COMMENTARY

Unraveling the mystery of the ring: Tracking heme dynamics in living cells

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Heme is an essential protein cofactor used by nearly all forms of life to perform a wide range of tasks, from shuttling electrons to keep photosynthesis running to moving the oxygen we breathe from our lungs throughout our bodies (1). Most of the heme in humans is produced in erythroid cells, which can contain up to 1 billion heme molecules per cell (2). Because free heme that is not bound to proteins is toxic, many students are taught that all heme in erythroid or other cells is bound or being degraded. At best, this picture is incomplete, and, at worst, it obscures fundamental biological questions. One might wonder how bound heme is first loaded into proteins in the various organelles of cells. In addition, new data suggest that unbound or labile heme may be an important signaling molecule (3, 4). Although there have long been tools to study heme structure and synthesis in vitro, as well as tools to study the total heme content in populations of cells, there are few tools to study the dynamics of labile heme in individual, live cells (5). In recent work in PNAS, Hanna et al. (6) develop a new genetically encoded fluorescent sensor for heme, HS1, and uncover the dynamic regulation of resting labile heme, document the mobilization of labile heme in response to NO, and identify glyceraldehyde phosphate dehydrogenase (GAPDH) as a heme buffer in yeast.

Previous Methods of Detecting Heme in Cells

Traditionally, heme has been studied in cells by homogenizing tissue, acidifying and heating the isolated proteins to release bound heme, and then measuring the fluorescence of the protoporphyrin ring to quantify the concentration of heme from the cells spectroscopically. Such experiments have been useful in identifying changes in heme status by comparing healthy and diseased cells (7). However, there are several limitations to this method. Determination of total heme in an organelle might be possible if the organelle of interest can be isolated from the rest of the cell, but some organelles, particularly the secretory pathway, cannot be physically isolated and studied. Because a population of cells is combined into a bulk measurement, any information about the heterogeneity of heme levels across cells, as well as temporal information about changes in heme concentration, is lost. Finally, in these experiments, there is no way to differentiate between labile and bound heme (6). To gain greater insight into the details of heme biology, it is necessary to detect heme in live cells in a noninvasive manner. The development of fluorescence-based sensors for various analytes, coupled with microscopy imaging, provides a means to witness the fate of the analyte of interest in real time and on a cell-by-cell basis (8).

Takeda et al. (9) developed the first fluorescent, protein-based heme sensor. This sensor consisted of green fluorescent protein (GFP) fused to cytochrome b_{562}, providing a heme-binding site tethered closely to GFP. Because the absorption spectrum of heme overlaps with the emission spectra of GFP, increasing the concentration of heme-bound fusion led to quenched GFP emission. This sensor would be difficult to use in live imaging for two reasons. First, it is a turn-off sensor, becoming dimmer with increasing analyte concentration, making it challenging to define a maximum saturation with high precision. Second, it is easier to quantify analyte concentration if a sensor signal is ratiometric, or includes an internal standard that allows for correction of sensor concentration, sample thickness, and small sample movement. The next generation of sensors was developed by Song et al. (10). This sensor is a fusion of CFP, the bacterial heme capture proteins, iron-regulated surface determinant (Isd) proteins, and YFP [heme chaperone-based Förster resonance energy transfer (FRET) sensor (CISDY) sensors]. When heme binds CISDY at the Isd domain, there is conformational change of the sensor that increases the FRET between CFP and YFP. These ratiometric sensors were applied to the organelles of various cell types with success (10). In engineering HS1, Hanna et al. (6) combine design elements of both of the previous sensors to create a new family of sensors for the detection of heme in cells. HS1 is a fusion of GFP; cytochrome b_{562}; and a red fluorescent protein, Katushka (mKate), that is not quenched by heme. Because the emission of mKate is insensitive to heme binding at the

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cytochrome b_{562} domain, the red emission signal of the sensor becomes an internal standard that can be used to normalize the altered emission of GFP upon heme binding (6).

Specific Challenges in Building a Heme Sensor

One of the challenges in designing a sensor for heme compared with other metals is that the sensor must contact and distinguish both the protoporphyrin ring and the metal center. HS1 was selective for heme over uncoordinated metals, free protoporphyrin IX, and the cellular heme degradation products bilirubin and biliverdin. Although selectivity is essential for a useful sensor, it is also necessary for sensors to detect small changes in analyte concentration in cells. To gain this sensitivity and accurately quantify the concentration and dynamics of an analyte, sensors must be partially saturated with the analyte in the organelle of interest when the cell is at rest (8). By mutating the axial coordinating amino acid ligand in cytochrome b_{562} from methionine to alanine (HS1-M7A), it was possible to achieve partial saturation of the sensor when the sensor was targeted to the cytosol of yeast cells, while maintaining the selectivity for heme and strength of signal response. Currently, heme sensors bind both ferrous and ferric heme, albeit with different affinities, which complicates cellular measurements. The ability to distinguish the redox state of the labile heme in cells more precisely would be a useful feature for future iterations of these sensors.

Key Advances in Sensor Development and Heme Biology

The application of HS1 and HS1-M7A to yeast provides lessons in both sensor development and heme biology that are worth highlighting. The application of chemical sensors to cells requires careful controls to ensure that the sensors are functioning well and measuring the analyte of interest selectively and specifically, without perturbing the concentration or function of that analyte. Two commonly used methods for validating sensor efficacy are to express analogous sensors of varying affinities to ensure the measured concentration of analyte is not an artifact of sensor affinity and to measure sensor saturation as a function of sensor expression level (11–13). Hanna et al. (6) were able to use the high-affinity and low-affinity HS1 sensors in both WT yeast and a yeast strain in which heme levels were manipulated by perturbing the heme biosynthetic pathway to verify sensor functionality. Moreover, because yeast genetics are well studied, HS1 could be put under the control of different promoters that give rise to high, medium, and low levels of expression. Both the sensor saturation and cell growth were unperturbed by increasing concentrations of sensor, providing evidence that the sensor reports on the concentration of labile heme without altering heme biology significantly.

An advantage of genetically encoded sensors over small-molecule sensors is that it is relatively straightforward to fuse a peptide sequence to genetically encoded sensors to target the sensor to various compartments of cells (14). Hanna et al. (6) use this aspect of their genetically encoded sensor to examine the mobilization of labile heme directly in the nucleus, cytosol, and mitochondria of yeast by NO. They find that NO increases the concentration of labile heme in the cytosol and nucleus, but not the mitochondria, of yeast cells. This direct evidence of NO mobilization of heme will help to provide new insight into the biological role of heme and NO.

In addition to being able to study live cells, light-based techniques allow for the accurate screening of small changes in the analyte of interest across many experimental conditions rapidly (15). Hanna et al. (6) use this principle and leverage the power of yeast genetics to identify a buffer of labile heme in the cytosol. By expressing the HS1-M7A in the cytosol of a knockout collection of yeast and screening for altered sensor saturation in resting cells, the group found that the cytosolic concentration of heme increased in cells that lacked an isoform of GAPDH. The group then asked if this increased concentration of heme in the GAPDH knockout cells alters the mobilization of heme by NO, but find that the mobilization of heme in the knockout cells is unaffected, thereby leaving questions of the mechanism of NO mobilization of heme open for further study. These experiments demonstrate the role the application of fluorescent sensors can play in opening up new avenues of biological study.

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