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The free heme concentration in healthy human erythrocytes

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ABSTRACT

Heme, the prosthetic group of hemoglobin, may be released from its host due to an intrinsic instability of hemoglobin and accumulate in the erythrocytes. Free heme is in the form of hematin (Fe^{3+} protoporphyrin IX OH) and follows several pathways of biochemical toxicity to tissues, cells, and organelles since it catalyzes the production of reactive oxygen species. To determine concentration of soluble free heme in human erythrocytes, we develop a new method. We lyse the red blood cells and isolate free heme from hemoglobin by dialysis. We use the heme to reconstitute horseradish peroxidase (HRP) from an excess of the apoenzyme and determine the HRP reaction rate from the evolution of the emitted luminescence. We find that in a population of five healthy adults the average free heme concentration in the erythrocytes is 21 ± 2 μM, ca. 100× higher than previously determined. Tests suggest that the lower previous value was due to the use of elevated concentrations of NaCl, which drive hematin precipitation and re-association with apoglobin. We show that the found hematin concentration is significantly higher than estimates based on equilibrium release and the known hematin dimerization. The factors that lead to enhanced heme release remain an open question.

1. Introduction

Heme, Fe^{2+}protoporphyrin IX, is the prosthetic group of hemoglobin. Each hemoglobin molecule carries four hemes, one for each of its sub-chains. One or more (less likely) of the hemoglobin hemes may be released to the solution [1,2]. The release occurs predominantly after its autoxidation to methemoglobin, in which the iron is in its Fe^{3+} form [2,3]. Hence, the heme released from hemoglobin is in the form of hematin, i.e., Fe^{3+} protoporphyrin IX liganded to OH^-. Hematin is a substance with proven toxicity, which triggers lyses of human erythrocytes [4–7] and other cell types [8,9]. It catalyzes the generation of toxic lipid peroxidation products by several mechanisms [10]. It generates redox reactive substances such as H_2O_2, superoxide radicals, and the hydroxyl radical, which directly mediates lipid peroxidation [11,12]. The heme released from hemoglobin binds to the red cell membrane causing membrane damage [13] and higher adhesion of sickle red cells to leucocytes and the endothelium [14–16]. Exposure of human B-cell lymphoma to free heme leads to high binding affinity to multiple autoantigens due to the heme's redox potential [17]. In some of these cases, the deleterious effects of free heme are exhibited after its release during hemoglobin catabolism in malaria infected cells [11,12], in others, due to the intrinsic instability of hemoglobin [4–7].

Despite the abundant evidence of the damage inflicted by free heme, we are aware of a single determination of the concentration of free heme in malaria-free erythrocytes [18]. Most of the recent work on heme quantification has focused on the determination of the total heme content, including quantities bound to globins, often in the context of forensics [19]. The methods employed in these studies were mostly based on the spectroscopic response of heme and heme-containing proteins [20]; HPLC [21], electrochemical methods such as capillary electrophoresis [22,23], and mass-spectrometry [19,24] have been used in recent work.

The objective of the investigations reported here is to determine the concentration of the free heme in healthy human erythrocytes. For this, we developed a new sensitive and selective analytical method. We carry out extensive tests to evaluate the bias in this determination that may be introduced by continued hemoglobin dissociation and interactions between heme, apoglobin and the erythrocyte membrane. We discuss the possible reasons why our results exceed the concentrations found previously [18]. We compare the found values of free heme to the predictions of several models that assume equilibrium between free heme and methemoglobin present in the red blood cells.

Abbreviations: HPLC, high performance liquid chromatography; HRP, horse radish peroxidase; Tris, Trisaminomethane, or 2-Amino-2-hydroxymethyl-propane-1,3-diol; SDS, sodium dodecyl sulfate.

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2. Materials and methods

2.1. An enzymatic method with luminescent detection for heme quantification

To determine the concentration of free heme in human erythrocytes, we use donor blood drawn according to institutional and NIH regulations. We lyse the red blood cells and separate the heme from hemoglobin by dialysis. Details about these procedures and the materials and solutions used are provided as Supplementary material.

The separation of free heme from hemoglobin by dialysis has the disadvantage that it lowers the concentration of free heme to below 1 nM. At concentrations below 0.5 μM, hematin and hemin solutions are colorless. The highest absorbance of such solutions, in the Soret band near 400 nm, is below 0.1. Hence, spectroscopic determination of concentrations below this limit is prone to high error [25].

To quantify such low concentrations of hematin, we modified a recent method that relies on the reconstitution of apo-horseradish peroxidase (apo-HRP) in the presence of hemin (Fe^{3+} protoporphyrin IX Cl) or hematin (tests, discussed in the Supplementary Material, revealed that the method yields identical results with these two compounds) [26]. The sequence of reactions that underlie the method is:

$$\text{Apo}-\text{HRP} + \text{Heme} \rightarrow \text{HRP} \tag{1a}$$

$$\text{HRP} + \text{H}_2\text{O}_2 \rightarrow \text{HRP} + 2\text{HO}^{\bullet} \tag{1b}$$

$$\text{HO}^{\bullet} + \text{luminol} \rightarrow \text{Aminophthalate} + \text{hv}. \tag{1c}$$

Reaction (1c) is accompanied by luminescence at 428 nm [27,28]. The luminescence intensity is proportional to the rate of catalytic decomposition of peroxide. This rate, in turn, is proportional to the concentration of reconstituted peroxidase. In excess of apo-HRP, the concentration of the reconstituted enzyme equals that of the initial heme. Hence, the luminescence intensity is proportional to the concentration of free heme. The transformation of the analyte to a catalyst of a reaction with a well-detectable product underlies the high sensitivity of this method. Furthermore, the luminol reaction is an example of very strong chemiluminescence [27,28]. The strong amplification of the signal allows detection and quantification of hematin or hemin levels as low as 50 pM, see tests of sensitivity below.

For experimental statistics, we used 96 well plates and filled each column of eight wells with identical solutions. Six of the columns were filled with solutions of known hemin concentration in the same buffer as the one used in the dialysis of the hemolysate and the other six—with samples of dialysate produced as discussed above. The six known concentrations were distributed in a range that bracketed the unknown dialysate concentration. The volume of standard solution added to each well varied from 0 to 40 μL so that the final heme concentrations was from 0 to 600 pM. For experimental statistics, a tested solution was divided into two, four, or six samples and each sample subdivided into the wells of one eight-well column. We added 40 μL of the tested solution to each well.

After we loaded the heme solutions, we added Tris buffer to each well in volume calculated to bring the total to 50 μL. Then we added 50 μL of apo-HRP stock solution yielding a concentration of 600 pM in each well. We incubated the microplate for 30 min at room temperature, ca. 23 °C, to reconstitute HRP [26]. The rate constant for heme binding to 35 different apoglobins is approximately $k_H = 1 \times 10^8\ M^{-1}s^{-1}$, regardless of the structure or overall affinity of the apoprotein for iron-porphyrin [29]. We assume that this value applies for apo-HRP. Then, the half-time for hematin and hemin binding to apo-HRP $t_{1/2} \approx (k_H C_{\text{apo-HRP}})^{-1} \approx 20$ s. Thus, 30 min is sufficient time for quantitative binding of free heme to apo-HRP.

After incubation, we added Immobilon (10 μL luminol and 10 μL peroxide) to the wells. The final solution volume in each well was 120 μL. We incubated the microplate for 15 min at room temperature, ca. 23 °C, and loaded it into the microplate reader. We monitored the evolution of the intensity of the luminescence emitted from each well over 30 min. For each moment of the reaction, the luminescence intensities were averaged over the eight wells with identical composition. An example of the intensity evolution over 30 min for six standard hemin concentrations is displayed in Fig. 1A.

2.2. Definition of the calibration curve in terms of rate constants

The average luminescence intensity from a tested sample could be compared to that from solutions with known concentration at the same reaction time and the unknown concentration could be determined by interpolation between the two bracketing values [26]. We developed a new method of determination of the heme concentration based on calibration curves in terms of the rate constant for luminesce intensity decay.

The breakdown of peroxide catalyzed by HRP and other peroxidases is well described by the Michaelis–Menten rate law for the catalytic process [30]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \overset{k_2}{\rightarrow} E + P, \tag{2}$$

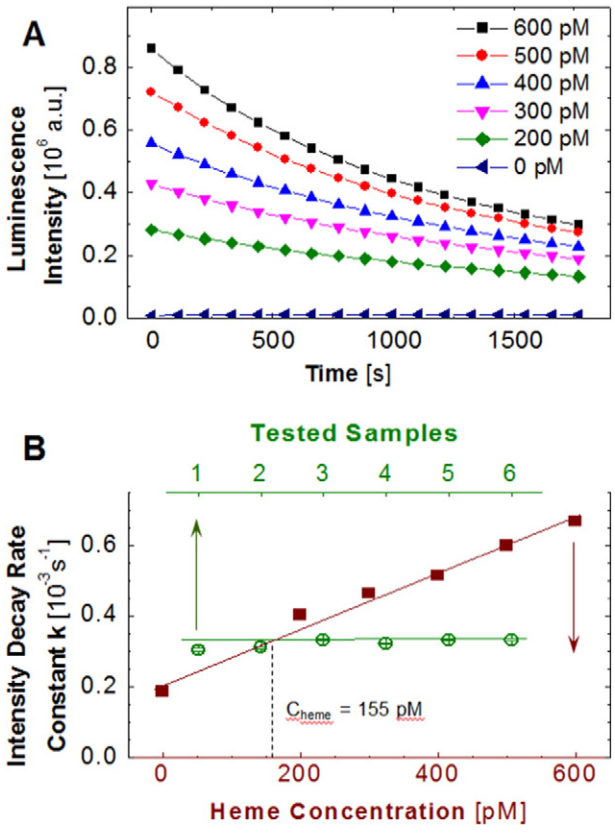

Fig. 1. An enzymatic method for the determination of the concentration of free heme. **A**, The evolution of luminescence intensity emitted by the reactions in Eq. (1a,b,c) at five concentrations of heme and in its absence. **B,** Illustration of the determination of the concentration of free heme in the dialysate. The standard curve, shown in solid symbols, relates known concentration of heme to the rate constant k of decay of the luminescent intensity determined from evolution curves similar to those shown in **A**. The constant k is determined for six samples of dialysate. The unknown concentration of free heme in the dialysate is determined from the average value of k.