Heme dynamics and trafficking factors revealed by genetically encoded fluorescent heme sensors

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Heme is a critical cofactor and signaling molecule. Heme acquisition by proteins and heme signaling are ultimately reliant on the ability to mobilize labile heme (LH). However, the properties of LH pools, including concentration, oxidation state, distribution, speciation, and dynamics, are poorly understood. Herein, we elucidate the nature and dynamics of LH using genetically encoded ratiometric fluorescent heme sensors in the unicellular eukaryote Saccharomyces cerevisiae. We find that the subcellular distribution of LH is heterogeneous; the cytosol maintains LH at ∼20-40 nM, whereas the mitochondria and nucleus maintain it at concentrations below 2.5 nM. Further, we find that the signaling molecule nitric oxide (NO) can initiate the rapid mobilization of heme in the cytosol and nucleus from certain thiol-containing factors. By integrating our heme sensors with genetic screens, we also find that the glycolytic enzyme glyceraldehyde phosphate dehydrogenase (GAPDH) is responsible for buffering intracellular heme and regulating the activity of the nuclear heme-dependent transcription factor heme activator protein (Hap1p). Altogether, these results reveal fundamental aspects of heme trafficking and dynamics, providing fresh insight into the cellular management of this essential nutrient.

Results

Design and Characterization of Heme Sensors. The first-generation heme sensor, HS1, consists of a heme-binding domain, the His/Met coordinating 4-alpha-helical bundle hemoprotein cytochrome b562 (Cyt b562) (13), fused to a pair of fluorescent proteins, EGFP and Katushka 2 (mKATE2), that are expected to exhibit heme-sensitive and -insensitive fluorescence, respectively (Fig. 1A). Holo-Cyt b562 is a fluorescence resonance energy transfer (FRET) acceptor for EGFP (14, 15) [excitation (ex.) = 488 nm, emission (em.) = 510 nm] but not mKATE2 (16) (ex. = 585 nm, em. = 620 nm) (SI Appendix, Fig. S1). Thus, HS1 was designed as an excitation-emission ratiometric probe (17) in which the ratio of heme-sensitive EGFP fluorescence to heme-insensitive mKATE2 fluorescence

Significance

All heme-dependent functions require the mobilization of labile heme (LH), of which there is little understanding of its nature and dynamics. To probe LH pools, we developed genetically encoded fluorescent heme sensors and deployed them in the unicellular eukaryote Saccharomyces cerevisiae. We find that LH is relatively abundant in the cytosol, but exceedingly low in the mitochondria and nucleus. Further, we find that LH can be mobilized by signaling molecules like nitric oxide. We also find that the glycolytic enzyme glyceraldehyde phosphate dehydrogenase constitutes a major heme buffer and is responsible for regulating the activity of a heme-dependent transcription factor. Altogether, our work will have profound implications for understanding the mechanisms of heme utilization.
provides a readout of cellular heme independent of sensor concentration. HS1 was adapted from a previously reported EGFP-Cyt_562 integral fusion protein, CG6, that exhibits >99% efficient FRET between EGFP and heme (18). mKate2 was appended to the N terminus of CG6 with a GlySer linker. Titration of ferric and ferrous heme into an aqueous buffered solution [20 mM NaP, 100 mM NaCl (pH 8.0)] of 0.5 μM purified HS1 resulted in visible absorbance spectra indicative of heme coordination (SI Appendix, Fig. S2), and quenched EGFP fluorescence (~10-fold decrease in signal), with minimal perturbation to mKate2 fluorescence (~20% decrease in signal) (Fig. 1B–D). Both oxidized and reduced heme quench the fluorescence of EGFP and mKate2 to similar degrees and the change in fluorescence as a function of [heme] evinces a 1:1 heme/HS1 stoichiometry (Fig. 1D and F).

HS1-ferric heme dissociation constants, $K_{d}^{II}$, were determined by direct titration of heme chloride into HS1 over a broad pH range (pH 5–8), and found to be 3 nM between pH 6.0 and 7.5 (SI Appendix, Fig. S3A–C). At pH 8.0, the 10 nM $K_{d}^{III}$ value for HS1 is identical to the $K_{d}^{III}$ values previously reported for Cyt_562 (13) and CG6 (18). HS1-ferrous heme dissociation constants, $K_{d}^{II}$ values, were too tight to measure by direct titration and are less than 1 nM.

Because previous estimates for the concentration of the LH pool are highly varied between nanomolar and micromolar values (12), we generated heme sensors with altered heme binding affinities. One variant, HS1-M7A, which was generated by replacing the heme axial Met$_{1}$ ligand of Cyt_562 with Ala (SI Appendix, Fig. S2), exhibits fluorescence properties similar to the fluorescence properties of HS1 and binds heme in a 1:1 stoichiometry (Fig. 1E and F). HS1-M7A binds ferrous heme with a $K_{d}^{II}$ value of 25 nM between pH 6.0 and 7.5 (SI Appendix, Fig. S3D–F). The HS1-M7A $K_{d}^{II}$ values are very weak, determined to be 0.5–2 μM over a pH range of 5.0–8.0 (SI Appendix, Fig. S3F). The $K_{d}^{II}$ value of HS1-M7A is similar in magnitude to previous estimates of the “regulatory” heme pool, 10–100 nM (12, 19), as well as the affinities of proteins that may respond to this pool, including the heme-dependent transcription factor Rev-erbβ (20) and constitutive heme oxygenase-2 (HO-2) (21) (SI Appendix, Fig. S3G).

Both HS1 and HS1-M7A are selective for ferrous heme over other metals, protoporphyrin IX, and the heme degradation products bilirubin and biliverdin (SI Appendix, Fig. S4 A and B). Further, apo- and ferrous heme-bound HS1 and HS1-M7A exhibit pH-independent EGFP/mKate2 fluorescence ratios between pH 6.5 and 9.0, but are markedly pH-dependent below pH 6.5 (SI Appendix, Fig. S4 C and D). The fluorescence ratio of HS1 and HS1-M7A is independent of protein concentration between 10 nM and 1 μM, suggesting that the sensors do not aggregate over these concentrations (SI Appendix, Fig. S4E). Ferrous heme binding to HS1 and HS1-M7A is reversible because competition with excess apo-Cyt_562 restores the fluorescence ratio to apo-sensor (SI Appendix, Fig. S5).

**Cellular Heme Imaging: Cytosol.** Using fluorimetry (Fig. 2A), flow cytometry (Fig. 2B), and fluorescence microscopy (Fig. 2C), we find that HS1 and HS1-M7A can be used to sense endogenous LH in a quantitative manner. Fluorimetry (Fig. 2A) and fluorescence microscopy (Fig. 2C) of WT yeast cells expressing cytosolic HS1 and HS1-M7A indicate an EGFP/mKate2 fluorescence ratio in between pH 6.5 and 9.0, but are markedly pH-dependent below pH 6.5 (SI Appendix, Fig. S4 C and D). The fluorescence ratio of HS1 and HS1-M7A fluorescence ratios in WT cells reflects their differential heme binding affinities (vida infra).

The HS1 and HS1-M7A EGFP/mKate2 fluorescence ratios in hem_1Δ cells decrease when heme synthesis is initiated due to supplementation with 5-ALA (Fig. 2A and C) in a dose-dependent manner (SI Appendix, Fig. S6 C and D), which is consistent with increased heme binding. Excess 5-ALA does not reduce HS1-M7A fluorescence ratios to the same extent as HS1. This effect is because excess 5-ALA (1.5 mM) results in intracellular heme levels similar to WT cells (SI Appendix, Fig. S6E); strict control of heme biosynthesis does not allow for endogenous heme levels that are sufficient to saturate the low-affinity heme sensor HS1-M7A. Conversely, the HS1 and HS1-M7A EGFP/mKate2 fluorescence ratios in WT cells increase upon supplementation with the heme biosynthesis inhibitor succinylacetone (SA) (Fig. 2A–C) in a dose-dependent manner (SI Appendix, Fig. S6 A and B), which is consistent with decreased heme binding (2). A variant of HS1 with His$_{562}$ and Met$_{1}$ heme-coordinating ligands mutated to Ala, HS1-M7A,H102A, and an mKate2-EGFP fusion protein lacking the Cyt_562 domain do not exhibit heme-dependent changes in fluorescence ratio, indicating that heme iron coordination to HS1 is required for cellular heme sensing (SI Appendix, Fig. S7).

The heme sensor does not itself perturb heme metabolism and is a reliable reporter for endogenous LH. Titration of
HS1-M7A expression using weak (pr<sub>ADH1</sub>), medium (pr<sub>TEF1</sub>), and strong (pr<sup>GPD</sup>) promoters does not result in a change in the observed concentration of the bioavailable heme pool, and pr<sup>GPD</sup>-HS1-M7A does not affect cell growth or heme-regulated functions like total heme, catalase activity, or respiration (SI Appendix, Fig. S8 A-F).

For quantitative heme monitoring, an in situ method to calibrate the sensor was developed (19). The concentration of [heme] accessible to the sensor is governed by the following expression (23):

\[
[heme] = K_d \times \frac{R_{\text{expt}} - R_{\text{min}}}{R_{\text{max}} - R_{\text{min}}} \left( \frac{F_{\text{mKATE2}}}{F_{\text{mKATE2}}^{\text{max}}} \right),
\]

where \(K_d\) is the heme-sensor dissociation constant, \(R_{\text{expt}}\) is the EGFP/mKATE2 fluorescence ratio under any given condition, \(R_{\text{min}}\) is the EGFP/mKATE2 fluorescence ratio when 0% of the sensor is bound to heme, \(R_{\text{max}}\) is the EGFP/mKATE2 fluorescence ratio when 100% of the sensor is bound to heme, \(F_{\text{mKATE2}}\) is the mKATE2 emission intensity when 0% of the sensor is bound to heme, and \(F_{\text{mKATE2}}^{\text{max}}\) is the mKATE2 emission intensity when 100% of the sensor is bound to heme. Determination of \(R_{\text{max}}\) and \(F_{\text{mKATE2}}^{\text{max}}\) involves recording EGFP and mKATE2 fluorescence after digitonin permeabilization of cells and incubation with 50 \(\mu\)M heme. Determination of \(R_{\text{min}}\) and \(F_{\text{mKATE2}}\) involves recording EGFP and mKATE2 fluorescence after cells are treated with SA or from hema1Δ cells cultured in parallel. Because the ferrous heme binding affinities are tighter than ferric affinities for both HS1 and HS1-M7A, \(R_{\text{max}}\) and \(F_{\text{mKATE2}}^{\text{max}}\) are determined in the presence of the reducing agent ascorbate (1 mM) to drive heme saturation of the sensors.

Using fluorimetry, in situ calibration of HS1 in WT cells indicates that it is >95% saturated (Fig. 2A and SI Appendix, Fig. S9), which is not ideal for imaging. On the other hand, HS1-M7A is typically 20-50% bound in exponential-phase WT cells and well poised to monitor changes in heme availability (Fig. 2A and SI Appendix, Fig. S9). Estimation of the concentration of LH using Eq. 1 depends on assumptions made about its oxidation state. In the two limiting cases of LH being 100% reduced or oxidized, cytosolic LH can be estimated to be buffered between ~30 nM and ~1 \(\mu\)M, respectively, after considering the data depicted in Fig. 2A and the \(K^{\text{II}}_d\) or \(K^{\text{III}}_d\) value of 25 nM or 1 \(\mu\)M at pH 7.0 (SI Appendix, Fig. S3), which is the pH of the yeast cytosol (24). Given the relatively reducing cellular environment, \(E_{\text{cyt}}\) ~ 320 mV vs. normal hydrogen electrode (NHE) (25), which is governed by the ratio of oxidized-to-reduced glutathione, and the reduction potential of aqueous heme, estimated to be between ~50 mV and ~220 mV vs. NHE (26, 27), we propose that LH is biased toward the reduced state and assume herein that LH is 100% reduced. However, the actual fraction of LH that is reduced is dependent on its speciation and the degree to which it equilibrates with the glutathione redox buffer, both of which are unknown. SI Appendix, Fig. S10 outlines estimates of [LH] for a sensor that is between 20% and 50% bound because the assumption in the oxidation state deviates from being 100% reduced. Given the weak ferric heme affinities of HS1-M7A, if [LH] < 100 nM, HS1-M7A cannot sense oxidized LH (SI Appendix, Fig. S10 A-F).

Flow cytometry of log-phase WT cells expressing HS1-M7A reveals that there are three populations of cells with distinct concentrations of cytosolic LH: 40 nM, 20 nM, and <1 nM heme (Fig. 2B). These LH concentrations correspond to 1,200, 600, and <50 molecules of heme, respectively, assuming a cytosolic volume (\(V_{\text{cyt}}\)) of 50 fl (28). By comparison, total cellular heme was determined to be ~1 \(\mu\)M (SI Appendix, Fig. S8D). Although this trimodal distribution is highly reproducible, the relative abundance of cells with these three distinct heme levels is quite variable between experiments (SI Appendix, Fig. S11). This observation suggests that heme availability may be dynamically regulated. In contrast, HS1 exhibits only one population due to complete heme saturation as a result of its high heme binding affinity (Fig. 2B and SI Appendix, Fig. S12).

**Cellular Heme Imaging:** Nucleus and Mitochondria. To probe the subcellular distribution of heme, we targeted HS1 and HS1-M7A to the mitochondria and nucleus by appending N-terminal COX4 mitochondrial matrix or C-terminal SV40 nuclear localization sequences, respectively (25, 29) (Fig. 3A). The localization tags do not affect heme binding to the sensors (SI Appendix, Fig. S13). Fluorimetry (Fig. 3B) and fluorescence microscopy (Fig. 3C) indicate that nuclear and mitochondrial HS1 exhibit EGFP/mKATE2 fluorescence ratios in WT cells that are distinctly lower than in hema1Δ cells, indicative of heme binding. In contrast, nuclear and mitochondrial HS1-M7A EGFP/mKATE2 fluorescence ratios
are unaltered between hem1Δ and WT cells (Fig. 3 B and C). To
determine the fractional saturation of our sensors in these compart-
ments, we developed an in situ sensor calibration method analogous
to the one described for the cytosol. However, because digitonin
cannot permeabilize nuclear or mitochondrial membranes (19), we
first had to enzymatically digest the yeast cell wall with zymolyase
and then incubate the resulting spheroplasts with 0.1% Triton X-100,
50 μM heme, and 1 mM ascorbate to heme-saturate the sensors. As
shown in Fig. 3B, this in situ calibration method resulted in limiting
Rmax values for HS1 and HS1-M7A that are identical within
experimental error.

In total, the data indicate that HS1 is ~100% heme-saturated in
the mitochondria and nucleus, whereas HS1-M7A is ~0% heme-
saturated in these organelles. Given the HS1-M7A Kd of 25 nM,
we estimate that heme is buffered at an upper limit of 2.5 nM in
the mitochondria and nucleus. This concentration corresponds
to fewer than six molecules in the nucleus [Vmax ~ 5 fl (30)] and ~0.6
molecules in the mitochondria [Vmax ~ 0.5 fl (28, 31)].

NO Mobilizes Cytosolic and Nuclear Heme. Previous work demonstrated
that NO could regulate heme transfer between certain protein pairs
(32). Could NO also mobilize LH pools? Using cytosolic, nuclear,
and mitochondrial targeted HS1-M7A, we found that NO derived from
the small molecule NOC-7 (33) rapidly increases cytosolic (Fig. 4A)
and nuclear (Fig. 4B) LH in WT cells, but not in the mitochondria (Fig. 4C)
or in heme-deficient hem1Δ cells (SI Appendix, Fig. S14A). In the cytosol, LH increases from 17 nM to
40 nM within 20 min of adding 25 nM NOC-7 (t1/2 = 10 min at
22 °C), which corresponds to an increase from 500 to 1,200 mol-
cules of heme, assuming Vct ~ 50 fl (28). This rapid increase is
followed by the reestablishment of the initial steady-state heme
levels over the course of ~80 min. In the nucleus, there is an even
more pronounced change in the HS1-M7A EGFP/mKATE2 ratio
upon NOC-7 treatment. NO results in an increase in nuclear LH
from <2.5 nM to a maximum of 218 nM, corresponding to a change
from fewer than six to ~650 heme molecules assuming Vmax ~ 5 fl
(30). NO-mediated heme mobilization is dose-dependent (SI Ap-
pendix, Fig. S14B), and the observed changes in fluorescence ratios
are not due to NO interactions within HS1-M7A because in-
cubation of purified heme-bound HS1-M7A with NOC-7 does not
alter sensor fluorescence (SI Appendix, Fig. S15).

Given that cellular thiols are targets of NO, we sought to determine
the effect of a thiol-specific alkylating agent [e.g., iodoacetamide
(IAM)] on heme mobilization. Although IAM does not initiate
the mobilization of heme, it does prevent the reestablishment of
the steady-state LH pool after NO treatment (SI Appendix,
Fig. S14C). This observation suggests that IAM blocks the
rebinding of heme from sites of mobilization, presumably certain
thiol-containing factors.

To differentiate the effects of NO and general oxidative stress on
heme mobilization, we probed the role of various oxidative insults.
Diamide, a thiol-specific oxidant; paraquat, a superoxide-generating
agent; and H2O2 had no impact on LH pools, suggesting that heme
mobilization is specific to NO (SI Appendix, Fig. S14D).

GAPDH Regulates Cytosolic Heme Availability and the Activity of the
Nuclear Heme-Dependent Transcription Factor Hap1p. To identify
genes that regulate LH, we transformed cytosolic HS1-M7A into
the yeast knockout collection and screened for mutants that exhibited
altered sensor fluorescence ratios. After partial screening, we
identified TDH3, which encodes an isof orm of the glycolytic enzyme
GAPDH, to be a determinant of LH. The tdh3Δ cells exhibit an
approximately fourfold greater concentration of labile cytosolic heme
(13 nM in WT cells and 57 nM in tdh3Δ cells) (Fig. 5A) without
affecting total heme levels (SI Appendix, Fig. S16B). Deletion of
TDH3 homologs TDH1 and TDH2 does not affect LH pools (Fig.
5A), likely because these isoforms contribute to a small fraction
of total GAPDH (SI Appendix, Fig. S16A). Episomal expression
of TDH3 in tdh3Δ cells restores LH to WT levels (SI Appendix,
Fig. S16 D and E).

To determine the role of Tdh3p on other heme-dependent
functions, we measured the activity of cytosolic heme catalase, Ctt1p,
and the activity of the heme-dependent transcription factor, Hap1p.
The tdh3Δ cells have normal Ctt1p activity as determined by a

![Diagram showing nuclear and mitochondrial heme monitoring with HS1 and HS1-M7A.](Image)

Fig. 3. Nuclear and mitochondrial heme monitoring with HS1 and HS1-M7A. (A) To label DNA, hem1Δ cells expressing nuclear or mitochondrial targeted HS1-
M7A were stained with DAPI. Live cells were imaged as described in Materials and
Methods at a magnification of 63×. “Merge” is the merged images of DAPI, EGFP,
and mKATE2. (B) Heme-dependent EGFP/mKATE2 fluorescence ratios of nuclear
and mitochondrial targeted HS1 and HS1-M7A in WT and hem1Δ yeast cells as
measured by fluorimetry. “Heme-saturated” is the ratio recorded upon Triton
X-100 permeabilization of yeast spheroplasts and incubation with excess heme as
described in the main text for in situ calibration for the mitochondrial and nu-
clear sensors. (C) Confocal microscopy of WT and hem1Δ cells expressing nuclear
and mitochondrial targeted HS1 and HS1-M7A. Fluorimetry data represent the
mean ± SD of triplicate mid-log-phase cultures grown in SC-EU. Microscopy
images are representative of at least two independent cultures. DIC, differential
interference contrast.
native in-gel colorimetric assay for catalase (34) (SI Appendix, Fig. S16C). On the other hand, tdh3Δ cells have a defect in Hap1p activity as measured using a transcriptional reporter that employs EGFP driven by the CYC1 promoter, a Hap1p target gene (9) (Fig. 5B). In total, our data indicate GAPDH is involved in buffering cytosolic heme and regulates the activity of Hap1p, presumably by acting as a source for heme.

Validation of Sensors in Escherichia coli and HEK293 Cells. To broaden the utility of HSI, we tested its applicability in other cell lines, including E. coli and human embryonic kidney HEK293 cells (SI Appendix, Fig. S17). In HEK293 cells, flow cytometry and fluorescence microscopy demonstrate that HSI and HSI-M7A exhibit EGFP/mKATE2 fluorescence ratios that are distinctly lower when cells are cultured in heme-replete media relative to heme-deficient (HD) media containing SA (HD + SA) (SI Appendix, Fig. S17A–E). Similarly, heme sensor fluorescence ratios in E. coli exhibit heme-dependent changes. A heme-deficient hemA strain lacking glutamyl-tRNA reductase, which catalyzes the first step in porphyrin synthesis, is a 5-ALA auxotroph. The ΔhemA strain showed a higher EGFP/mKATE2 fluorescence ratio after growth shift to media lacking 5-ALA (SI Appendix, Fig. S17F). In total, HSI can be used to probe heme physiology across multiple model organisms and cell lines.

Discussion

The mobilization of LH underlies all heme-dependent processes, including heme acquisition by client proteins and heme-based signal transduction. However, the properties of the LH pool are poorly understood. To address this fundamental gap in heme cell biology, we developed genetically encoded ratiometric heme sensors and deployed them in Baker’s yeast to probe key characteristics of the LH pool, including its concentration, subcellular distribution, buffering factors, and dynamics. The cytosol has more LH (~20–40 nM) than the nucleus or mitochondria (~2.5 nM). Further, the cell-to-cell heterogeneity in cytosolic heme suggests its availability may be dynamically regulated (Fig. 2B). Given the subcellular distribution of heme, we propose that once heme is biosynthesized in the mitochondrial matrix, a fraction of heme transits to the cytosol and acts as a reservoir for signaling and/or a heme source for proteins.

The mitochondria, which have a very high demand for heme and are the site of heme biosynthesis, have exceptionally low quantities of LH, less than 2.5 nM or fewer than one molecule. By comparison, total ferrous heme in the yeast mitochondria has been estimated to be ~30 μM, or ~9,000 molecules (28). Taken together, this low amount of mitochondrial LH suggests that mitochondrial heme is tightly regulated and trafficked in a manner that limits its availability. This observation is consistent with the identification of mitochondrial heme metabolism complexes that traffic heme via transient protein–protein interactions, thereby circumventing the LH pool (35).

The nucleus also limits LH to less than 2.5 nM or fewer than six molecules. This low amount of nuclear LH is not surprising, given the cytotoxicity of heme and proximity to genetic material (5, 12). However, this observation raises the question of how nuclear heme-regulated transcription factors acquire heme, given that the heme-regulatory motifs (HRMs) of many heme-dependent transcription factors, including Hap1p in yeast, exhibit micromolar affinities for heme (11). Factors like Hap1p may acquire heme from transient increases in LH due to active signaling processes. Indeed, this concept is supported by our demonstration that signaling molecules like NO can result in a >10-fold increase in nuclear LH (Fig. 4B). An alternative possibility is that the heme affinities of many HRM-containing proteins are mischaracterized. For instance, a reevaluation of the heme dissociation constants of the HRM-containing transcription factor Rev-erbβ found that heme binds 100-fold tighter than previously estimated, with $K_d^{III}$ and $K_d^D$ of ~20 nM vs. 2–6 μM (20).

Our observation that subcellular [LH] is heterogeneous stands in striking contrast to a recent report by He and coworkers (19) that used a heme chaperone-based FRET sensor (CISDY-9) for heme in human cell lines. In that work, cytosolic, mitochondrial, and nuclear heme were all reported to be ~25 nM. The deviation between our two results needs to be clarified but could reflect organismal variation, difficulty in calibrating CISDY-9 in organisms, or uncertainties in LH oxidation state.

We demonstrate, for the first time to our knowledge, that LH is dynamic and can be mobilized by signaling molecules like NO (Fig. 4 and SI Appendix, Fig. S14). Although previous studies have indicated that H$_2$O$_2$ and NO can regulate heme transfer between specific protein pairs (32, 36), our results suggest that NO can mobilize cell-wide LH pools to regulate heme-dependent processes in multiple compartments. The mechanism of NO-mediated heme mobilization is unknown, but likely involves S-nitrosation of certain hemeoproteins and heme dissociation (32, 37).

NO-mediated heme mobilization provides insights into NO physiology, including NO-dependent inflammatory responses. Both heme and NO regulate inflammation, but through distinct mechanisms (8, 38, 39). Our work indicates that NO can promote inflammation via heme mobilization. Indeed, previous observations in endothelial cells found that NO elevates cellular iron due to enhanced activity of the heme-degrading enzyme, HO (40). This observation was proposed to occur as a result of NO-induced heme release from hemeoproteins. Our current studies are the first, to our knowledge, to provide direct support for this model.

Fig. 4. NO-dependent mobilization of LH. WT cells expressing cytosolic (A), nuclear (B), or mitochondrial (C) HSI-M7A were incubated with 50 μM NOC-7, and EGFP/mKATE2 fluorescence ratios were monitored by fluorometry. The data represent the mean ± SD of triPLICATE mid-log-phase cultures grown in SCE-LEU. The red and black lines indicate the fluorescence ratios of HSI-M7A when it is 0% and 100% saturated with heme as derived from hemAΔ cells expressing HSI-M7A or WT cells expressing HSI, respectively. This approach for determining $R_{max}$ is validated by the fact that the EGFP/mKATE2 fluorescence ratio of HSI-M7A that is saturated with heme from the in situ calibration methods is identical within experimental error to HSI (Fig. 3B and SI Appendix, Fig. S9).

Fig. 5. Tdh3p regulates intracellular heme availability and Hap1p activity. (A) Fluorimetric determination of EGFP/mKATE2 fluorescence ratios of the indicated yeast strains expressing HSI-M7A. $R_{max}$ and $R_{min}$ were determined to be 6.1 (from the hemAΔ strain) and 0.2 (from in situ calibration with digitonin), respectively. (B) Hap1p activity in the indicated strains as measured by a transcriptional reporter that used EGFP driven by the CYC1 promoter, a Hap1p target gene. All data represent the mean ± SD of triPLICATE mid-log-phase yeast extract-peptone-dextrose media supplemented with ergosterol and tween-80 (YPDE) cultures.

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Our findings that GAPDH and NO regulate heme availability and mobilization are highly reminiscent of and supported by previous studies demonstrating that GAPDH and NO cooperate to control heme insertion into nitric oxide synthase (32). As such, it is tempting to speculate that Tdh3p is the source of NO-mobilized heme. However, NO-dependent heme mobilization is unaffected in tdh3Δ cells (SI Appendix, Fig. S18).

Altogether, the HS1s can be applied across prokaryotes and eukaryotes to probe heme trafficking, signaling, and dynamics in various physiological contexts.

Materials and Methods

All cell lines, culture conditions, reagents, and methods are described in SI Appendix, SI Materials and Methods. Yeast strains used in this study were in the BY4741 genetic background. The ΔhemA E. coli strains were in the MG1655 background. HEK293 cells were obtained from A. Oyelere (Georgia Institute of Technology). The genes for mKATE2 and C6G used to construct HS1 were purchased from GENESCRIPT and codon-optimized for expression in E. coli and S. cerevisiae or humans. Standard published methods for protein expression and purification (18), heme binding studies (20, 27), fluorimetry, UV/visible spectroscopy, microscopy, flow cytometry, immunoblotting (41), catalase activity (34), oxygen consumption (41), total heme analysis (42), and cell growth are outlined in SI Appendix, SI Materials and Methods.

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