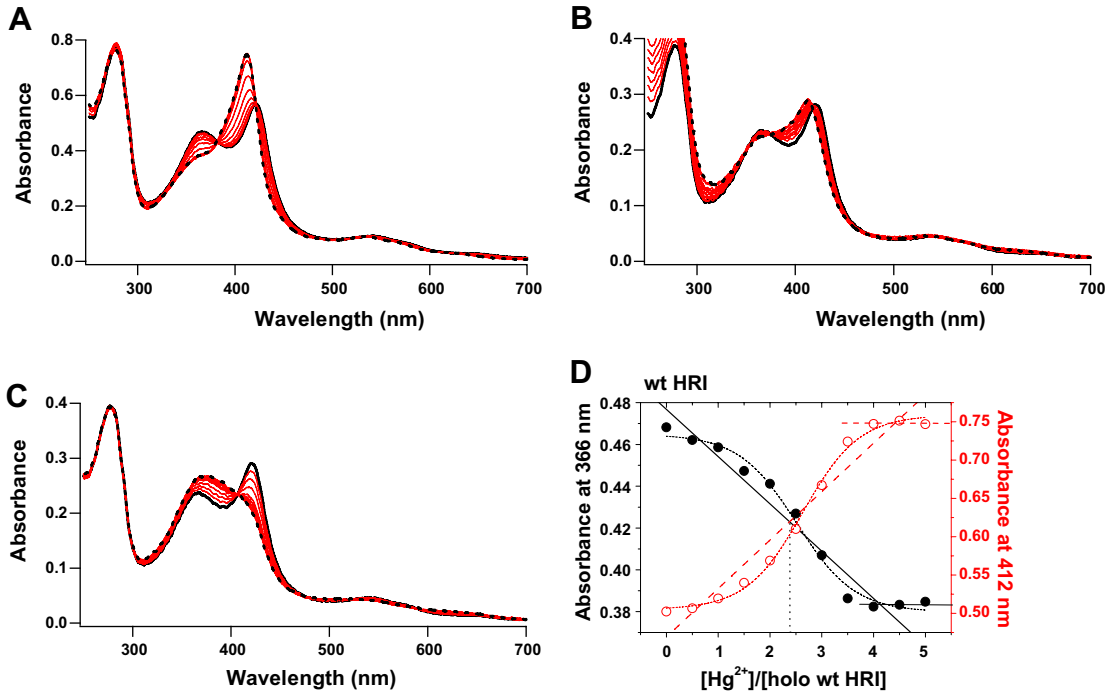

Fig. 3. Effects of Hg^{2+} (A), Cu^{2+} (B), and Zn^{2+} (C) on the Soret absorption spectrum of full-length wild-type HRI (10 μM in A and 5 μM in B, C in 50 mM Tris–HCl, pH 8.0). The metal cations were added in ten steps till they reach the concentration the same as HRI. (D) Titration data of Hg^{2+} suggest that four Cys residues are involved in the interactions between Hg^{2+} and HRI. Titration of Cu^{2+}, Cd^{2+}, and Zn^{2+} (data not shown) did not reveal any correlation between absorption changes and metal-to-protein ratios.

HRI, using optical absorption spectroscopy. The Soret absorption spectrum of full-length wild-type HRI was markedly altered in the presence of metal cations. Specifically, addition of Hg^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+} led to an increase in absorption at 412, 413, 366 and 370 nm, respectively, and a concomitant decrease in the band at 421 nm (Fig. 3). These spectral changes indicate that the metal ions induce a shift in the heme coordination state of the major species from the 6-coordinate low-spin to the 5-coordinate high-spin complex. Changes in the Soret region of the spectrum for full-length wild-type enzyme induced by Hg^{2+} were distinct from those in the presence of Cu^{2+} and Zn^{2+} (Fig. 3). In particular, the decrease in absorption at 366 nm is characteristic of Hg^{2+} competition with heme. Absorption at around 370 nm was suggested to involve in the thiol-Fe^{3+}-hemin bond of heme-sensor proteins [11,15]. We propose that the metal ions trigger a switch from the axial Cys ligand bound to heme to a water molecule or OH-anion, resulting in a decrease in absorption at 366 nm. Finally, the stoichiometry of Hg^{2+} binding indicates that full-length HRI contains four reactive Cys residues that interact with Hg^{2+} (Fig. 3, panel D), of which at least one is the heme axial ligand.

3.5. Evidence of Cys binding to Hg^{2+}

To further establish the role of reactive Cys residues, we examined the effects of dithiothreitol and mercaptoethanol on metal-induced changes in the Soret absorption spectra of heme-bound HRI. Spectral changes induced by Hg^{2+} and Cu^{2+} were restored by dithiothreitol and/or mercaptoethanol, but not EDTA (Fig. 4). These results confirm that Hg^{2+} or Cu^{2+} interacts with Cys residue(s) in HRI, disrupting the heme Fe–thiol bonds. On the other hand, EDTA, but not dithiothreitol, restored spectral changes by Cd^{2+} and Zn^{2+} (Fig. 4), suggesting that these metal ions are coordinated to amino acid residue(s) other than Cys, possibly His.

4. Discussion

In the current study, we show that heavy metal cations (in particular Hg^{2+}) effectively inhibit HRI. Since HRI is active under conditions of cellular stress, it is reasonable to assume that metal contamination initiates cellular protein synthesis, a response important for cell survival. We additionally show that micromolar NO can fully reverse the inhibition of HRI by Hg^{2+}, but not other metal cations. Under conditions of cellular stress, micromolar levels of NO are generated by inducible NO synthase [10], and eIF2α kinases, including HRI, are activated to promote cellular survival. Therefore, both Hg^{2+} and NO may competitively regulate protein synthesis. Thiol anions form a complex with Hg^{2+} [13] and S–NO species with NO [16–20]. Accordingly, we propose that protein synthesis is upregulated in cells contaminated with Hg^{2+} as a result of Hg^{2+}–thiol complex formation in HRI, which is reversed or reduced by NO due to the generation of S–NO species.

The well-known mercury sensor protein, MerR, senses Hg^{2+} with an apparent dissociation constant of about 0.5 μM [21]. It is possible that HRI functions as a Hg^{2+} sensor protein that is as competent as MerR in vivo.

A variety of cell stress conditions may lead to rapid transcriptional activation of inducible NO synthesis, resulting in the production of micromolar concentrations of NO [10]. Furthermore, both nitrate and nitrite anions play important

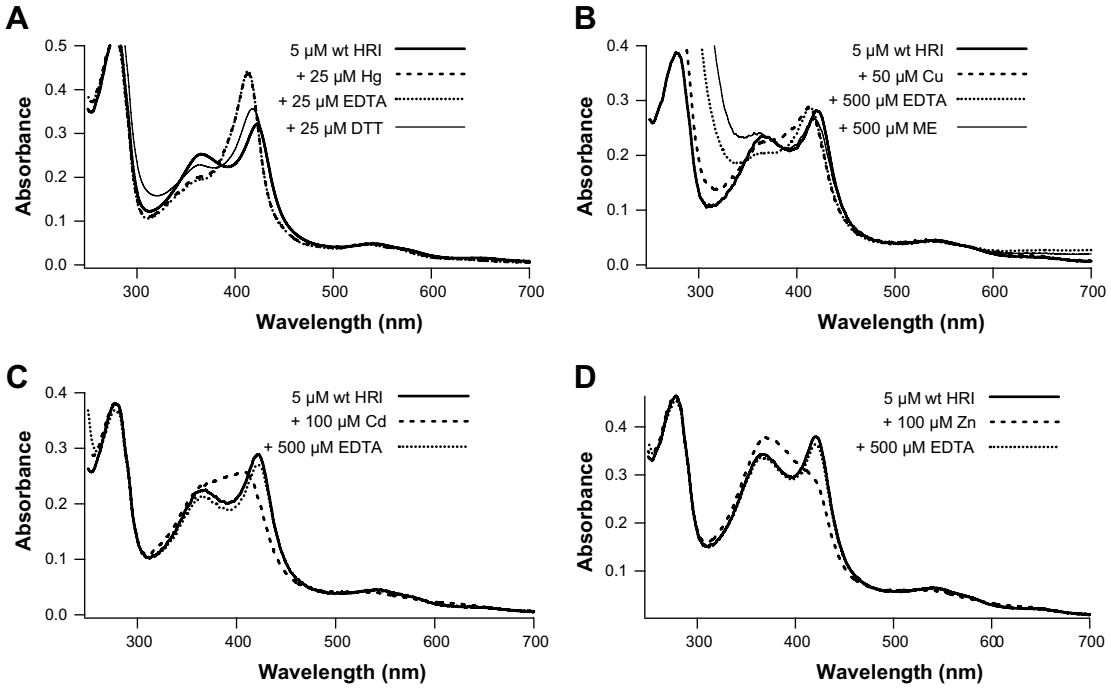

Fig. 4. Addition of dithiothreitol or methyl mercaptoethanol to full-length wild-type HRI. The (A) Hg^{2+}- or (B) Cu^{2+}-induced bands (broken lines) at 412 and 413 nm, respectively, were shifted back to the original position (i.e., in the absence of metal ion) at 421 nm (bold and thin lines). EDTA did not affect spectra in the presence of Hg^{2+} or Cu^{2+} (dotted lines). Addition of EDTA to full-length HRI shifted the (C) Cd^{2+} and (D) Zn^{2+}-induced bands at 366 and 370 nm, respectively, back to the original position (i.e., in the absence of the metal ion) at 422 nm.

roles in various physiological processes [14]. However, in our analyses, neither of these anions affected the catalytic activity of HRI in the presence of heme and metal cations.

Interestingly, the NO transport protein displays heme-assisted S-nitrosation of a proximal thiolate [22]. In the current study, NO did not reverse Hg^{2+}-mediated inhibition of the catalytic activity of N-terminal truncated HRI. Notably, the full-length wild-type HRI is a hexamer, whereas the N-terminal truncated mutant is a trimer [11]. Therefore, the hexameric state of HRI may be important for the reversal of Hg^{2+} inhibition of activity by NO, probably due to intersubunit interactions.

Our results obtained using purified enzyme in a reconstituted system directly contradict previous reports that heavy metal cations inhibit protein synthesis by affecting HRI [23,24]. Moreover, in contrast to an earlier publication that HRI plays an essential role in mediating arsenite stress-induced phosphorylation [12], arsenite had little effect on HRI activity in our experiments.

Based on the data, we suggest that global structural changes in the HRI protein upon heme or metal cation association/dissociation regulate catalysis, analogous to other heme-sensor proteins [25,26]. Global protein structural changes, including axial ligand switching, occur for several heme-sensor proteins upon signal binding [27,28].

Acknowledgements: This work was supported, in part, by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to M.M. and T.S.) and a Postdoctoral Fellowship for Foreign Researchers to M.M. from the Japan Society for the Promotion of Science (JSPS).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.07.055.

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