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ApoHRP-based Assay to Measure Intracellular Regulatory Heme

Hani Atamna\(^1\), Marmik Brahmbhatt\(^1\), Wafa Atamna\(^1\), Gregory A. Shanower\(^1\), and Joseph M. Dhahbi\(^3\)

\(^1\)Department of Basic Sciences, TCMC, Scranton, PA. \(^2\)Department of Medical Education, California Northstate University (CNSU), Elk Grove, CA. \(^3\)Department of Biochemistry, University of California at Riverside, Riverside, CA, USA.

**Key Words:** Heme, Amyloid-\(\beta\), toxic metal, peroxidase.

**Running title:** Regulatory heme.

**Abbreviations:**

RH, Regulatory Heme; HRM, Heme Regulatory Motif; NMP, N-methyl protoporphyrin IX; ALA, \(\delta\)-Aminolevulinic acid; holoHRP, holo horseradish peroxidase; apoHRP, apo-horseradish peroxidase; heme (ferro-protoporphyrin IX), iron’s redox state is \(Fe^{2+}\); hemin (ferriprotoporphyrin ix), redox state of \(Fe^{3+}\) and chloride as a ligand; A\(\beta\), Amyloid-\(\beta\) \(\sqsubset\) Pb, Lead; Hg, Mercury.

* Correspondence;
  Hani Atamna
  hani.atamna@cnsu.edu

Address for Proofs:
Hani Atamna
Department of Medical Education
California Northstate University
9700 West Taron Drive
Elk Grove, CA. 95757
Abstract:

The majority of the heme-binding proteins possess a “heme-pocket” that stably binds with heme. Usually known as housekeeping heme-proteins, they participate in a variety of metabolic reactions (e.g., catalase). Heme also binds with lower affinity to the “Heme-Regulatory Motifs” (HRM) in specific regulatory proteins. This type of heme binding is known as exchangeable or regulatory heme (RH). Heme binding to HRM proteins regulates their function (e.g., Bach1). Although there are well-established methods for assaying total cellular heme (e.g., heme-proteins plus RH), currently there is no method available for measuring RH independently from the total heme (TH). The current study describes and validates a new method to measure intracellular RH. The method is based on the reconstitution of apo-horseradish peroxidase (apoHRP) with heme to form holoHRP. The resulting holoHRP activity is then measured with a colorimetric substrate. The results show that apoHRP specifically binds RH but not with heme from housekeeping heme-proteins. The RH assay detects intracellular RH. Furthermore, using conditions that create positive (hemin) or negative (N-methyl protoporphyrin IX) controls for heme in normal human fibroblasts (IMR90), the RH assay shows that RH is dynamic and independent from TH. We also demonstrated that short-term exposure to subcytotoxic concentrations of lead (Pb), mercury (Hg), or amyloid-β (Aβ) significantly alters intracellular RH with little effect on TH. In conclusion the RH assay is an effective assay to investigate intracellular RH concentration and demonstrates that RH represents ~6% of total heme in IMR90 cells.
Introduction:

Heme is a prosthetic group that binds tightly with the heme-pocket in many of "housekeeping" heme-proteins in both prokaryotic and eukaryotic cells (e.g., catalase, cytochromes)\(^1\). Housekeeping heme-proteins are essential for cellular function due to their ability to catalyze specific biochemical reactions\(^2,3\). These housekeeping heme-proteins can bind heme irreversibly by covalent bond (e.g., cytochrome c) or non-covalently (e.g., cytochrome b). When non-covalently bound, heme affinity to the apoprotein is usually very high (\(K_d\) ranges between \(10^{-12}\)-\(10^{-15}\) M), indicating that the heme moiety is mostly protein-bound. Interestingly, exchange of heme moiety can occur when a heme-protein is interacting with an other protein that exhibits a higher affinity for heme\(^4\).

In addition to the housekeeping function, a role for heme as a regulator for specific proteins (heme-responsive proteins) is emerging\(^1,5\). For example, heme regulates its own synthesis as well as several additional biological functions\(^5a\). In this role heme can regulate specific proteins by binding in an ON/OFF mechanism to Heme Regulatory Motif (HRM) in specific proteins\(^5a,6\). Contrary to heme in housekeeping heme-proteins, HRM-dependent proteins reversibly bind with Regulatory Heme\(^5a,6b,7\). Miksanova et al. showed that the binding of RH to HRM is weaker than heme binding with the heme-pocket in heme-proteins\(^7b\). An additional type of regulation by heme involves changes to heme ligation status (e.g., binding NO) or by altering the redox status of heme-iron in specific heme-proteins\(^5-6\).

The source of heme in the HRM-dependent type of regulation is believed to originate from "free heme", "exchangeable" heme (from low affinity heme-binding proteins), or newly synthesized heme, which collectively we will refer to as Regulatory Heme (RH). Furthermore, the biophysical nature of RH has not been established\(^6a, b, 7a, 8\). Regardless of being "free" or "exchangeable", several lines of evidence demonstrate the transient effect of heme on the activity of specific HRM-dependent protein factors, which is consistent with a regulatory role\(^6a, b, 7a, b, 8-9\).

The HRM-dependent proteins include the heme activator protein (Hap1)\(^10\), \(\delta\)-aminolevulinic acid synthase (ALAS)\(^11\), iron regulatory protein-2 (IRP2)\(^12\), heme-regulated eukaryotic initiation factor 2 (\(e\)IF2) kinase (HRI) (protein translation)\(^13\), DGCR8 (RNA processing)\(^14\), REV-erb-\(\alpha\)\(^9\), and the transcriptional repressor Bach1\(^15\). In general, the interaction of heme with these proteins influences regulatory steps of the cellular metabolism rather than providing a direct catalytic
activity. For example, RH regulates its own synthesis by controlling ALAS import into the mitochondria, which is the rate-limiting enzyme of heme synthesis\textsuperscript{11}.

The current study is concentrating on Regulatory Heme. RH has not been directly investigated in cells due to the lack of a specific method for measuring RH independently from heme moiety of the housekeeping heme-proteins\textsuperscript{16}. Most of our knowledge about RH comes from indirect observations of protein factors that transiently bind heme. For example, Sassa et. al has proposed that the intracellular RH concentration is in the range of low nM\textsuperscript{16}. Therefore, we adapted and modified a method for the determination of free heme in chloroplasts, which involves extraction with organic solvents\textsuperscript{17}, to develop an assay to measure cellular RH. The purpose of the current study is to provide a simple assay to measure intracellular RH (RH assay) independently from the heme moiety of the housekeeping heme-proteins, which accounts for the majority of the total heme.

The RH assay is described in details in the following sections. In order to validate the method we performed control experiments as well as examined the effect of specific treatments that are known to alter the intracellular level of heme and used these treatments to create conditions of positive or negative controls in human lung fibroblasts (IMR90). Then, we used the RH assay to investigate the susceptibility of RH to Pb and Hg as well as to amyloid-β (Aβ). These agents are known to interfere with heme metabolism. The RH assay provides accurate and quantitative information about the intracellular concentration of RH, which may facilitate future investigations into RH in different cell types and tissues.
Methods:

**Materials:** Pb(NO$_3$)$_2$, Hg(NO$_3$)$_2$, HNO$_3$, human hemoglobin, catalase, and sodium dithionite were obtained from Sigma (St. Louis, MO). Amyloid-β$_{40}$ (Aβ) was from American Peptides Company (Sunnyvale, CA). ApoHRP was from Calzyme Labs (San Luis Obispo, CA). Hemin and N-methyl protoporphyrin IX (NMP) were from Frontier Scientific (Logan, UT). Peroxidase Substrate Kit TMB (SK-4400) was from Vector Labs (Burlingame, CA). Bio-Gel P2 gel filtration beads were from Bio-Rad (Hercules, CA). Protease inhibitors cocktail was from MP Biomedicals (Solon, OH). DNase, RNase, and protease free TritonX100 was from ACROS Organics (Pittsburgh, PA) and all the organic solvents were HPLC grade (Fisher Scientific). Normal human lung fibroblasts (IMR90) were from Coriell Institute for medical research (Camden, NJ). Cell culture media and reagents (DMEM, FBS, Pen/Strep, and trypsin-EDTA (0.05% trypsin/0.53 mM EDTA) were from Life-Technologies.

**IMR90 cell culture preparation and cell counting:** IMR90 cell were seeded at 0.5 million cells per plate in 10 ml DMEM supplemented with 10% fetal bovine serum (FBS) and 2 ml Penicillin and Streptomycin (Pen/Strep). The cells were harvested with 1.5 ml trypsin/EDTA and counted using Beckman-Coulter Z2 (Brea, CA) for particle count and size analysis. The cells were collected by centrifugation at 4 °C and used for lysate preparation or stored at -80 °C.

**Cell lysate preparation and protein quantification:** The lysates from 5-7 million IMR90 cells were prepared in 300 µl of ice-cold PBS/0.1% Triton-X100 supplemented with 20 µl of protease inhibitors. The lysates were then sonicated and centrifuged at 18000 xg for five minutes at 4 °C. The protein content of the lysate was determined using Quick Start Bradford Dye Reagent (Bio-Rad, Hercules CA) and fatty acid free Bovine Serum Albumin. This lysate preparation produces a protein concentration of about 2-3 µg/µl.

**Stock solution of hemin:** Hemin stock solution was freshly prepared by dissolving 3.27 mg into 5 ml 0.1 N NaOH and stored in dark. The solution was centrifuged to remove insoluble material and the concentration was determined from the absorbance at 385 nm and the mM extinction coefficient of 58.4 using spectrophotometer DU800 (Beckman Coulter, Brea, CA).
Preparing stock solution of ApoHRP: The commercial apoHRP was extracted by acid acetone solution (250 µl concentrated HCl (36%) in 10 ml acetone) in order to eliminate any possible residual HRP activity. Briefly, 8 mg apoHRP were dissolved in 2 ml DDW and extracted with 40 ml acid acetone. The pellet was then collected by centrifugation at 2000xg for 2 min, and dissolved in 2 ml PBS. The residual HRP activity was measured by the peroxidase TMB assay kit. The cycle of extraction can be repeated if necessary. The concentration of apoHRP was confirmed using a molar extinction coefficient at 280 nm of 20,000. A stock of 50 µM apoHRP was prepared in PBS and saved in aliquots at -20 °C.

HPLC analysis for total cellular heme: The total heme (TH) of cellular lysates, which includes both heme in heme-proteins (except heme c) and RH, was extracted with acid acetone (250 µl concentrated HCl in 10 ml acetone) and measured as described earlier. Hemin from the working solution of 10 nM was similarly extracted with acid acetone and used as HPLC standard. Fifteen microliters of each sample and the standard were injected into an ODS Hypersil (C18) 250x4 mm column (particle size 5µm) and separated with a gradient of two mobile phases using Waters Binary HPLC Pump-1525; Waters 2489UV/Vis detectors; Waters 2707 autosampler with temperature control; and Empower-Pro software control and data analysis. Hemin separation was accomplished at room temperature by utilizing a gradient made by mixing the two mobile phases A and B. Mobile Phase A: was made from 30% acetonitrile; 70% DDW; and 0.05 % heptafluorobutyric acid (HFBA), Mobile Phase B: was made from 85% Acetonitrile; 15% DDW; and 0.05 % HFBA. The separation cycle was 39 min and depended on two mobile phases as follows: 2 min wash with 100% mobile phase A; within 22 min switch to 100% mobile phase B; maintain 100% mobile phase B for 2 min; within 5 min switch to 100% mobile phase A; maintain 100 % mobile phase A for 8 min. Heme elution was monitored at 398 nm.

The assay for Horseradish Peroxidase (HRP): The Peroxidase Substrate TMB kit for measuring peroxidase activity depends on the oxidation of 3,3’,5,5’-tetramethylbenzidine (TMB). The oxidation of TMB by holoHRP was measured at 652 nm with the plate reader SpectraMax M5 (Molecular Devices, Sunnyvale, CA).

Establishing the standard curve of ApoHRP reconstitution with hemin (RH assay): The stock
solution of hemin was diluted in DDW to 25 nM, which was immediately used for the regulatory heme assay. The apoHRP working solution of 50 µM was also freshly prepared in PBS or from frozen aliquot. The time-dependence of apoHRP reconstitution with hemin was determined by mixing 10 µl apoHRP (final concentration 5 µM) with 10 µl hemin (final concentration 2.5 nM) in a tube that already contains 80 µl PBS followed by incubation at 4 °C. The final volume of the reconstitution reaction was 100 µl. (The 5µM apoHRP were determined in a separate experiment that is described in the following section). At different intervals a 10 µl aliquot was removed from the reconstitution reaction and used to measure the resulting holoHRP activity with the TMB peroxidase substrate kit. The holoHRP activity, presented as absorbance at 652 nm, was then plotted against the reconstitution time (min). This experiment establishes the minimal time that is required for apoHRP to reconstitute with hemin.

A hemin standard curve was then prepared as follows and as summarized in Table 1. A 10 µl of 50 µM apoHRP were added to each one of pre labeled seven Eppendorf tubes followed by adding increasing concentrations of hemin using the 25 nM hemin solution to give the following final concentrations: 0, 0.25, 0.5, 1, 1.5, 2, 2.5 nM (i.e., 0, 25, 50, 100, 150, 200, 250 fmole) after bringing the final volume in each tube to 100 µl with PBS as described in Table 1. The reconstitution reactions were then mixed and incubated for at least ten minutes in 4 °C. At the end of the incubation, the holoHRP activity was measured by transferring 10 µl out of each reconstitution reaction to 96-well plate wells followed by 200 µl of TMB working solution using. The enzymatic oxidation of TMB was allowed to progress for 5-10 min (or as needed). The absorbance was read using a plate reader (SpectraMax M5, Molecular Devices) set at 652 nm. The absorbance data at 652 nm was plotted against the fmole of hemin in each well to establish the standard curve. The final concentration of apoHRP in each of the seven tubes is 5 µM (i.e., 500 pmole). Thus, the ratio of apoHRP to hemin at each concentration of hemin is 2x10^4, 1x10^4, 5x10^3, 3.3x10^3, 2.5x10^3, and 2x10^3, respectively (Table 1). It is important to indicate that PBS can be replaced with 80 mM KCl; 20 mM sodium phosphate, and 20 mM HEPES at a pH 7. Furthermore, the hemin working solution can be prepared in 50% DMSO. Although we found that both buffers work equally well, the data we present was collected using PBS and DDW.

**Measuring RH in cellular lysate using the RH assay:** The procedure of RH quantification using cellular lysate was identical to the procedure of preparing the standard curve, except that hemin
was replaced with cellular lysate as summarized in Table 1 and presented here in more details.

To Eppendorf tubes 25 µl of PBS were added followed by 10 µl of 50 µM apoHRP and 10 µg of protein from the lysate (usually 3-5 µl of the lysate). (The 10 µg apoHRP were determined in a separate experiment that is described in the following section). The final volume of the reconstitution reaction is then brought to 100 µl with PBS (Table 1). After mixing, the reconstitution reaction was incubated for at least ten minutes at 4 °C. At the end of the incubation, the activity of holoHRP was measured as described previously. Briefly, up to 30 µl (3 µg protein) were transferred from each of the 100 µl reconstitution reactions to separate wells of a 96-well plate followed by transferring 200 µl of TMB working solution using a multi channel pipette. The absorbance was measured using plate reader set at 652 nm (SpectraMax M5, Molecular Devices). The RH in the lysate sample was calculated with the help of a standard curve that was simultaneously prepared as described in the previous section (Figure 1B) and normalized to 1 mg protein. The development time of holoHRP activity depends on the RH concentration in the sample and is usually 5-10 min. Regardless of the precise development time of holoHRP assay, it is important that both the standard curve and the lysate samples be allowed to develop simultaneously for the same duration. The contribution of the lysate the absorbance at 652 nm (i.e., background) was measured by simulating the reconstitution reaction while omitting apoHRP (Table 1 note 5). This contribution is usually close to zero, however, it is important to subtract the background from the signal.

**Determining the optimal concentrations of apoHRP and protein lysate for the RH assay:** The concentration of apoHRP that is required for optimal reconstitution with lysate was determined by using increasing concentrations of apoHRP. Each of the apoHRP concentrations was mixed with 10 µg of the lysate protein and incubated for 10 min. The procedure was identical to that of the RH assay as described in table 1, except that the increase in the volume of apoHRP was accounted for by similar decrease in the volume of PBS.

The linearity of the RH assay with increasing protein was examined by mixing 5 µM apoHRP with increasing concentrations of protein lysate. The rest of the assay was performed as described in table 1, except that the increase in the volume of the lysate was accounted for by similar decrease in the volume of PBS. The activity of holoHRP that result from the
reconstitution of apoHRP in both experiments was plotted against the concentration of apoHRP or the concentration of the protein, respectively.

**Examining the specificity of ApoHRP towards regulatory heme:** Catalase and hemoglobin (Hb) are two typical heme-proteins that bind heme tightly in an almost irreversible manner. Furthermore, these are typical heme-proteins that have had their binding properties extensively studied and binding constants clearly determined. Thus, in order to test if apoHRP is capable of removing heme that is already complexed with heme-proteins, we substituted hemin in the RH assay with either catalase or hemoglobin. Stock solutions of catalase or hemoglobin were prepared in PBS and then purified by gel filtration using Bio-Gel P2 beads in order to remove possible unbound (exchangeable) heme contamination, which is usually found in commercially prepared heme-proteins. The concentration of hemoglobin and catalase were calculated using the extinction coefficients (mM$^{-1}$ cm$^{-1}$) of 524 at 415 nm for Hb$^{23}$ and of 290 at 405 nm for catalase$^{24}$. Hb and catalase were used at a final concentration of 60 nM each.

Commercial Hb is essentially auto-oxidized to methemoglobin (metHb), which has lower affinity for heme$^{25}$. MetHb can be reduced to Hb with sodium dithionite. Thus, we decided to compare heme transfer to apoHRP from Hb-O$_2$ that was or was not reduced by sodium dithionite. MetHb was reduced by adding few grains of sodium dithionite to the stock solution of 50 µM metHB. The reduction of metHb in open air forms Hb-O$_2$, which was determined by the red-shift of the Soret band (from 406 to 412 nm) and the appearance of a 543 nm and 577 nm peak (data not shown). We then used these preparations to compare the ability of apoHRP to extract heme moiety from Hb-O$_2$ and metHb (i.e., before and after reduction with sodium dithionite), or catalase. The procedure was the same as for the RH assay as described in table 1, except that the lysate was substituted with Hb-O$_2$, metHb, or catalase. The reconstitution reaction with each of the heme proteins (Hb-O$_2$, metHb, or catalase) was allowed to proceed for different time points. At each time point a sample of the reconstitution reaction was removed and used to measure the resulting holoHRP with the TMB peroxidase assay kit. The holoHRP activity was plotted against the reconstitution time. The potential interference of sodium dithionite with the peroxidase assay was examined by adding few grains of dithionite to PBS (without metHb). Then we diluted the dithionite solution 833 times (simulating the dilution from 50 µM to 60 nM) and incubated in ice until it is tested with the TMB assay kit (mimicking the steps of the RH
A sample from this preparation was tested for its effect on holoperoxidase activity using the TMB assay kit. Holoperoxidase was prepared separately using hemin as described in table 1.

Furthermore, in order to examine if heme moieties from the many heme-proteins that are native to the lysate would transfer to apoHRP, the reconstitution reaction between the lysate (10 µg protein) and apoHRP was extended beyond 2 min. The reconstitution reaction was allowed to proceed for various intervals and at the completion of each reconstitution time point the resulting holoHRP activity was measured and the activity was plotted against the reconstitution time.

**The effect of positive and negative controls on intracellular RH:** Inhibition of heme synthesis by N-methyl protoporphyrin IX (NMP), a specific inhibitor of heme synthesis, is expected to lower RH (negative control) while adding hemin to the growth medium is expected to increase RH (positive control). For the negative control, IMR90 cell culture was incubated up to 72 hours with 8 µM NMP. At each time point the cells were harvested and prepared for measuring total heme and RH. NMP has no cytotoxicity at the conditions used in this experiments. For the positive control of RH, increasing concentrations of hemin were added to the growth medium of IMR90 cells for 45 min. At the end of the incubation, the medium was removed, each culture was washed with ice-cold PBS, and the cells were harvested, and pelleted by centrifugation. The cells were washed again three times with PBS, and stored at -80 °C until use.

**The effect of Hg, Pb, and Amyloid-β (Aβ) on intracellular RH:** Hg and Pb are known to interfere with heme synthesis, thus we tested their effect on RH. Hg and Pb were dissolved in HNO₃ at 3 mM stock solutions. The cultures of IMR90 cells were incubated with increasing concentrations of Hg, Pb, and HNO₃ for 24 hr. The concentrations of Hg were: 0.5, 50, 500, and 5000 nM while the concentrations of Pb were: 250, 500, 750, and 1000 nM. After 24 hr the cultures were harvested and the cells were stored at -80 °C until use. The lysates were prepared and RH and total heme were determined using the RH assay and HPLC, respectively. The effect of the vehicle HNO₃ on RH was similarly examined using the respective concentrations of HNO₃ to which the cells were exposed when treated with Pb or Hg (ranged from 22.4 nM to 44.8 µM).

Aβ₄₀ peptide binds heme tightly and interferes with heme synthesis. Aβ₄₀ was dissolved in NANOpure water to give a final concentration of 300 µM and stored as aliquots in -80 °C. IMR90 cell cultures were treated with 500 nM Aβ for up to 24 hr. At different time
points, the cultures were harvested and the cells were stored at -80 °C until use. The lysates were prepared as above followed by determining RH and total heme as described above.

**Statistical analysis:** Graphing and statistical analysis using *t*-tests, Mann-Whitney nonparametric tests, or one-way ANOVA were performed using Prism 6.0 software (GraphPad, San Diego, CA, USA). Significance was defined at 95% confidence interval when *P* < 0.05. Prism 6.0 software (GraphPad, San Diego, CA, USA) was also used for linear and non-linear regression analysis of the data sets using equations built in Prism 6.0.
Results:

Establishing the standard curve for the RH assay utilizing the reconstitution of ApoHRP with hemin to form active HoloHRP:

The time-dependent reconstitution of hemin with apoHRP to form holoHRP is shown in figure 1A. The reconstitution reaction reached plateau within 2 min and the resulting holoHRP activity remained constant until the end of the experiment, indicating the stability of holoHRP. We estimated that 10 min was sufficient to bind all the free hemin in the solution and to complete the formation of holoHRP. We then examined if the reconstitution of apoHRP with increasing concentrations of hemin correlates with the formation of holoHRP activity (Table 1). A linear correlation between hemin concentration and holoHRP activity is shown in figure 1B, which established the standard curve for hemin. The data from the standard curve showed that the apoHRP-heme reconstitution assay (RH assay) could detect low femtomole levels of hemin. The limit of detection (LD) of the RH assay was 0.13 ± 0.06 fmole/200µl (0.65 ±0.3pM) and the quantification limit was 0.4 ± 0.2 fmole/200 µl (2 ± 1 pM). Precision and accuracy were determined by calculating the relative standard deviation (RSD) as 6 ± 1.5 %.

The majority of the hemin in aqueous solutions is in the form of dimers, which is in equilibrium with monomers, raising the question whether the dimer form of hemin interferes with the reconstitution reaction. Thus, the efficiency of the reconstitution step was evaluated under conditions where hemin was prepared in an aqueous solution (which forms mainly dimers) or in 50 % DMSO (which forms mainly monomers). The standard curves were very similar regardless if the working solution of hemin was prepared in PBS or 50% DMSO. A sample of fitting equations of the linear regression for the standard curves data were y=0.059*x+0.13 (R²=0.985) and y=0.062*x+0.128 (R²=0.99) for PBS and 50% DMSO, respectively. A likely explanation for the high efficiency of apoHRP’s reconstitution with hemin in PBS is that hemin dimers dissociate into monomers, a process that could be enhanced by the high ratio of apoHRP (5 µM) to hemin (Table 1). ApoHRP can further drive the dissociation of hemin dimers by the high stability of holoHRP, which prevents the dissociation of heme from holoHRP.

ApoHRP reconstituted with hemin and RH but not with heme moiety from housekeeping heme-proteins:
The cellular milieu contains housekeeping heme-proteins in addition to exchangeable or regulatory heme (RH). Thus, the possibility that heme may transfer from a housekeeping heme-protein to apoHRP was tested using catalase, hemoglobin, metHb as well as heme proteins native to the cellular lysate.

There was a transfer of heme from Hb-O₂ to apoHRP when apoHRP (5 µM) was incubated with Hb-O₂ (60 nM) in the absence of sodium dithionite. This was evident in the continued formation of holoHRP with time (Figure 2A, − sodium dithionite). This transfer of heme to apoHRP was probably due to the fact that the commercial Hb is oxidized to metHb (Hb with oxidized heme-iron), which binds heme at lower affinity than Hb. Thus, heme from metHb transfers to apoHRP, albeit at very slow rate as seen in figure 2A (− sodium dithionite) after 2 min of incubation. Sodium dithionite reduces heme-iron and converts metHb to Hb-O₂ (in the presence of O₂), which binds the heme moiety at higher affinity than metHb (see Discussion). Interestingly, contrary to metHb there was no transfer of heme from Hb-O₂ to apoHRP (Figure 2A, + sodium dithionite). Additionally, there was no time-dependent increase in the reconstitution of apoHRP with Hb-O₂ after 2 min (Figure 2A, + sodium dithionite). These observations demonstrated that apoHRP did not extract heme from Hb-O₂, the normal form of Hb in vivo.

Incubating apoHRP with catalase (a different heme-protein) showed negligible formation of peroxidase activity, which did not develop further with increasing incubation time (Figure 2B). The small burst of holoHRP activity that was seen at the 2 min in figures 2B and 2A could be the result of a small amount of hemin that remained after gel filtration.

In addition to Hb and catalase, we examined the ability of apoHRP to extract the heme moiety of the housekeeping heme-proteins native to the lysate. Consistent with the results from figure 1A, the reconstitution of apoHRP with RH from the lysate was complete within 2 min (Figure 2C). Furthermore, similar to Hb or catalase, extended incubation of apoHRP with the lysate did not cause additional formation of holoHRP (Figure 2C). Taken together, these observations indicate that apoHRP was unable to extract heme from the heme-proteins of the lysate, Hb, or catalase and supports the conclusion that apoHRP-based RH assay specifically reconstitutes with RH or exchangeable heme (as the case in metHb) and not with heme moiety of housekeeping heme-proteins. We estimated that 10 min would be sufficient to bind all the RH or exchangeable heme to form holoHRP.
Determining the optimal concentrations of apoHRP and protein lysate for the RH assay:

The concentration of apoHRP for the optimal reconstitution reaction was determined by plotting the dose-response of apoHRP concentration against holoHRP activity using 10 µg protein lysate (Figure 3A). The plot showed that holoHRP formation reached plateau at 5-6 µM apoHRP. Thus, the concentration of apoHRP for reconstitution with hemin or with lysate was set at 5 µM. We also determined the optimal concentration of the lysate protein for the RH assay. The RH assay was linear with increasing concentration of the lysate proteins (Figure 3B). Linear regression analysis for the relation between RH and protein (µg) produced the linear equation: \( Y=3.9*X+16.4 \) \((R^2=0.95)\). Thus, the concentration of protein lysate between 5-60 µg in the reconstitution reaction was optimal for the RH assay.

The RH assay detected changes to intracellular regulatory heme:

The data from the controls of several experiments were used to calculate the averages of RH and total heme in IMR90 cells. The average of RH in IMR90 cells was found to be 4.3 ± 1.5 pmol RH/mg protein (mean ± SD), while that of total heme was found to be 67 ± 15 pmol/mg protein (mean ± SD, Table 2). These averages were used to calculate the percent of RH out of the total extractable heme, which was found to be \( \approx 6 \% \) (Table 2). Furthermore, we used the RH values in conjunction with the cell volume of human fibroblasts to calculate the intracellular concentration of RH. The cell volume of human fibroblasts was reported to be about 2500 fl\(^{30}\), which we confirmed by directly measuring IMR90 cell volume using the Beckman-Coulter Z2. We also found that on average four million IMR90 cells contain 1 mg protein (data not shown). Therefore, RH in IMR90 cells can be presented as 4.3 ± 1.5 pmols RH / 4 million cells. Thus, the average volume of four million IMR90 cells is \( \approx 10 \mu l \) (2500 fl x 4 x10\(^6\) cells). Using 4.3 ± 1.5 pmol RH, the total cell volume of 1 mg protein (i.e., 10µl protein volume), and that 0.7 is the water fraction of a living cell\(^{31}\), we calculated the concentration of RH as follows: (pmole RH) \( \div \) (10 \( \mu l \)) \( \div \) (0.7). Using the previous equation, the concentration of RH in IMR90 is 614 ± 214 nM (mean±SD). The intracellular concentration of RH in IMR90 cells is about 20 times higher than that was previously proposed\(^{6a, b, 7a, 8, 16}\). Future investigations that include additional cell types as well as in vivo experiments will expand our current understanding of RH homeostasis under physiologic and pathologic conditions and could provide insight regarding its biological significance.
The effect of hemin and heme synthesis inhibitors on intracellular regulatory heme:

The effect of inhibiting heme synthesis by NMP on RH was used as a negative control to examine the specificity of the RH assay. The cells were harvested, proteins were measured, and RH assay was performed as described in Table 1. NMP did not affect total heme as measured by HPLC (Figure 4A). However, there was an insignificant decline in total heme after an hour of treatment with NMP. The level of total heme remained stable afterwards. At this time we do not have explanation for this brief effect of NMP. However, within 10 hours of exposure to NMP a significant 29% decline in RH is observed, which advanced to 71% decline within the next 24 hr (Figure 4B). No further decline in RH was observed in extended incubation time with NMP.

The effect of adding hemin to the growth medium on RH was used as a positive control. IMR90 cell cultures were incubated with increasing concentrations of hemin (0 to 6 µM). After 45 minutes of incubation, excess extracellular hemin was thoroughly washed, and RH was measured using the RH assay. Extracellular hemin caused a significant 3 pmol/mg protein increase in RH, which reached plateau at 2 µM extracellular hemin (20 nmols hemin/10 ml) (Figure 4C). Thus, after adding hemin to the extracellular medium RH level in IMR90 cells was more than doubled.

Lead (Pb) and mercury (Hg) lower intracellular concentration of regulatory heme:

Heme synthesis is subject to interference by variety of exogenous toxic agents such as Pb or Hg. IMR90 cell cultures were incubated for 24 hr with increasing concentrations of Pb or Hg followed by measuring total heme-proteins and RH. Pb and Hg exhibited only a weak effect on total heme-proteins that was not significant (Figure 5A and 5C, respectively). However, the RH level significantly declined by 26%, 34%, and 40% after 24 hr at concentrations of 750, 1000, and 5000 nM of Pb, respectively (Figure 5B). Similarly, Hg caused a 20-23% decline in RH at concentration higher than 500 nM (Figure 5D).

Pb and Hg where dissolved in HNO₃. As a result the final concentration of HNO₃ that the cells were exposed to ranged from 22.4 nM to 44.8 µM. Thus, the effect of HNO₃ on RH was tested using these concentrations and compared to the control cells. HNO₃ did not affect RH in IMR90 cells (Data not shown).

Amyloid-β (Aβ) peptide lowers intracellular concentration of regulatory heme:
Amyloid-β peptide (Aβ) forms a complex with hemin \textit{in vitro} \cite{21,28a}. Aβ induces heme synthesis probably due to its ability to bind heme \cite{28a}. Thus, it has been presumed that RH is the target of Aβ, which could explain the induction of heme synthesis and iron uptake by Aβ \cite{28a}. However, there was no experimental evidence to support that Aβ binds with RH. Aβ peptide is formed inside the cell \cite{32} and it also readily penetrates the cell membrane from extracellular sources \cite{33}. Therefore, we tested if Aβ affects the level of RH in IMR90 cells. IMR90 cells were incubated with 500 nM Aβ for various intervals. The level of RH significantly declined by 35%, 50%, and 60% after 2, 7, and 24 hr incubation with Aβ, respectively (Figure 6A). Total heme-proteins, on the other hand, were not affected by the Aβ peptide (Figure 6B).
Discussion:

Heme is a prosthetic group that exhibits a regulatory function \(^{6a, b, 7a, 8}\). In general, the fraction of regulatory heme (RH) is referred to as RH, “free heme”, newly synthesized heme, or “exchangeable” heme \(^6\). In this study we described a quantitative assay for RH that is based on the reconstitution of RH with apoHRP to form holoHRP (Table 1 and Figure 1A). The kinetic of apoHRP reconstitution with hemin shows that the formation of holoHRP is complete within 2 min (Figure 1A, 2C). However, we chose the reconstitution time with apoHRP and lysate to be at least 10 min to ensure complete reconstitution.

The specificity of the RH assay was examined by measuring the transfer of the heme moiety of metHb, Hb-O\(_2\), or catalase to apoHRP. There was no transfer of the heme moiety of the housekeeping heme-proteins (Hb-O\(_2\), catalase, or heme-proteins native to the lysate) to apoHRP (Figure 2A, B, C). These findings demonstrate that apoHRP selectively and specifically reconstitutes only with exchangeable heme and “free heme”. These observations are consistent with that equilibrium dissociation constants (K\(_d\)) of heme binding with the heme-pocket of heme-proteins (ranges between 10\(^{-12}\)-10\(^{-15}\) M (pM–fM) \(^{34}\)), which is much lower than the K\(_d\) of apoHRP (estimated to be 15.5 x 10\(^{-8}\) M \(^{35}\)). MetHb is formed from oxidation of heme-iron (Fe\(^{2+}\)) to heme-iron (Fe\(^{3+}\)) in red blood cells or \textit{in vitro}. MetHb, as opposed to Hb-O\(_2\), has a low affinity for heme \(^{25}\), thus explaining the transfer of heme from metHb to apoHRP (Figure 2A). However, when Fe\(^{3+}\) in metHb was reduced to Fe\(^{2+}\) by sodium dithionite, which increases heme affinity to globin \(^{25}\), heme transfer to apoHRP is eliminated (Figure 2A, open circles). The differential reconstitution of apoHRP with metHb and Hb-O\(_2\) demonstrates that the RH assay measures free heme or/and exchangeable heme that weakly bound with specific heme-proteins.

The concentration of Hb or catalase (60 nM) that we used in figure 2 mimics the concentration of heme-proteins in the lysate. This concentration was calculated based on the HPLC data that show total heme in the lysate is 67 ± 15 pmol /mg protein. In the RH assay we used 10 \( \mu \)g protein, which contains 0.67 pmol heme proteins in 100 \( \mu \)l final volume. Thus, the heme concentration is 67 nM. We selected 60 nM of catalase and Hb to examine if heme transfers from heme-proteins to apoHRP, creating similar ratios of apoHRP to: Hb, catalase, or lysate.

Furthermore, we have also examined if the heme moiety from heme-proteins that are native to the whole lysate transfers to apoHRP (Figure 2C). The reconstitution of apoHRP with
heme from native heme-proteins in the lysate is fast and found to be complete within 2 min, which is consistent with the idea that apoHRP binds only with RH (Figure 2C). Importantly, extending the incubation time of apoHRP with the lysate does not lead to additional formation of holoHRP beyond that formed during the first two min. This observation indicates that the heme moiety from the native heme-proteins of the lysate does not transfer to apoHRP (Figures 2C and Figure 3). In conclusion, apoHRP is unable to remove heme from Hb, catalase, or heme-proteins from the lysate. These observations demonstrate that apoHRP binds with RH independently from heme of the heme-proteins. Furthermore, these observations provide support that the fraction of RH (or exchangeable heme) is different from the heme moiety of the heme-proteins; however, the biophysical nature of the RH fraction is under investigation.

Following the validation of the assay, we investigated the effects of various conditions on RH including: hemin, NMP, Hg, Pb, and A\ldots. Using the RH assay we showed that the level of RH in control cells is \(4.3 \pm 1.5\) pmol/mg protein in IMR90 cells (Table 2), while HPLC showed that total heme is \(67 \pm 15\) pmol/mg protein. The variability in RH and total heme that we found could be explained, in part, by the fact that IMR90 cells in tissue culture progress toward senescence. Furthermore, a new IMR90 cell culture must be prepared once the cells senesce. These factors, in conjunction with the typical variability in cell culture preparations, might have contributed to the variation in RH. Taking into consideration the fraction of total heme-proteins, we found RH to be 6% of total heme. Since heme is a pro-oxidant that can catalyze a Fenton-like reaction producing highly reactive oxygen species that may cause oxidative damage, the steady-state concentration of RH is likely to be maintained under tight balance. Possible factors that contribute to the RH level include the rate of heme synthesis, the level of expression of heme-proteins, heme export (e.g., FLVCR)\(^{36}\), and heme oxygenase\(^{37}\).

Increasing the concentration of hemin in the growth media leads to an increase in cellular RH (Figure 4C) until it reaches saturation at 2 \(\mu\)M hemin. The induction of heme oxygenase could also contribute to the plateau in RH. Furthermore, inhibiting heme synthesis by NMP causes rapid decline in RH, which within 24 hr reaches a plateau at about 30% of the control level (Figure 4B). Inhibiting heme synthesis has little effect on total heme (Figure 4A,B). The slow turnover of heme-proteins could, in part, explain the resistance of total heme-proteins to inhibition of heme synthesis by NMP. The positive (adding hemin) and negative (using NMP) control experiments provide validation for the ability of the RH assay to detect intracellular RH.
They also show that RH is dynamic and its intracellular level changes on a time scale of minutes (Figure 4B,C). The data also suggests that RH is independent from total heme-proteins (Figure 4A,B).

The toxic heavy metals Hg and Pb inhibit heme synthesis\textsuperscript{38}. We treated IMR90 cells with subtoxic concentrations of Hg and Pb for only 24 hr in order to avoid the complications of cell death. The concentrations of Pb or Hg that decrease RH are much lower than the cytotoxic concentrations for both metals as experiments on fibroblasts and other cell types showed\textsuperscript{39}. Pb causes significant decline of 26%, 34%, and 40% in RH at concentrations of 750, 1000, and 5000 nM, respectively (Figure 5B). Similarly, Hg causes a 20-23% decline in RH at concentrations higher than 500 nM (Figure 5D). The decrease in RH by Pb or Hg reaches a plateau (Figure 5B, D). One possible explanation for the plateau is that RH is a negative regulator of the heme synthetic pathway. RH blocks the import of ALAS into the mitochondria, which is a key step in the induction of heme synthesis\textsuperscript{11}. Thus, a decline in RH by Hg and Pb could induce heme synthesis by increasing ALAS import into the mitochondria\textsuperscript{11}, preventing a further decrease in RH.

Interestingly, total heme-proteins appear more resistant to Pb and Hg (Figures 5A,C). These observations also suggest that the impact of treatment with Hg and Pb on RH is more pronounced on RH than on total heme-proteins. Pb inhibits ALAD\textsuperscript{38a}, the second enzyme in heme synthetic pathway, while Hg oxidizes specific SH groups in specific enzymes (such as ferrochelatase), thus altering their activity\textsuperscript{28b, 40}. RH seems more susceptible to Pb (Figure 5B) than to Hg (Figure 5D) probably due to the strong inhibition of ALAD by Pb. The effect of Hg and Pb on heme synthesis, may explain in part the fast decline in RH by Pb and Hg, as compared to total heme-proteins. The relative resistance of total heme-proteins to Hg and Pb (as is the case with NMP) could be due to the slow rate of turnover of heme-proteins, which maybe measured in days.

The neurotoxic A\textsubscript{β} peptide possesses a specific motif that tightly binds with heme to form the complex A\textsubscript{β}-heme\textsuperscript{21, 28a, 41}. Since A\textsubscript{β} peptide readily penetrates the cell\textsuperscript{32b, 33, 42}, we investigated the effect of A\textsubscript{β} on RH. Within 8 hr of treatment, A\textsubscript{β} caused a 60% decline in RH (Figure 6). The decrease in RH could explain in part the induction of heme synthesis and the increase in iron uptake that is induced by A\textsubscript{β}\textsuperscript{28a, 43}. Total heme, on the other hand, was not affected by A\textsubscript{β} (Figure 6B). Since A\textsubscript{β}-heme exhibits a weak peroxidase-like activity\textsuperscript{41, 43}, we
examined if Aβ and Aβ-heme interfere with the RH assay and found no effect of either form of Aβ on the RH assay in cell-free system (data not shown). A possible explanation for the lack of interference of the peroxidase activity of Aβ-heme on RH assay could be that the peroxidase activity of Aβ-heme as compared to HRP is very weak. 

Apomyoglobin reconstitutes with hemin or exchangeable heme in a cell-free system to form myoglobin. Myoglobin is then measured at 410 nm, 600 nm, or full spectra. However, the apomyoglobin approach to measure RH in cellular lysate is limited since it relies on an endpoint absorbance at 410 nm, 600 nm, or full spectra. Furthermore, the use of apomyoglobin in lysate would require the separation of the resulting myoglobin from the lysate proteins in order to accurately measure the “free/exchangeable” heme. This requires additional protein isolation procedures. These limitations make the apomyoglobin approach less sensitive, a subject to interference from existing heme-proteins in the lysate, and labor intensive. The apoHRP assay, on the other hand, is based on the catalytic activity of the holoperoxidase. The holoperoxidase propagates the signal, thus enhancing the sensitivity (fmole) of the assay. Furthermore, the high sensitivity and specificity of the apoHRP assay allow the use of a small amount of the biological material. Therefore, the apoHRP assay can be used in biological samples.

Additional research is needed to further our understanding of the cell biology and the biophysical nature of RH. All types of cells synthesize heme (except mature RBC) in order to provide the prosthetic group for heme-proteins as well as RH. The RH assay we described here has the potential to serve as a tool to investigate the various aspects of the cell biology and biochemistry of RH in both eukaryotic as well as prokaryotic cells.

**Acknowledgment:**

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Table 1: Outline of the steps and the reagents for performing the RH assay.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>fmole, Hemin</th>
<th>Sample (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- PBS¹ (µl)</td>
<td>0 25 25 25 25 25 25 25</td>
<td>25</td>
</tr>
<tr>
<td>2- Lysate² (µl)</td>
<td>0 0 0 0 0 0 0 3-5</td>
<td></td>
</tr>
<tr>
<td>3- ApoHRP³ (µl)</td>
<td>10 10 10 10 10 10 10 10</td>
<td>25</td>
</tr>
<tr>
<td>4- Hemin⁴ (µl)</td>
<td>0 1 2 4 6 8 10 0</td>
<td></td>
</tr>
<tr>
<td>5- PBS⁵ (µl)</td>
<td>65 64 63 61 59 57 55 60-62</td>
<td></td>
</tr>
<tr>
<td>6- Final volume⁶ (µl)</td>
<td>100 100 100 100 100 100 100 100</td>
<td></td>
</tr>
<tr>
<td>ApoHRP/Hemin</td>
<td>-- 2x10⁴ 1x10⁴ 5x10⁴ 3.3x10⁴ 2.5x10⁴ 2x10⁴ --</td>
<td></td>
</tr>
</tbody>
</table>

¹ PBS can be replaced by 80 mM KCl; 20 mM sodium phosphate, and 20 mM HEPES at a pH 7.
² The optimal range of lysate’s protein concentration is less than 40 µg.
³ apoHRP working solution is 50 µM.
⁴ Hemin working solution is 25 nM hemin. It can be prepared in NANOpure water or 50% DMSO.
⁵ Background was measured by replacing apoHRP with PBS.
⁶ Usually 30 µl are taken into 200 µl TMB working solution to assay holoHRP activity.
Table 2: The levels of total heme and regulatory heme in IMR90 cells.

The intracellular level of regulatory heme (RH) and total non-covalently bound heme (TH) in human fibroblasts (IMR90) were measured by the RH assay and HPLC, respectively. The data from the controls of the many experiments (at least 13 different experiments) was combined to calculate the averages of total heme and RH in IMR90 cells. The Mann Whitney statistical test was used to analyze the data. (P<0.0001).

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Std. Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total heme</td>
<td>67.35</td>
<td>14.68</td>
</tr>
<tr>
<td>Regulatory heme</td>
<td>4.288</td>
<td>1.522</td>
</tr>
</tbody>
</table>
Legends:

**Figure 1: The time and the dose-dependent reconstitutions of apoHRP with hemin.**
ApoHRP was mixed with hemin in PBS at final concentrations of 5 µM and 2.5 nM, respectively, as described in Table 1. At different time points a sample was removed and the resulting enzymatic activity of holoHRP was measured using the TMB assay kit and presented as absorbance at 652 nm. (A) The time-dependent reconstitution of apoHRP with hemin. The resulting absorbance at 652 nm from holoHRP activity was plotted against the reconstitution time (min) using one-binding site hyperbola (Prism 6.0 software, GraphPad, San Diego, CA, USA). The data are the mean ± sem of four different experiments. (B) The dose-dependent reconstitution of apoHRP with increasing concentrations of hemin. ApoHRP (5µM) was mixed with increasing concentrations of hemin (0-2.5 nM) in PBS as described in Table 1. The resulting activity of holoHRP was measured using the TMB assay kit and the absorbance at 652 nm was plotted against the respective concentration of hemin. Linear regression analysis of the data shows $R^2 = 0.98$ (Prism 6.0 software, GraphPad, San Diego, CA, USA). The data are the mean ± SD of triplicates of the representative experiment.

**Figure 2: The time-dependent reconstitution of apoHRP with specific housekeeping heme-proteins.**
The transfer of the heme moiety of hemoglobin (60 nM), methemoglobin (60 nM), catalase (60 nM), or heme proteins from the lysate (10 µg) to apoHRP was investigated using the RH assay as described in Table 1, except hemin was replaced with the respective heme-protein as described in the Methods section. The resulting enzymatic activity of holoHRP was measured using the TMB assay kit. HoloHRP activity (as absorbance at 652 nm) was plotted against the reconstitution time using one-binding site hyperbola (Prism 6.0 software, GraphPad, San Diego, CA, USA). (A) MetHb (Closed circles, - sodium dithionite), hemoglobin (Hb-O₂, Open circles, + sodium dithionite); (B) Catalase; and (C) heme-proteins from cell-free lysate. Shown the mean ± sem of at least three different experiments for each condition.

**Figure 3: The dependence of the RH assay on apoHRP and protein concentrations.**
(A) Increasing concentrations of apoHRP (0.5 to 6 µM) were incubated with 10 µg protein from the cell-free lysate for 10 min. The resulting holoHRP activity at 652 nM was converted to RH
content using standard curve similar to the one described in figure 1B. The data was plotted against apoHRP concentration (Prism 6.0 software, GraphPad, San Diego, CA, USA). (B)

Different protein concentrations (5-60 µg) from lysate were mixed with 5 µM apoHRP for 10 min. The resulting holoHRP activity was converted to RH concentration using a standard curve similar to the one presented in Figure 1B and as described in Materials and Methods. The calculated RH/mg protein was then plotted against the protein concentration using linear regression (Prism 6.0 software, GraphPad, San Diego, CA, USA). Shown the mean ± sem of four different experiments for A and B.

Figure 4: The effect of inhibiting heme synthesis or adding hemin to the growth medium on intracellular RH.

The time-dependent effect (0.5 to 72 hr) of inhibiting heme synthesis with 8 µM N-methyl protoporphyrin IX (NMP) on RH and total heme was determined in the cellular lysate using HPLC as described in Table 1 and the Methods. (A) Shows the linear regression analysis of the effect of NMP on total heme. (B) Shows the plot of the effect of NMP on RH using best-fit one-phase exponential decay. The data are the mean ± sem of three independent experiments. *P<0.01, **P<0.001, ***p<0.0001, One-way ANOVA test using Dunnett's multiple comparisons. (C) The dose-dependent effect of adding hemin (0.5 to 6 µM) to the growth medium of IMR90 cell culture for 45 min. After washing hemin, the cells were harvested, the lysate was prepared, and the RH content was measured as described Materials and Methods. **P<0.001, One-way ANOVA test using Dunnett's multiple comparisons. Shown is the mean ± sem of three independent experiments.

Figure 5: The effect of lead and mercury on regulatory and total heme.

IMR90 cell cultures were incubated for 24 hr with increasing concentrations of Pb (250 to 1000nM) or Hg (0.5 to 5000nM). The cells were harvested and the lysates were prepared from each condition. Total heme from lead-treated cells (A) or mercury-treated cells (C) were measured by HPLC as described in Materials and Methods. RH was measured using the lysates by the RH assay in lead (B) or mercury (D) treated cells. *P< 0.01, **P<0.001, ***p<0.0001, One-way ANOVA using Dunnett's multiple comparisons. Data are mean±sem of at least six independent experiments.
Figure 6: The effect of Amylod-β (Aβ peptide on regulatory and total heme.
IMR90 cell cultures were incubated with 500 nM Aβ40. At different time points (0.5 to 24 hr) the cells were then harvested, lysates prepared, and RH and total heme were measured by the RH assay and HPLC, respectively. (A) Nonlinear regression analysis of the time-dependent effect of Aβ on RH (one-phase exponential decay) (Prism 6.0 software, GraphPad, San Diego, CA, USA). *p<0.01, **p<0.001, One-way ANOVA using Dunn's multiple comparisons. The data are the mean ± sem of four independent experiments. (B) The time-dependent effect of Aβ on total heme. No statistical significance was observed. Data was plotted using linear regression (R²=0.02). The data are the mean ± sem of at least three independent experiments.
References:


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33. (a) A. Khan, J. G. Quigley, Heme and FLVCR-related...


Figure 1A

![Graph showing reconstitution time vs Reconstituted Holo-HRP (Abs 652 nm)]
Figure 1B

![Graph showing the relationship between Hemin, fmols and Reconstituted Holo-HRP (Abs 652 nm)](image)

\[ Y = 0.043 \times X + 0.08 \quad R^2 = 0.96 \]
Figure 2B

![Graph showing reconstitution time (min) vs. reconstituted Holo-HRP (Abs 652 nm)]
Figure 2C

Reconstitution time (min)

Cell-free lysate

Regulatory Heme, pmole / mg Protein

0 1 2 3 4 5 6 7 8 9 10
Figure 3A

 apoHRP (µM) vs. Regulatory Heme, pmole / mg Protein
Figure 3B

![Graph](image)

Y = 3.9 * X + 16.4  \( R^2 = 0.95 \)
Figure 4A

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Figure 4B

Regulatory Heme pmole/mg of Protein

Time (hr)
Figure 4C

![Graph showing the relationship between hemin concentration (µM) and regulatory heme content (pmole/mg Protein). The graph demonstrates a positive correlation with a peak at around 2 µM hemin.](image)

**Note:** Significant increases are indicated by **.
Figure 5A

The effect of lead

Total Heme, pmoles/mg protein

Cont. 250 500 750 1000 5000 Pb (nM)
Figure 5B

The effect of lead

Pb, (nM)

Regulatory Heme pmole/mg of Protein

Cont. 250 500 750 1000 5000

*  **  ***
Figure 5C

The effect of mercury

Total Heme, pmoles/mg protein

Cont. 0.5 50 500 5000

Hg, (nM)
Figure 5D

The effect of mercury

Regulatory Heme
pmole/mg of Protein

Cont.       0.5           50          500        5000
Hg, (nM)

*   
**  

Metallomics Accepted Manuscript
Figure 6A

The effect of Amyloid-β

Regulatory Heme, pmole/mg protein

Time (hr)
Figure 6B

The effect of Amyloid-$\beta$

Time (hr)

Total Heme, pmole/mg protein