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Quantification of Active Apohemoglobin Heme Binding Sites via Dicyanohemin Incorporation

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Abstract

Apohemoglobin (apoHb) is produced by removing heme from hemoglobin (Hb). Unfortunately, preparations of apoHb may contain damaged globins, which render total protein assays inaccurate for active apoHb quantification. Fortunately, apoHb heme-binding sites react with heme via the proximal histidine-F8 (His-F8) residue, which can be monitored spectrophotometrically. The bond between the His-F8 residue of apoHb and heme is vital for maintenance of fully functional and cooperative Hb. Additionally, most apoHb drug delivery applications facilitate hydrophobic drug incorporation inside the apoHb hydrophobic heme-binding pocket in which the His-F8 residue resides. This makes the His-F8 residue a proper target for apoHb activity quantification. In this work, dicyanohemin (DCNh), a stable monomeric porphyrin species, was used as a probe molecule to quantify active apoHb through monocyanoheamin-His-F8 bond formation. ApoHb activity was quantified via the analysis of the 420 nm equilibrium absorbance of DCNh and apoHb mixtures. His-F8 saturation was determined by the presence of an inflection point from a plot of the 420 nm absorbance of a fixed concentration of apoHb against increasing DCNh concentration. Various concentrations of a stock apoHb solution were tested to demonstrate the precision of the assay. The accuracy of the assay was assessed via spectral deconvolution, confirming His-F8 saturation at the inflection point. The effect of the heme-binding protein bovine serum albumin and precipitated apoHb was not significant on assay sensitivity. An analysis of the biophysical properties of reconstituted Hb confirmed heme-binding pocket activity. Taken together, this assay provides a simple and reliable method for determination of apoHb activity.

Graphical Abstract

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Author Contributions

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INTRODUCTION

ApoHemoglobin (apoHb) is a dimeric protein produced via heme extraction from the tetrameric protein hemoglobin (Hb). In Hb, the heme group is rigidly bound to the proximal histidine (F8) in the hydrophobic heme-binding pocket¹. The bond between this site-specific residue, located inside the hydrophobic heme-binding pocket of Hb, is a requirement for gaseous ligand binding to Hb^{2,3}. ApoHb is a precursor for *in vivo* Hb synthesis and recombinant Hb assembly during which apoHb binds heme into its hydrophobic heme-binding pocket forming native Hb. Additionally, due to apoHb's ability to bind hydrophobic molecules in the vacant hydrophobic heme-binding pocket, this protein has been used for heme detection⁴ and drug delivery applications^{5,6}. However, since the main applications of apoHb target its heme-binding pocket, further development of such applications relies on accurate quantification of the number of functional heme-binding sites. These heme-binding sites can become damaged during production or from apoHb's intrinsic instability in aqueous solution. Thus, assessing the number of the site-specific His-F8 residues that maintain heme-binding activity yield a suitable target to quantify the number of active heme-binding sites in apoHb preparations.

Heme can be extracted from Hb in acidified organic solvents to yield apoHb after extensive dialysis⁷. When active, apoHb can bind a variety of ligands in the unoccupied hydrophobic heme-binding pocket^{8–11}. Unfortunately, the harsh heme extraction environment (i.e. acidic organic solvents used for protein unfolding during preparation) and the intrinsic instability of apoHb in aqueous solution (i.e. protein aggregation) results in denatured or improperly folded globins. These unstable globins are prone to aggregation and precipitate from solution^{12,13}. Initially developed methods for producing apoHb led to mostly inactive apoHb in solution^{14,15}. Yet, implementation of controlled cold acetone and liquid-liquid extraction methods resulted in highly active apoHb⁷. Even though the soluble globin can exist as either active or inactive apoHb in solution, no definitive assay has been described and validated in the literature to determine the amount of active apoHb in solution. Heme can also bind nonspecifically to locations other than the heme-binding pocket of apoHb and inactive apoHb, forming globin-heme complexes which do not replicate any of the biological functions of Hb^{16–19}. These complexes can easily lose the bound heme or precipitate from solution^{16,17,20–22}. Therefore, to enable proper analysis of apoHb biochemistry or for analyzing mutant Hb production an assay that determines the number of active heme-binding pockets would be useful.

The instability of apoHb is evident by the protein's gradual precipitation in aqueous solution^{7,13}. This implies that the molar extinction coefficient at 280 nm and total protein assays commonly used to quantify total protein concentration inaccurately represent the true concentration of active apoHb in solution. In addition, absorbance quantification methods are limited by the requirement for constant removal of protein aggregates to prevent light scattering and residual heme not fully extracted during production^{23,24}. Finally, apoHb solutions gradually form disulfide bonds in solution²⁵. These bonds absorb at 280 nm which will interfere with quantification via its extinction coefficient²⁶. The inaccuracy of apoHb quantification via absorbance is apparent in the literature, where the extinction coefficient ranges widely from 12.7 to 16.2 mM⁻¹ cm⁻¹^{7,27}. Colorimetric total protein assays such as Bradford's assay also have inherent drawbacks. These assays depend on the use of a standard calibration curve, which can lead to inaccurate measurement of the sample's concentration²⁸.

A possible protocol for quantifying the activity of apoHb would be to reconstitute it with heme into reconstituted Hb (rHb). The rHb could then be quantified via Hb quantification methods present in the literature^{29,30}. Fully reconstituting apoHb into Hb can be done by reacting apoHb with hematin (ferric hydroxide-bound heme) followed by reduction of the reconstituted methemoglobin (met-rHb), thereby recovering the activity of native ferrous Hb¹¹. However, multiple processing steps are required to yield rHb, leading to potential protein damage and yield loss. Additionally, heme can bind to denatured globins that do not recover native Hb properties and do not correctly insert into the apoHb hydrophobic heme-binding pocket¹⁶⁻¹⁹. These inactive globin-heme complexes convolute the UV-visible spectra^{22,31,32}. Therefore, a heme addition/reduction quantification protocol is a lengthy process, which requires multiple processing steps. Furthermore, measuring the final rHb concentration would not accurately quantify the initial apoHb concentration before reconstitution. As such, an alternative assay is necessary to assess the concentration of active apoHb in solution on a per heme basis.

Heme has a naturally high affinity and specificity for the hydrophobic heme-binding pocket of apoHb⁷. Additionally, heme incorporation into the apoHb heme-binding pocket causes a significant increase in the Soret peak absorbance compared to pure heme in solution. Therefore, a spectrophotometrically monitored titration of apoHb with heme could assess the amount of heme required to saturate the active heme-binding pockets of apoHb. A similar assay has been used to quantify the binding capacity of heme oxygenase for hematin³³.

In previous studies, apoHb has been titrated against heme to determine if apoHb could be reconstituted to yield native Hb¹¹. However, heme-binding was only used to determine the reaction stoichiometry between apoHb and heme, and did not quantify the amount of active apoHb in solution. The protocol for these titrations was to successively add heme to a single reaction vessel with a fixed concentration of apoHb, while measuring the absorbance after each addition. In previous studies and in the kinetic data presented here, the reaction of apoHb with heme may take hours to reach completion¹¹. Thus, successive heme additions after a reaction time on the order of minutes is not sufficient for the reaction to reach

equilibrium. This issue is discussed in more detail in the discussion and conclusion section of this current study.

Additionally, previous studies have used hematin or CO-heme as the apoHb probe molecule, which could result in inaccurate and irreproducible active apoHb quantification. For example, hematin aggregates in aqueous solution, which complicates its binding to apoHb^{34,35}. In addition, hematin dimerization and polymerization alters its absorbance spectra, which leads to inaccurate measurement of the free heme concentration^{36,37}. This is also demonstrated by the varying extinction coefficients of hematin observed in the literature ranging from 50 mM⁻¹ cm⁻¹ at 390 nm³⁸ or 385 nm³⁹ to 58.4 mM⁻¹ cm⁻¹ at 385 nm⁴⁰. It has also been shown that the hematin Soret band in neutral aqueous solution does not obey Beer's law. In addition, hematin also binds to the surface of glasses and plastics⁴¹. Even though CO-heme is a monomeric porphyrin species, it requires the use of carefully maintained CO saturated solutions to maintain the CO-heme equilibrium. This adds to the complexity of the assay. In addition, preparation of CO-heme uses powerful reducing agents such as sodium dithionite, which can introduce unknown reaction by-products that can interfere with the heme-binding reaction^{35,37,42,43}. Finally, both hematin and CO-heme can bind both to denatured and nonspecifically to globins, thereby convoluting spectral analysis^{16,32,44}. Studies analyzing such heme-globin binding showed that up to thirty heme molecules can bind to denatured globins^{16,17}.

Unlike hematin, dicyanohemin (DCNh) exists as a stable monomeric porphyrin species in aqueous solution, which simplifies the reaction kinetics between DCNh and apoHb. Furthermore, DCNh absorbance follows Beer's law⁴¹. When reacted with apoHb, DCNh is first inserted into the hydrophobic heme-binding pocket. After insertion, a cyanide ligand must be displaced from DCNh in order for the heme iron to bind the His-F8 residue of apoHb. The resulting complex is reconstituted cyanohemoglobin (rHbCN). Thus, in rHbCN, monocyanohemin (MCNh) is bound to the His-F8 residue. In the presence of excess cyanide, DCNh, unlike CO-heme and hematin, does not bind to denatured globins⁴⁵. This occurs because the excess cyanide can displace the nonspecific bonds between the heme and globin, but not the bond between MCNh and His-F8⁴⁵. Additionally, a major benefit of using DCNh versus other forms of heme include the fact that MCNh bound Hb is one of the most stable forms of Hb⁴⁶. The MCNh-His-F8 bond could even prevent irreversible denaturation of Hb (i.e. prevents formation of bis-histidine complexes)⁴⁷.

Although MCNh should not readily bind to most globin residues, a bond to the distal histidine (His-E7) residue might be possible. However, some evidence in the literature indicates that MCNh preferentially binds to His-F8. The affinity of heme for the His-F8 residue has been shown to be a major factor regulating association between the heme and globin chain³¹. Additionally, mutated Hbs lacking the His-F8 residue were shown to exhibit much lower heme-binding affinity, faster heme loss, or absence of heme^{48–50}. Additionally, studies with bis-histidine Hbs have shown that the cyanide ligand displaces the His-E7 and not the His-F8 bond, which would indicate that the His-F8 bond is stronger than the His-E7 bond^{51,52}. Finally, ¹HMR studies of rHbCN and heme-binding modules have shown that DCNh binds to His-F8 and not His-E7^{53–55}.

Even though the MCNh-His-E7 bond is not favorable, the MCNh-His-F8 bond can occur in a reversed heme-orientation along the α - γ meso axis of the heme group⁵⁶. Yet, such heme orientations should not affect the spectrophotometric detection of MCNh-His-F8 bond formation. Additionally, this altered heme orientation is not an indicator of apoHb inactivity, since these reversed heme groups occur at ~1:1 molar ratio upon heme binding and gradually reorient themselves towards the correct orientation in rHb^{54,56,57}. Based on these studies, the change in absorbance of the Soret peak generated from the reaction between DCNh and globins can be attributed to bond formation between MCNh and His-F8 of active apoHb.

As seen in Figure 1, the reaction between either DCNh or hematin with apoHb leads to an absorbance increase in the Soret peak. When hematin instead of MCNh binds to apoHb, larger absorbance changes occur. Such an intense absorbance change is due to the formation of the sharp Soret band of met-rHb from the broad Soret band of aqueous dimeric hematin^{41,58}. Although more significant absorbance changes would be favorable for spectrophotometric detection of the reaction between heme and active apoHb, the previously mentioned issues with hematin make DCNh the more favorable form of heme for analyzing the reaction. In light of these facts, we developed a spectrophotometric titration assay to determine the molar concentration of active apoHb in solution on a per heme basis. In this study, we outline an analytical method for quantifying active apoHb concentration and validated it with spectroscopy, as well as compared the biophysical characteristics of rHb to native Hb.

MATERIALS AND METHODS

Materials

Na₂HPO₄ (sodium phosphate dibasic), NaH₂PO₄ (sodium phosphate monobasic), NaHCO₃ (sodium bicarbonate), and hemin chloride were procured from Sigma Aldrich (St. Louis, MO). KCN (potassium cyanide), HCl (hydrochloric acid), acetone, nylon syringe filters (rated pore size 0.2 μ m), and dialysis tubing (rated pore size: 6–8 kDa) were purchased from Fisher Scientific (Pittsburgh, PA), while Millex-GP PES syringe filters (rated pore size: 0.2 μ m) were purchased from Merck Millipore (Bellerica, MA). Expired human red blood cell (RBC) units were generously donated by the Transfusion Service in the Wexner Medical Center at The Ohio State University (Columbus, OH).

Hb Preparation

Hb (P68871,P69905) for this study was prepared via tangential flow filtration as described by Palmer *et. al.*⁵⁹. The concentration of Hb was determined spectrophotometrically based on the Winterbourn equation²⁹.

ApoHb Preparation

The apoHb used in this study was prepared according to the protocol outlined by Fanelli⁶⁰. Briefly, 1.5 mL of a 25 $\pm$ 5 mg/mL Hb solution was rapidly mixed with 45 $\pm$ 0.5 mL of an acidified acetone solution (6 mM HCl) at -80 ± 1 °C. The resultant mixture was then centrifuged at -9 ± 0.5 °C for 15 min at 1080 g in an Accuspin 3R (Fisher Scientific,

Pittsburgh, PA) to yield a pellet of precipitated apoHb protein. The apoHb pellet was then suspended in deionized water and then further dialyzed against deionized water (6 hours), sodium bicarbonate (1.60 ± 0.05 mM) (22 hours), and phosphate buffer (7.0 ± 0.05 pH, $0.1 \pm$ M) (6 hours). At the end of dialysis, any residual protein precipitate (i.e. aggregated apoHb) was removed via centrifugation on a table top centrifuge. All samples used for analysis were verified for any residual heme by measuring the ratio of absorbance between 404 nm and 280 nm to ensure it was less than 0.1 AU, which indicates less than 1% residual heme⁷.

Total Protein Assays

To estimate the total protein concentration of the apoHb solution, a Bradford assay was used employing the Coomassie Plus Protein assay kit (Pierce Biotechnology, Rockford, IL)⁶¹. Additionally, spectrophotometric analysis of the 280 nm peak for each sample was used to estimate the total protein concentration using the millimolar extinction coefficients for apoHb found in the literature^{7,11,62–64}.

DCNh Preparation

The DCNh solution was prepared daily, kept refrigerated in either an ice bath or in a $4 \pm 0.5^\circ\text{C}$ environment away from direct light. All apoHb solutions that received DCNh also contained 35 ± 0.5 mM KCN to maintain monomeric DCNh throughout the reaction with apoHb. To prepare this solution, 2 ± 0.1 mg of hemin chloride was dissolved in 400 ± 6 μL of NaOH (0.1 M). The resultant hematin solution was well mixed and filtered through a 0.2 μm filter to remove any undissolved solids. After filtration, 200 ± 3 μL of the hematin solution was diluted with 800 ± 6 μL of a 50 mM KCN in phosphate buffer (pH 7.0 ± 1 , 0.1 ± 0.05 M) to a final volume of 1 ± 0.006 mL. The absorbance of the sample was then measured at 420 nm using UV-visible spectroscopy with a computerized HP 8452 diode array spectrometer (Olis Spectralworks, Bogart, GA). A molar extinction coefficient of $85 \text{ mM}^{-1}\text{cm}^{-1}$ at 420 nm was used to determine the concentration of DCNh in solution⁶⁵.

DCNh-ApoHb Kinetics

All kinetic measurements were carried out on a HP 8452 diode array spectrometer. The pathlength for each well was 10 mm. All measurements were performed in phosphate buffer (pH 7.0, 0.1 M) at 21°C . A 2 mL 7.6–8.6 μM DCNh solution was prepared. ApoHb was then added to the prepared DCNh solution at a 4:1, 2:1, 1:1, and 0.75:1 apoHb to DCNh estimated molar ratio. Each kinetic time course consisted of at least three runs. The Soret peak absorbance at 420 nm was measured in 5–10 s intervals for 1 hour. It was assumed that DCNh was inserted to a single heme-binding pocket in apoHb in a single step reaction with second order reaction kinetics of the form $\text{apoHb} + \text{DCNh} \rightarrow \text{DCNh} - \text{apoHb complex}$ (Phase I). This was followed by local rearrangement (Phase II), and a global conformational response (Phase III). The focus of this study was the binding of the iron from the heme to the proximal histidine (His F8 corresponding to residue 92 and 87 of the β and α chains, respectively) (Phase IV) and the additional slow phase (Phase IV+) to reach steady state. First order rate constants were determined via standard plots of the log of the change in absorbance at 420 nm versus time with curve fitting performed using Igor Pro 6.32 (Wavemetrics, Lake Oswego, OR).

DCNh-ApoHb Titration

The maximum heme-binding site concentration (obtained via the initial Hb concentration) and approximate results from the total protein assay were used to initially estimate the theoretical maximum concentration of active apoHb on a per heme basis. Thirty uniform increments of DCNh were added to a 8× diluted apoHb solution whose theoretical maximum concentration was approximately 120 μM (per heme basis). The uniform increments were added to individual wells. The uniform DCNh increments ranged from 0.1:1 to 1.5:1 molar ratio of DCNh to theoretical maximum heme (1.46–21.8 μM). This theoretical stock apoHb dilution was selected to yield ~ 10 μM of active apoHb hydrophobic heme-binding pockets to ensure accurate and linear absorbance readings in the multiwell plate reader. Each apoHb + DCNh solution was loaded in triplicate into a 96 well plate in a cold room maintained at 4 ± 0.5 °C and allowed to react for 40 minutes at room temperature. Additionally, 5 evenly spaced DCNh increments over the concentration range (1.46–21.8 μM) had their absorbance read without any apoHb (DCNh control). The 420 nm absorbance of the wells in the plate was measured every two and a half minutes on a VersaMax multiwell plate reader (Molecular Devices, SunnyVale, CA) for a total of 40 minutes.

Effect of Total ApoHb Concentration

The aforementioned apoHb activity assay was performed on varying dilutions of a stock apoHb solution in triplicate to evaluate the precision of the apoHb assay. DCNh increments remained the same for comparison (1.46 to 21.8 μM). Dilution factors of 22×, 8× and 4.1× were used to ensure that the total active apoHb in the sample was within the concentration limits of DCNh increments added to the sample. The results from the diluted sample were corrected by the dilution factor. These results were further used to determine the precision of the assay by analyzing the standard deviation of the determined apoHb stock solution concentration for each dilution.

Effect of Contaminants

The presence of other heme-binding proteins could interfere with the apoHb assay; thus, bovine serum albumin (BSA) (Albumin Standard, Pierce Biotechnology, Rockford, IL) was used as a test contaminant using the same mass of protein as a typical apoHb trial. In addition, a trial with a mixture of apoHb and BSA was performed to analyze the effect of a direct heme-binding contaminant on the accuracy of the results. Finally, this assay was performed on both an un-centrifuged apoHb sample and the supernatant from a centrifuged sample to demonstrate that the assay, unlike previous quantification methods, was not affected by the presence of aggregated protein in solution.

Spectral Deconvolution

To determine if excess DCNh was present in solution before saturation of the active apoHb heme-binding pockets during the apoHb titration assay, the concentration of each chemical species following incremental addition of DCNh was determined using a spectral deconvolution algorithm developed in MATLAB (Mathworks, Natick, MA). The analysis was performed in 10 mm quartz cuvettes with 47.6 μM apoHb in triplicate. This concentration maintained the 500–600 nm absorbance readings between 0.1 to 1 AU so that

the spectra of DCNh and rHbCN could be differentiated. The absorbance spectrum for each of the pure species was obtained from 450 to 700 nm. For comparison to the 420 nm absorbance plots, the solutions were further diluted by 7× so the absorbance at 420 nm ranged between 0.1 and 1 AU. With the 420 nm absorbance data, the DCNh titration analysis was performed as previously described in this study. To estimate the molar fraction of each species in each DCNh apoHb solution, the MATLAB algorithm squared the difference between the Fourier transforms of the raw absorbance spectra between 510 nm - 585 nm for each sample with a linear combination of Fourier transforms of the spectra of pure DCNh or HbCN. The linear combination of the pure species' spectra with the lowest error was used to estimate each species' concentration in solution. The wavelength range was selected based on two criteria: (1) all absorbance values ranged from 0.1 to 1 AU, and (2) regions where potassium ferricyanide does not absorb⁶⁶.

Abridged DCNh Assay

To reduce the time and resources required to determine the concentration of active apoHb in solution via the aforementioned apoHb assay, we also developed and tested a simplified assay. First, a DCNh solution was prepared as described previously. Then $600 \pm 6 \mu\text{L}$ of the DCNh solution prepared previously and $600 \pm 6 \mu\text{L}$ of a 1.5 M KCN solution were added to 50 mL phosphate buffer (7.0 pH, 0.1 M). $2 \pm 0.012 \text{ mL}$ of this solution was then added to a 10 mm quartz cuvette, and the absorbance measured at 420 nm (A_o). A small volume of an apoHb sample was then added to the cuvette and incubated at room temperature for 1 hour. After 1 hour, the absorbance was measured at 420 nm (A_f). Using Beer-Lambert's law ($A = A_{\text{CNHb}} + A_{\text{dicyanohemin}}$), we derived Equation 1 to calculate the concentration of rHbCN in the cuvette.

$$C = \frac{A_f - (A_o/D)}{(\epsilon_{\text{HbCN}} - \epsilon_{\text{dicyanohemin}}) \cdot L} \quad (1)$$

Where ϵ_{HbCN} is the extinction coefficient of HbCN at 420 nm ($\epsilon_{\text{HbCN}} = 120 \text{ mM}^{-1} \text{ cm}^{-1}$), $\epsilon_{\text{dicyanohemin}}$ is the extinction coefficient of DCNh at 420 nm, and L is the pathlength of the cuvette and D is the dilution of the DCNh solution due to apoHb sample addition⁶⁷. A linearity test was performed via analysis of a serial dilution of a stock apoHb solution of known concentration in triplicate.

Statistical Data Reduction Protocol

A statistical data reduction algorithm was employed in MATLAB (Mathworks, Natick, MA) to avoid operator bias when removing any data outliers and fit the best lines to the experimental data for the apoHb assay. A flow chart of the algorithm is shown in Figure 2.

The MATLAB program consists of two subprograms. The first subprogram estimates the equimolar inflection point (i.e. DCNh concentration that completely saturates all active apoHb heme-binding sites) by adding data points to linear fits beginning from the lowest (i.e. major line) and highest (i.e. minor line) DCNh concentrations. For each data point added to a linear fit, goodness of fit (GOF) values were calculated and stored. When a data

point from the opposing linear fit was added to the linear fit, the GOF of the linear fit decreased, thus the point at which GOF values began to significantly decrease was set as an estimated equimolar inflection point. Afterwards, the data point with the lowest root mean square error (RMSE) was removed from the analysis. Then, a new estimated equimolar inflection point was determined using the previously described GOF analysis. The process of deleting the data point with the lowest RMSE, and estimating the equimolar inflection point was repeated until the estimate reached a stable value (i.e. deleting another data point did not change the location of the estimated equimolar inflection point). The stable estimated equimolar inflection point was then sent to the second phase of the program. In this subprogram none of the raw data points from the data reduction algorithm were initially removed. Then major and minor lines were fit to the three closest experimental values on either side of the estimated equimolar inflection point. For each of the linear fits, the highest residual point was deleted until the average error of the linear fits was equivalent to the accuracy of the multiwell plate reader used. From the reduced data set, the set of major and minor linear fits with the lowest RMSE was selected as the most appropriate linear fits to the experimental data.

Fully Functional Hb Reconstitution and Preparation

To regenerate the oxygen-binding properties of Hb (i.e. reconstituted Hb, rHb), samples of apoHb were reconstituted with hematin to yield met-rHb and then reduced to yield rHb. First, hematin was added in excess to apoHb to yield met-rHb. The reaction was left overnight at 4 ± 0.5 °C to go to completion. Met-rHb was centrifuged and passed through a $0.22 \mu\text{m}$ filter before any experiments were conducted. Reduction of met-rHb to yield deoxy-rHb was achieved by adding sodium dithionite at 1.5 mg/mL to met-rHb. The solution was then passed through a HiPrep 26/10 desalting column (GE Healthcare Bio Sciences, Pittsburgh, PA) to remove excess sodium dithionite and hematin, yielding oxy-rHb.

rHb Analysis

Various liganded forms of rHb used in this study were analyzed via UV-Vis spectroscopy and compared against native Hb. The oxy-rHb and Hb equilibrium binding curves were measured using a Hemox analyzer (TCS Scientific Corp., New Hope, PA) at 37 °C as described in the literature^{59,68,69}. The instrument simultaneously measures O₂ saturation via dual wavelength spectrophotometry and the dissolved O₂ concentration via a Clark O₂ electrode⁶⁹. rHb was buffer exchanged into Hemox buffer (TCS scientific Corp.) to a total volume of 5 mL and concentration of (31 μM , per heme basis). Prior to the measurement, 20 μL of Additive-A (TCS Scientific Corp.), 10 μL of Additive-B (TCS Scientific Corp.), and 10 μL of antifoaming agent (TCS Scientific Corp.) were added to the solution. Compressed air was bubbled through the rHb/Hb solution and allowed to saturate at a pO₂ of 147 ± 1 mm Hg. After O₂ saturation, nitrogen gas was bubbled through the solution to deoxygenate rHb/Hb. The O₂ saturation of rHb/Hb was plotted as a function of the partial pressure of O₂ (pO₂). The O₂ equilibrium curve was then fit to the Hill Equation (Equation 2) in Igor Pro 7 (Wavemetrics, Lake Oswego, OR).

$$Y = \frac{ABS - A_0}{A_\infty - A_0} = \frac{pO_2^n}{pO_2^n - P_{50}^n} \quad (2)$$

Where ABS is the measured absorbance of the sample, A_0 is the absorbance at 0 mm Hg, and A_∞ is the absorbance at maximum saturation ($pO_2 \rightarrow \infty$). The P_{50} (partial pressure of O_2 at which 50% of the rHb/Hb is saturated with O_2) and the cooperative coefficient (n) of Hb were regressed by fitting the O_2 equilibrium curves to Equation 2.

RESULTS AND DISCUSSION

The kinetics of the reaction between DCNh and apoHb was monitored by following the absorbance at 420 nm for one hour. The absorbance was found to plateau by the end of an hour. An example of a kinetic time course and rate plots for the reaction monitored at 420 nm are shown in Figure 3.

Vasudevan and McDonald previously determined that DCNh incorporation into apoHb occurred in four distinct phases: heme insertion (Phase I), local structural rearrangement (Phase II), global conformational response (Phase III), and irreversible histidine-iron bond formation (Phase IV)²⁷. They also noted the presence of an additional slow phase (Phase IV +). As expected from Phase I kinetics, we observed that the absorbance at 420 nm initially decreased with the Soret peak absorbance maxima moving towards 425 nm⁷⁰. However, this phase occurred in an interval too short for the spectrometer to properly resolve for appropriate kinetic analysis. After this initial phase, the Soret peak maxima increased while shifting back from 425 nm back to 420 nm.

Unfortunately, we did not use a stopped flow type spectrometer to detect the kinetic events resulting from the Phase I - III reactions of DCNh with apoHb. Thus, only Phase IV and Phase IV+ reactions were analyzed in this study. However, previous work determined that the rate of Phase I, II, and III reactions lead to insignificant absorbance changes at large time scales used in this study (>15 minutes)²⁷. The rapid kinetics of these initial reactions prevent apoHb precipitation during analysis since the structural configuration changes after heme insertion make the DCNh-globin complex more stable. If these initial reactions took longer, the addition of excess DCNh to apoHb at room temperature would not result in 1:1 reconstitution due to loss of some of the initial apoHb in the sample while the reaction was taking place.

The reaction constant for the Phase IV reaction was determined to be approximately $0.00636 \pm 0.00033 \text{ s}^{-1}$. This value was significantly lower than literature values ($0.008\text{--}0.012 \text{ s}^{-1}$)²⁷. It has been shown that the rate limiting step for the binding of the iron in DCNh to the proximal histidine (His F8 corresponding to residue 92 and 87 of the β and α chains, respectively) in the Hb heme-binding pocket corresponds to dissociation of a single cyanide ligand from DCNh³¹. Thus, the lower reaction constants observed here may result from the excess KCN in solution, which may decrease the dissociation rate of a single cyanide ligand from DCNh. A later gradual increase in the absorbance at 420 nm was also observed, which

was indicative of a Phase IV+ reaction. The estimated rate constant for Phase IV+ was $0.00103 \pm 2.85 \times 10^{-5} \text{ s}^{-1}$. Since DCNh preferentially binds to the α -globin (isolated α -chain without the heme) versus the β -globin, it is expected to yield different reaction rates following insertion into each type of globin⁴². As noted by Vasudevan and McDonald, when reacting DCNh with isolated α -globin, the Phase III reaction was not observed, indicating that the Phase II reaction represents structural rearrangement of the α -globin alone. Therefore, Phase III represented structural rearrangement of the β -globin. Extending this idea of individual reaction rates corresponding to each globin subunit, the iron binding to the histidine residue of each subunit could explain detection of the different Phase IV and IV+ reactions. It was also observed that there was no statistically significant difference ($p < 0.05$) of the apoHb to DCNh molar ratio on the first order reaction rate constants. In addition, all reactions reached steady-state at approximately 40 minutes after addition of DCNh.

Titration of DCNh against a fixed concentration of apoHb initially led to a linear increase in the equilibrium absorbance at 420 nm, which corresponds to the major line fit. At a certain DCNh concentration (i.e. the equimolar inflection point), the equilibrium absorbance of successive DCNh increments deviated from this major line resulting in a sharp cut-off followed by a second linear fit with a lower slope (i.e. minor line fit). This minor line was observed to be parallel to the absorbance at 420 nm versus DCNh concentration plot. From these observations, it was concluded that points on the major line had excess apoHb, thus any added DCNh reacted to form only rHbCN (i.e. MCNh-His-F8 bond) and unreacted apoHb. When sufficient addition of DCNh led to the presence of both rHbCN and excess DCNh in solution, the equilibrium absorbance at 420 nm data point would be expected to deviate from this initial major line, indicating that the cutoff point represented the concentration of DCNh that saturated all the heme-binding pockets of active apoHb (i.e. equimolar inflection point). After the equimolar inflection point was attained, further addition of DCNh resulted in excess DCNh along with a fixed concentration of rHbCN. Thus, since the rHbCN concentration (equal to the amount of active apoHb) was constant for all DCNh increments on the minor line, any absorbance difference was due to excess DCNh in solution. Given that excess DCNh has the same absorbance as pure DCNh, the minor line was parallel to the 420 nm absorbance versus DCNh concentration linear fit. Additionally, DCNh has a lower extinction coefficient at 420 nm versus rHbCN, thus the slope of the minor line was lower than the slope of the major line. To further elucidate this inflection point, it is possible to subtract the absorbance of pure DCNh from the 420 nm equilibrium absorbance. By referencing the DCNh absorbance, for each DCNh addition, only the increase in absorbance due to rHbCN formation will be monitored. On the major slope line, the addition of more DCNh lead to the formation of more rHbCN. However on the minor slope line, the maximum amount of rHbCN was already formed. Therefore, a linear positive slope will be observed for points on the major slope line, while a slope close to zero will be observed for points of the minor slope line.

A MATLAB program was used to perform linear fits on the major line and minor line data sets in this study. The inflection point of these two linear fits indicated the concentration of active apoHb heme-binding sites in solution on a per heme basis. The residuals for the fitted data indicated that there were no significant trends deviating from the major and minor lines.

A plot of the final processed data is shown in Figure 4. The absorbance of pure DCNh was subtracted from each point in Figure 4A, and the resulting plot is shown in Figure 4B. If curvature in the absorbance data was observed, it would indicate that the apoHb-DCNh reaction had not reached equilibrium.

To assess the precision and linearity of the assay, a stock apoHb sample was diluted 22×, 8×, 6× and 4.1× and each of these dilutions were assayed for active apoHb. The plot of each trial is shown in Figure 5A and the result from these trials as well as the final stock solution concentration obtained from each dilution is shown in Figure 5B. After correcting for the dilution factor, the average concentration of active apoHb in the stock solution was $75.54 \pm 0.30 \mu\text{M}$ on a per heme basis. The low relative standard deviation of the calculated concentration of the stock apoHb solution indicated the high specificity of the apoHb-DCNh reaction, which led to the high precision of the assay.

From the standard deviation of each apoHb dilution, it was noted that the precision of the DCNh titration assay was dependent on both the absorbance of the point where the slope change occurs, and the number of data points in the major and minor lines. As seen in Figure 5B, the standard deviation of intermediate dilutions was significantly lower than the extremities (0.63 and $0.88 \mu\text{M}$ for 8× and 6× compared to 3.75 and $7.90 \mu\text{M}$ for 22× and 4.1×, respectively). Thus, fewer data points in either the major or minor line led to a significantly higher standard deviation for the 22× and 4.1× dilutions. This lack of precision was compounded in the 4.1× trial in which DCNh additions exceeding $16 \mu\text{M}$ led to higher than 1 AU absorbance. To maintain linearity in the absorbance values as a function of DCNh concentration, the values should be maintained between 0.1–1 AU. For linearity, Beer's Law assumes that all particles have an equal probability of absorbing light; thus at absorbance values greater than 1 AU, the measured absorbance will deviate from linearity (lower than expected). This will be apparent by an asymptotic flattening of the Beer's Law curve at high absorbances^{71–73}. This may be addressed by diluting the wells that exceed the maximum specifications determined by the spectrometer with a known volume of the buffer solution. Additionally, the assay could be redone at a lower apoHb concentration to ensure that all points on the major and minor lines are in the linear range. There was no significant difference for each of the slopes for the major line fits, which would be expected since all these data points correspond to rHbCN and apoHb. Despite the decreased slope for the 4.1× dilution, there was no significant difference for the minor line fits and the DCNh line for each dilution. Analysis of the line fits indicated that the apoHb heme-binding pockets were saturated with DCNh at the calculated equimolar inflection point.

The assay was also performed in the presence of BSA or small amounts of precipitated apo-protein to examine the effects of these contaminants. BSA is a heme-binding protein with lower affinity for DCNh compared to apoHb; thus, it would be expected to compete for DCNh binding. Yet, when DCNh was added to pure BSA, no apparent slope change of the 420 nm absorbance plot was seen. This result is shown in Figure 6A. Therefore, when analyzing the data using the MATLAB program, the solution would not converge. In addition, a linear fit to the BSA spiked DCNh sample was found to be parallel to the pure DCNh line (Figure 6A). To further investigate the effect of BSA and other possible contaminants on the DCNh-titration assay, the assay was performed on an apoHb sample

spiked with BSA and prior to centrifugation and the results compared to the standard assay. The data showed that these contaminants had no significant effect on the result of the assay. The result from the DCNh-titration assay of the BSA spiked apoHb sample is shown in Figure 6B. When apoHb was mixed with BSA, the estimated equimolar inflection point was shifted from 9.37 μM (no BSA) to 9.39 μM (with BSA). This increase was expected because, as stated before, BSA is a heme-binding protein thus an increase in the detected number of heme-binding sites was observed. Yet, the effect of BSA, and other potentially similar proteins, was negligible. This was also expected since it has been shown that DCNh has a much higher affinity for apoHb versus BSA and does not exchange between BSA and apoHb⁴⁶. Thus, the equilibrium absorbance of a mixture of apoHb and BSA with DCNh would still be determined by the apoHb as described by the major line. Even though the minor line could have a different slope, the inflection point between these two lines would still occur saturation of the apoHb heme-binding sites, since the change in slope would still occur at the same concentration of added DCNh. In addition, centrifugation of the apoHb sample to remove apoHb precipitates reduced the predicted concentration from 7.56 μM to 7.39 μM which was also within the margin of error of the assay. Thus, contrary to total protein quantification at 280 nm, the presence of apoHb precipitates is not crucial for assay accuracy.

Spectral deconvolution was used to assess the accuracy of the apoHb quantification assay and its results are shown in Figure 7. The absorbance spectra of pure DCNh and HbCN are very similar with only absorbance changes at 420 nm and a slight blue-shift at 540 nm after DCNh incorporation (Figure 7A). However, this slight change was detectable by the spectral deconvolution software and indicated negligible unbound DCNh ($> 1.5 \pm 0.7\%$) before reaching the equimolar inflection point (Figure 7C). Conversely, DCNh was detected at significant levels at concentrations above the equimolar inflection point. An example of the fit from the algorithm is shown in Figure 7B.

The results from spectral deconvolution confirmed that the chemical species on the major line corresponds to pure rHbCN, while the chemical species on the minor line corresponds to a mixture of rHbCN and free DCNh (Figure 7C). Thus, as expected, the absorbance on the major line was linearly dependent on [rHbCN]. Above the equimolar inflection point, further addition of DCNh resulted in free DCNh in solution along with a constant concentration of rHbCN, making the absorbance on the minor line a linearly dependent on [DCNh].

During the study, it was observed that the offset between the pure DCNh line and the minor lines fit was constant. Yet, this offset was dependent on the initial amount of active apoHb in solution. Using this observation, a quick method to determine the concentration of active apoHb in solution was developed by adding excess DCNh to an apoHb sample, and using the resulting 420 nm absorbance offset to quantify apoHb activity. This abridged assay was evaluated by obtaining an apoHb solution with an active apoHb concentration previously determined by the described DCNh titration method. Varying concentrations of this apoHb solution was then titrated against a set concentration of DCNh. The amount of active apoHb (rHbCN formed) was calculated from the absorbance offset at 420 nm. The results from this analysis are shown in Figure 8. Using the estimated quantity of active apoHb from the

DCNh titration assay, we determined that this method predicted on average 0.2% more apoHb than the DCNh titration assay ($p < 0.05$). The adjusted R-square of the fit to the detected rHbCN concentration as a function of the estimated amount added was 0.996 with $p < 0.05$. Taken together, these results indicate that the linear fit was 1:1 and was statistically significant, confirming that the activity of the apoHb sample can be predicted from the offset. As expected, when more apoHb was added than the amount of initial DCNh on a molar heme basis, the amount of active apoHb detected was the same as the initial DCNh. This plateauing effect results from an insufficient quantity of DCNh in solution to react with the remaining apoHb in solution. Since the amount of DCNh used is known, the maximum amount of detectable apoHb could be determined by examining the ratio of the absorbance of the final rHbCN solution to the initial DCNh solution. If the ratio of the absorbance was equal to the ratio of the extinction coefficients of rHbCN to DCNh (approximately equal to 1.3), the sample was likely at the upper detection limit. If so, a more dilute apoHb solution would be required for accurate detection of active apoHb in solution.

The 280 nm quantification method was assessed by first diluting two different apoHb solutions and BSA so they each had the same absorbance at 280 nm (Figure 9A). All three solutions then received the same amount of DCNh. Using 280 nm quantification, the concentration of active apoHb and the resultant rHbCN concentrations should be the same. However, it was observed that the resulting rHbCN Soret band spectra differed significantly. The results from this analysis are shown in Figure 9B. This indicates that the 280 nm quantification method inaccurately quantifies the amount of active apoHb in solution.

Consequently, the DCNh-titration assay only quantifies the activity of an apoHb sample, since it relies on the high affinity of heme for the heme-binding pocket of apoHb and not the total amount of protein in solution. Therefore, the amount of active apoHb in solution had to be less than or equal to the total protein concentration. A higher amount of active apoHb would indicate that the titration method yielded inaccurate results. As shown in Figure 10, the DCNh-titration assay followed this trend, providing lower values than both the 280 nm and Bradford assay quantification methods. The 280 nm UV-visible quantification method provided values closer to the Bradford assay, which is expected given that both methods quantify total protein in solution. Additionally, 280 nm quantification can be influenced by the presence of inactive apoHb, residual heme, or apoHb precipitate contributing to a higher 280 nm absorbance. The variation from 280 nm quantification is showcased by the wide range of literature values for apoHb extinction coefficients, which vary from 12.7 to 16.2 $\text{mM}^{-1} \text{cm}^{-1}$ ^{7,11,62–64}, demonstrating the inconsistency in apoHb quantification through this method.

The apoHb samples used in this study were reconstituted with 1.5× excess hematin to confirm that the analyte of the assay was active apoHb, and that the properties of the reconstituted Hb (rHb) were recovered. As seen in Figure 11, the absorbance spectra of rHb (Figure 11B) matched that of native Hb (Figure 11A).

All the rHb spectra resembled that of native Hb. It is important to note that reconstituting apoHb into rHb, the raw spectra of the rHb species had an offset compared to native Hb. This difference may be explained by the presence of nonspecifically bound heme or inactive

heme-globin complexes in solution²⁹. These proteins may result from either denaturation of met-rHb or reduction with sodium dithionite^{21,74}. The appearance of these heme-globin complexes in the final rHb solution highlight the inaccuracy of quantifying active apoHb after full reconstitution. To remove these inactive proteins from solution, rHb samples were filtered on a 0.2 μm nylon filter, which, due to its' high hydrophobic binding capacity, could retain denatured protein aggregates and only pass through rHb. The same peak at 555 nm corresponding to deoxyHb was observed after reducing rHb with sodium dithionite. After passing reduced met-rHb through the desalting column, excess heme and sodium dithionite were removed from solution. Through exposure to an oxygenated buffer solution during desalting, the rHb solution became oxygenated. This is apparent in the change of the spectra to oxyrHb with the alpha and beta peaks at 540 nm and 575 nm, respectively.

The oxygen dissociation curve shown in Figure 12 indicated that rHb recovered the oxygen binding properties of native Hb. The lower P_{50} was expected due to possible incorrect heme insertion, the presence of heme-globin complexes which could interfere with the absorption spectra used by the HEMOX analyzer to determine the oxygen saturation of rHb. In addition, the lower cooperativity (n) demonstrated that the heme were likely incorrectly oriented as the cooperativity depends on heme orientation⁵⁴. In addition, it has been shown that reduction of native Hb can decrease the cooperativity value, which would also explain the lower value from our study⁷⁵. From spectral analysis and equilibrium oxygen binding properties, it was clear that the apoHb produced in the study had active heme-binding pockets and could reconstitute into a tetrameric cooperative conformation.

In previous studies, heme was titrated into apoHb to measure the stoichiometric ratio of heme:apoHb binding. However, these studies did not focus on apoHb quantification, only on determining the reaction stoichiometry, so large heme increments were used. Additionally, hematin was commonly used as the probe molecule in previous apoHb studies. This may increase assay error if there was insufficient time to overcome the heme monomerization rate limiting step^{34,35}. Furthermore, previous studies performed heme titration within a single vessel while monitoring the absorbance after each heme addition. This procedure may compound the error of insufficient reaction time, since it was not clear if the reaction had reached equilibrium between the successive heme increments. We hypothesize that the lack of resolution, use of aggregative forms of heme, and reactions that did not reach equilibrium restricted quantitative analysis of heme binding to apoHb⁶⁰.

In Vasudevan and McDonald's studies, a mixture of the titration and abridged assay was presented. After each DCNh addition, the difference between pure DCNh and reconstituted HbCN was determined. However, a gradual increase in the absorbance at 420 nm was observed even after their indicated saturation of all the heme-binding pockets in solution (determined via 280 nm quantified apoHb)^{70,76-78}. These results are consistent with the reaction not reaching equilibrium between successive DCNh increments. The absorbance continued to increase, since not enough time was given for the completion of the slower bond between monocyanohemin and His-F8.

Finally, in one of these studies, a 1:1 stoichiometry between the 280 nm quantified apoHb and DCNh was reported⁷⁰. However, the minor slope data appears to yield a greater slope

than the fit the authors used. It seems the minor line fit was forced to a 1:1 binding ratio between 280 nm quantified protein and DCNh added, and was not consistent with the raw data. Therefore, inaccurate quantification, slow reaction time, and large heme increments could also explain other inconsistencies in previous studies. Additionally, in studies that quantified apoHb via 280 nm absorbance, an offset at the equimolar absorbance point and varying heme:apoHb binding ratios have been presented^{70,75,79,80}.

Vasudevan and McDonald also reported a blue spectral shift (from 425 to 420 nm) while performing their DCNh titration²⁷. However, the data presented here and studies done with incorporation of DCNh into myoglobin indicated this shift was temporary. The spectra reverted to the normal peak wavelength (420 nm) at equilibrium³⁷. Additionally, this shift was shown by Vasudevan and McDonald to increase at lower temperatures^{76,78}. The temperature dependence of the shift is explained by the decreased reaction rate at lower temperatures. Consequently, at lower temperatures the slower reaction rate would imply that the lower temperature reactions had not yet reached completion. This may result in the elevated blue shift that was observed. In addition, Vasudevan, Fonseka and McDonald initially stated that the spectral shift was only observed until half saturation of the apoHb sample. They proposed that this indicates selective saturation of the α -globin of apoHb²⁷. This was contradicted in later studies where the spectral shift was observed until the equimolar equivalence point or only on the initial heme addition^{70,76–78}. There are also conflicting results from the temperature dependence of the spectral shift upon CN-deuterothemin (a modified version of DCNh) incorporation into apoHb. An initial study showed that at 5 °C, 10 °C, 15 °C and 30 °C, no shift was observed⁷⁶. Yet, in a later study, a spectral shift due to deuterothemin incorporation in the heme-binding pocket was observed from 2 °C to 14 °C but not at 10 °C⁴⁸. However, the authors fail to reference these inconsistencies, and only report the agreement between the 10 °C experiments^{76,78}.

CONCLUSIONS

The activity of apoHb was determined by quantifying the molar concentration of functional His-F8 binding-sites found in the hydrophobic heme-binding pocket of apoHb samples. This concentration was determined through spectrophotometric monitoring of the Soret band region during incremental DCNh additions to apoHb, until the presence of excess unbound DCNh indicated the saturation of the heme-binding pockets. The apoHb assay was found to be precise by assaying different dilutions of the same known concentration of active apoHb. The accuracy of apoHb assay was confirmed through spectral deconvolution, and it was shown to be insensitive to weak heme scavengers such as BSA. Although previous apoHb assays employed titration of unmodified heme, the use of aggregative forms of heme and insufficient time for the reaction to reach completion likely led to inaccurate results. Additionally, the previously used quantification methods have inherent errors that can be avoided using this assay, which provides reproducible results.

The apoHb activity assay presented here provided concentrations that did not exceed the values measured using total protein quantification assays. Not all proteins in an apoHb preparation display activity. Because of this, using total protein assays may inaccurately over-estimate the amount of active apoHb in solution⁴. Fortunately, total protein analysis

methods provide a concentration estimate for performing the DCNh titration assay. Given that the results from the DCNh titration assay, Bradford assay, and 280 nm UV-vis analysis were on the same order of magnitude, the concentration values determined from the DCNh-titration assay were likely in the correct range.

In summary, this work presents simple, precise and accurate assays to determine the concentration of active apoHb in solution. The standardization of apoHb quantification with these methods will lower variation in the results of apoHb analysis by only quantifying apoHb activity and not total protein concentration. Thus, future apoHb studies should utilize this assay for quantification of active hydrophobic heme-binding pockets in preparations of apoHb.

The presented DCNh titration assay range is primarily limited by the quantification range of the equipment used. With the current experimental setup, it was observed that the range of detection was from 3 to 18 μM . To decrease variation by a fitting sufficient number of data points to the major and minor lines and maintaining absorbance values less than 1 AU, we recommend testing the apoHb sample between 5 and 15 μM of active apoHb. Additionally, possible contaminants that react with DCNh or KCN will result in byproducts that may interfere with apoHb-heme binding, that absorb at alternate wavelengths, or that shift heme to aggregative forms. Therefore, the interaction of the buffers or other solution components with the DCNh and KCN reagents should be examined before use of the apoHb quantification technique described here. We recommend performing the effect of contaminants analysis described previously.

DETAILED PROCEDURE

Each of the following procedures outline a step-by-step detailed methodology for the analysis of apoHb via the previously described DCNh titration methods. In general, we recommend performing the titration procedure to first validate the method upon first use, especially if different buffers or reaction conditions may be required. Apart from the apoHb, a list of required supplies is shown in Table 1.

DCNh Solution Preparation Procedure

The following procedure outlines the preparation of the DCNh solution that is used for DCNh titration of apoHb. Keep this solution refrigerated at 4–6 °C or in an ice bath. All DCNh solutions should be kept closed and away from light.

1. Prepare a 5 mg/mL hemin chloride solution by dissolving hemin chloride in 0.1M NaOH. (For a standard set of experiments we recommend 1 mL of solution)
2. Pass solution prepared in step 1 through a 0.2 μm syringe filter to remove possible higher-order aggregates and undissolved hemin.
3. Prepare a concentrated DCNh solution by combining the following
 - a. 1530 μL 7.0 pH 3.75 mM Phosphate Buffer (or equivalent)
 - b. 70 μL 10 wt% KCN

- c. 400 μL DCNh solution prepared in step 2.
4. Combine 1530 μL of buffer (PB), 70 μL of 10% KCN, and 400 μL of the solution prepared in step 2 and mix thoroughly.
5. Measure the concentration of the solution prepared in step 3 using UV visible absorbance at 420 nm with an extinction coefficient of $85 \text{ mM}^{-1}\text{cm}^{-1}$.

ApoHb DCNh Titration Procedure

This procedure outlines the DCNh titration procedure to determine the quantity of active apoHb in solution. We recommend performing this procedure using a 96-well microplate; however, a series of cuvettes or other equipment may also be used. The following procedure was developed for use in a standard 96-well microplate. Prepare all solutions at 4–6 °C. We recommend performing this procedure whenever new conditions are used, such as different buffers or other components that may react with the KCN in the assay solution. The assay described here outlines a methodology to analyze a single sample at two dilution levels in triplicate in a single 96-well microplate. The selected DCNh increments may be adjusted or redesigned to best suit individual lab applications; however, the equimolar inflection point should allow for a sufficient number of points in the major and minor slopes.

1. Estimate the concentration of apoHb using a total protein assay. Use this value to dilute the apoHb solution to prepare two apoHb solutions with a concentration of 7 μM and 14 μM , respectively.
2. Using these values calculate the volume of each DCNh increment (1.5 μM to 16.5 μM) using the following equation:

$$V_{i,DCNh}(\mu\text{L}) = (1.5 * i) * W / (1000 * C)$$

3. Where C is the DCNh concentration (in mM) determined in step 5 of the DCNh solution preparation procedure and W is the well volume in microliters (200 μL recommended).
4. In each well in row 1 of the microplate add 200 (or W) μL of the blank buffer from the apoHb solution.
5. In each well in rows 2–4 of the microplate add 200 (or W) μL of the 14 μM apoHb solution.
6. In each well in rows 5–7 of the microplate add 200 (or W) μL of the 7 μM apoHb solution.
7. In each well of the i+1 column add $V_{i,DCNh}$ (μL) of the DCNh solution until the 11 DCNh additions are made.
8. Let the solutions react at room temperature for at least 1 hour and 30 minutes.
9. Measure the absorbance in each well using a microplate reader at 420 nm.
10. Analyze the data using a data reduction algorithm or other equivalent methods to determine the equimolar intersect of the major (rHbCN) line and the minor

(DCNh) line for each dilution. The blank sample in row 1 can be used to gauge the accuracy of the minor line fit.

Abridged DCNh Activity Procedure

The following procedure outlines an abridged DCNh assay procedure that was designed to decrease the time and resources involved in the apoHb DCNh titration procedure. Depending on equipment sensitivity and operator proficiency, the number of recommended replicates can vary from 3 to 6.

1. Estimate the concentration of apoHb using a total protein assay. This value will help to determine the volume of apoHb added to the DCNh solution.
2. Prepare a dilute DCNh solution using the following solutions
 - a. 48.85 mL 7.0 pH 3.75 mM PB (or equivalent)
 - b. 1.15 mL 10 wt% KCN
 - c. $\frac{37.5}{C - 0.75}$ μ L DCNh solution prepared in step 3 of the DNCh Solution Preparation Procedure (C is the concentration of the DCNh solution determined in step 6 of the DNCh Solution Preparation Procedure).
 - d. Place an adequate volume of the solution prepared from step 2 into a cuvette and measure the absorbance at 420 nm.
 - i. If the absorbance is greater than 0.8 AU at 420 nm, we recommend diluting the DCNh solution prepared in step 2 with a 0.22 % KCN solution in PB such that the final absorbance is between 0.6 and 0.8 AU at 420 nm.
 - ii. If the absorbance is less than 0.5 AU, we recommend adding additional DCNh to the DCNh solution prepared in step 2 such that the final absorbance is between 0.6 and 0.8 AU at 420 nm.

*Note this solution should produce enough material for 50/n individual abridged activity assays, where n is the volume of the cuvette. The solution may be scaled up or down to meet lab requirements. However, we do not recommend keeping it for more than 24 hours.

3. Place an adequate volume of the solution prepared from step 2 into a cuvette and measure the absorbance at 420 nm. Record this value as A_0 .
4. Add in a dilute apoHb solution to the cuvette. Estimate the concentration of the apoHb solution either via the DCNh titration assay or total protein assay so that the concentration is within the limit of detection of the assay.
 - a. The upper limit of detection (LOD) concentration can be determined using the following equation:

$$LOD (mM) = \left(\frac{A_0 * V_{DCNh}}{V_{DCNh} + V_{apo}} \right) / 85$$

V_{DCNh} is the volume of DCNh in the cuvette and V_{apo} is the volume of the diluted apoHb solution added to the cuvette.

5. Wait 1 hour and measure the absorbance at 420 nm. Record this value as A_f

*note: You may perform steps 3–5 for any number of samples as reasonable given the allotted time.

6. Calculate the ratio between A_f and $\frac{A_0 * V_{DCNh}}{V_{DCNh} + V_{apo}}$ where V_{DCNh} is the volume of DCNh in the cuvette and V_{apo} is the volume of the diluted apoHb solution added.

7. If the ratio is greater than 1.29, your sample is too concentrated. Repeat steps 3–5 with a more dilute apoHb solution. Optimal results have the ratio at about 1.2.

8. Calculate the activity by using the following equation:

$$C \text{ (mM)} = \frac{D \left(A_f - \frac{A_0 * V_{DCNh}}{V_{DCNh} + V_{apo}} \right)}{35 \cdot L \text{ (cm)}}$$

9. Where C is the heme binding site activity of the added apoHb solution in mM on a heme basis, D is the dilution factor of the apoHb sampled, V_{DCNh} is the volume of DCNh in the cuvette and V_{apo} is the volume of the diluted apoHb solution added and L is the cuvette path length in cm.

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ABBREVIATIONS

apoHb	apohemoglobin
Hb	hemoglobin
DCNh	dicyanohemin
rHbCN	reconstituted cyanoheemoglobin
MCNh	monocyanohemin
oxyHb	oxygenated hemoglobin
met-rHb	reconstituted methemoglobin
rHb	reconstituted hemoglobin

CO-heme	carboxyheme
HbCN	cyanohemoglobin
BSA	bovine serum albumin
GOF	goodness of fit
RMSE	root-mean-square error
pO_2	partial pressure of oxygen
P_{50}	partial pressure of O_2 at which 50% of the rHb/Hb is saturated with oxygen
n	cooperativity coefficient
deoxyHb	deoxygenated hemoglobin
metHb	methemoglobin
oxy-rHb	oxygenated reconstituted hemoglobin
deoxy-rHb	deoxygenated reconstituted hemoglobin
met-rHb	reconstituted methemoglobin
LOD	Limit of Detection

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Figure 1:
Comparison of absorption spectra of heme (DCNh and hematin) to rHb (rHbCN and met-rHb). The concentration of all species was set at 7.1 μ M on a per heme basis.

Figure 2. Flowchart outlining the statistical data reduction algorithm to eliminate data outliers and calculate the final equimolar inflection point.

Figure 3.

Absorbance and rate plots for the reaction of a 2:1 molar ratio of apoHb to DCNh. **(A)** shows the raw 420 nm absorbance reading over time, **(B)** shows the Phase IV reaction at an intermediate time interval, and **(C)** shows the Phase IV+ reaction that occurs at long time scales. The dashed lines represent the 95% confidence interval predicted from the fit and A_0 is the initial absorbance before apoHb addition into DCNh.

Figure 4:

Titration of a fixed apoHb concentration with DCNh. **(A)** Total equilibrium absorbance from each DCNh increment. Also included are the major and minor line fits generated by the MATLAB regression algorithm (solid black lines) and the pure DCNh absorbance (dashed line). The residuals from the calculated fits are shown below the graph. **(B)** Plot of titration data subtracting the absorbance of pure DCNh from each equilibrium absorbance.

Figure 5.

Serial dilution of a known concentration of apoHb changes the location of the equimolar inflection point (A). Results of the DcNh-titration assay from each diluted sample and corresponding stock apoHb concentration obtained from each dilution (B). The vertical dashed lines indicate the equimolar inflection point for each apoHb dilution (all adjusted R^2 values for the fitted data were above 0.992 with the exclusion of the minor line for the 4x dilution which was 0.964). The 8x dilution 420 nm equilibrium absorbance plot is shown in Figure 4 and was omitted from (A) for greater clarity.

Figure 6.

Analysis of contaminants in the DCNh-titration assay. **(A)** Effect of a lower affinity heme-binding protein contaminant (BSA) on the 420 nm absorbance of DCNh. **(B)** Result from DCNh-titration assay with and without BSA. Open symbols indicate outliers identified by the data reduction program.

Figure 7.

Spectral deconvolution of apoHb-DCNh mixtures. **(A)** Spectra of each apoHb solution (47.6 μM) after one hour of reaction with increasing concentrations of DCNh (labeled on each line) and the pure species spectra of DCNh and HbCN. **(B)** Deconvolution of the 57.3 μM DCNh increment along with the estimated contributing spectra of rHbCN and DCNh. **(C)** Estimated percentage of each species in solution estimated from the deconvolution algorithm (**right y-axis**) as a function of DCNh concentration. The 420 nm absorbance is also shown for comparison (**left y-axis**). Note that the estimated percent values increase after the equimolar inflection point (indicated by the dashed line at ~47 μM). The error bars depict the standard deviation from three trials performed at each DCHh concentration.

Figure 8:
Abridged DCNh assay results for the amount of rHbCN detected compared to a known amount of apoHb added to the DCNh solution. Note the shaded region corresponds to the 95% confidence interval. The dashed black line indicates the upper detection limit, where any additional apoHb added would not result in an increase in the amount of active apoHb detected.

Figure 9:
Absorbance of apoHb and BSA solutions (A) before addition of DCNh and (B) after addition of DCNh.

Figure 10:
Comparison between the apoHb DCNh titration assay to the Bradford assay and 280 nm quantification methods ($\epsilon = 12.7$ and $\epsilon = 16.2 \text{ mM}^{-1} \text{ cm}^{-1}$ (*)).

Figure 11:
Comparison of absorbance spectra of reconstituted Hb (rHb) and native Hb. (A) Spectra of different forms of native Hb. (B) Spectra of different forms of rHb after reduction and desalting.

Figure 12.
Oxygen dissociation curve, P_{50} and n of rHb compared to unmodified Hb.

Table 1:

Materials required for performing the DCNh apoHb titration procedure. This includes solution preparation, titration assay, and abridged assay.

Item	Item Specifics	#	Units
Phosphate Buffer	7.0 pH 0.1 M stored at 4 °C	1.53 48.85	mL Per DCNh Solution Prep mL Per Abridged DCNh Prep
Sodium Hydroxide	0.1 M Sodium Hydroxide	1	mL
Potassium Cyanide (KCN)	10 wt% stored at 4 °C in a closed opaque container	70 1.15	μL Per DCNh Solution Prep mL Per Abridged DCNh Prep
Hemin Chloride	Hemin Chloride	5	mg
Cuvettes for UV-Visible Spectrophotometer	10 mm pathlength quartz cuvette or non binding equivalent for UV-visible spectrophotometer	1 1 3 to 6	Per DCNh Solution Prep Per Abridged DCNh Prep Per Abridged DCNh Assay
UV-Visible Spectrophotometer	UV-visible spectrophotometer capable of reading at 420 nm.	N/A	
Microplate	96-well microplate with non-binding surface or equivalent.	1	Per titration assay
Microplate Reader	96 Well microplate reader capable of reading the absorbance at 420 nm.	N/A	