Sickle Cell Hemoglobin in the Ferryl State Promotes βCys-93 Oxidation and Mitochondrial Dysfunction in Epithelial Lung Cells (E10)∗

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Tigist Kassa1, Sirsendu Jana1, Michael Brad Strader1, Fantao Meng2, Yiping Jia3, Michael T. Wilson4, and Abdu I. Alayash1,2

From the 1Laboratory of Biochemistry and Vascular Biology, Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, Maryland 20993 and the 2Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, United Kingdom

Background: HbS oxidation is recognized as an important element in the pathophysiology of sickle cell disease.

Results: The ferric/ferryl redox cycle of HbS is compromised.

Conclusion: The inability of ferryl HbS to revert back results in oxidative damage and mitochondrial dysfunction in lung epithelial cells.

Significance: These oxidative pathways may contribute to the vasculopathy in sickle cell disease and can be targeted with antioxidants.

Polymerization of intraerythrocytic deoxyhemoglobin S (HbS) is the primary molecular event that leads to hemolytic anemia in sickle cell disease (SCD). We reasoned that HbS may contribute to the complex pathophysiology of SCD in part due to its pseudoperoxidase activity. We compared oxidation reactions and the turnover of oxidation intermediates of purified human HbS and HbA. Hydrogen peroxide (H2O2) drives a catalytic cycle that includes the following three distinct steps: 1) initial oxidation of ferrous (oxy) to ferryl Hb; 2) autoreduction of the ferryl intermediate to ferric (metHb); and 3) reaction of metHb with an additional H2O2 molecule to regenerate the ferryl intermediate. Ferrous and ferric forms of both proteins underwent initial oxidation to the ferryl heme in the presence of H2O2 at equal rates. However, the rate of autoreduction of ferryl to the ferric form was slower in the HbS solutions. Using quantitative mass spectrometry and the spin trap, 5,5-dimethyl-1-pyrroline-N-oxide, we found more irreversibly oxidized βCys-93 in HbS than in HbA. Incubation of the ferric or ferryl HbS with cultured lung epithelial cells (E10) induced a drop in mitochondrial oxygen consumption rate and impairment of cellular bioenergetics that was related to the redox state of the iron. Ferryl HbS induced a substantial drop in the mitochondrial transmembrane potential and increases in cytosolic heme oxygenase (HO-1) expression and mitochondrial colocalization in E10 cells. Thus, highly oxidizing ferryl Hb and heme, the product of oxidation, may be central to the evolution of vasculopathy in SCD and may suggest therapeutic modalities that interrupt heme-mediated inflammation.

Sickle cell disease (SCD)3 is a genetic disorder caused by a single point mutation at the β6 position of hemoglobin (Hb) (β6Glu → Val), which results in the formation of a hydrophobic (sticky) patch on the surface of the molecule. Under conditions of low oxygen tension, this leads to the accumulation of long fibers of deoxyHb molecules within red blood cells (RBCs) (1).

As RBCs travel to hypoxic regions they undergo the classic sickle cell shape change, and after several cycles of sickling and unsickling, they rupture releasing a mixture of Hb fibers and Hb molecules to circulation. During hemolytic episodes, heme and Hb released by lysed RBCs may accumulate in plasma (2). Heme exerts multiple adverse effects, including leukocyte activation and migration, adhesion molecule and cytokine up-regulation, and oxidant production (3). SCD is characterized by chronic hemolysis, inflammation, vaso-occlusion, and ischemia-reperfusion injury leading to strokes and organ infarctions (4). Another manifestation of SCD is severe painful crises, episodic acute lung injury (also known as acute chest syndrome), and in some cases persistent intravascular hemolysis, which over time leads to chronic vasculopathy (5).

HbS is known to be unstable in vitro and is particularly less stable than HbA upon exposure to heat, oxidants, and mechanical shaking (6). It has been suggested, however, that the susceptibility of HbS to oxidation-related mechanisms is most likely a contributing factor to the pathophysiology of the disease; HbS autoxidizes at faster rates than HbA in solution (6, 7) and has greater affinity than HbA to react with membrane aminophospholipids (8). Both processes result in conversion to metHb and the generation of reactive oxygen species (ROS), including superoxide ions (O2−), which dismutate to H2O2.

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1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed. Tel.: 240-402-9350; E-mail: abdu.alayash@fda.hhs.gov.

3 The abbreviations used are: SCD, sickle cell disease; ROS, reactive oxygen species; Hpx, hemopexin; sulfHb, sulfhemoglobin; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; XIC, extracted ion chromatogram; OCR, oxygen consumption rate; FCCP, carbonyl cyanide p-trifluoromethoxyphenylnitrazone; RP-HPLC, reversed phase HPLC; AHP, altered heme product; bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; ECAR, extracellular acidification rate; Hp, haptoglobin.
resulting in greatly enhanced Hb denaturation and partitioning of the released heme in the membrane bilayer (8).

Recent studies on sickle cell RBCs revealed a unique oxidant environment that includes the presence of up-regulated NADPH oxidase catalytic subunits (9). NADPH oxidase-derived ROS in sickle RBCs may cause direct oxidative damage to a variety of subcellular structures, reducing deformability and resulting in increased RBC fragility and hemolysis. Moreover, NADPH oxidase activity may deplete the cellular pool of NADPH, thus impairing the ability of the RBC to maintain its antioxidant defenses (9).

Another intriguing study recently reported the link between accelerated redox reactions associated with HbS (compared with HbA) and protection against malarial infection (10). Ferryl HbS (ferryl Hb, the iron is in the FeIV+ state) was found to inhibit actin polymerization in malaria-infected HbS RBCs, thereby preventing the malarial parasites from creating its own actin cytoskeleton within the host cell cytoplasm. Although this mechanism appears to explain how HbS confers protection against malaria, it also serves to show that Hb oxidative reactions, including the formation of ferryl Hb can be detected in the reducing environment of sickle RBCs (10).

We have recently investigated these oxidative pathways in transgenic sickle mice; specifically, we examined the effects of Hb oxidation products on venules as they undergo stasis (little or no blood flow) in the microcirculation (11). Infusion of Hb or heme triggered vaso-occlusion in sickle but not in normal mice. MetHb, but not heme-stabilized cyanometHb, induced vaso-occlusion, indicating heme liberation is necessary. Hb-induced vaso-occlusion was blocked by the metHb-reducing agent methylene blue, haptoglobin (Hp), and the heme-binding protein hemopexin (Hpx). It was further shown that heme, released from Hb, elicits vaso-occlusion (stasis) in transgenic sickle mice by binding to the endothelial Toll-like receptor-4 (TLR4). The heme–TLR4 complex activates NF-κB and triggers vaso-occlusion through Weibel-Palade body degranulation and adhesion molecule expression (11). Based on these observations, it is therefore not surprising that heme (derived from sickle RBC hemolysis) has recently been described as a damage-associated molecular pattern molecule driving inflammation (11, 12). This is consistent with the early proposed role of Hb oxidation and heme in the disease pathophysiology (3).

The reaction between the ferrous or the ferric forms of Hb with H2O2 is known to proceed via the formation of the highly reactive oxoferryl complex, HbFe4O, detected by optical spectroscopy and a globin-associated free radical HbFe4+=O detected by EPR. Ferryl Hb can spontaneously autoreduce back to the ferric form completing a catalytic cycle (Scheme 1). Both the ferryl heme and the protein cation radical induce a wide variety of oxidative reactions affecting both the protein itself and nearby molecules due to their high midpoint redox potentials (E1/2 = −1.0 V) (13). These internal reactions result in the modification of heme, its attachment to nearby amino acids, and the irreversible oxidation of “hot spot” amino acids in particular the βCys-93 side chain (14). Experimental evidence from animal studies supports the notion that these oxidative activities of Hb occur in vivo with some potentially serious consequences (11, 15).

In this study, we examined a potential mechanism that links oxidation reactions to oxidative HbS toxicity and instability. Specifically, we examined the hypothesis that ferryl HbS persists longer in solutions than its HbA counterpart due to a slower autoreduction pathway. We coupled this analysis with cellular toxicity studies using mouse lung type 1 epithelial cells (E10). E10 cells are major cells lining lung alveoli, which can potentially be a target for Hb toxicity due to their inherent sensitivity and anatomical predisposition, i.e. large surface area and close apposition with the capillary endothelium (16). These cells might therefore be exposed apically to acellular Hb and its oxidation products following alveolar hemorrhage. It is well accepted that cell fate is primarily regulated by functional mitochondria through maintenance of cellular energy homeostasis and regulation of death signaling pathways (17). However, functional bioenergetic analyses remain mostly unexplored in Hb-mediated cytotoxicity. Therefore, we employed a biochemical technique utilizing extracellular flux analyzer to access the bioenergetics in functional mitochondria in lung E10 cells.

We found that the ferryl form of HbS promotes a significant impediment of mitochondrial respiration and oxidative stress in lung cells. Accumulation of long-lived ferryl Hb may therefore contribute to the development of vascular inflammation and vaso-occlusion in SCD.

### Experimental Procedures

**Reagents**—Buffer solutions were prepared by mixing either K2HPO4/KH2PO4 or using sodium acetate trihydrate solutions (Fisher Scientific) dissolved in deionized water. Sickle cell Hb and heme/hemin5 were purchased from Sigma.

Hemin was dissolved in PBS with 0.1 N sodium hydroxide. Chromatography media, columns, and equipment were obtained from GE Healthcare and Sigma. Catalase (bovine) and trifluoroacetic acid (TFA, 99% purity protein sequencing grade) was purchased from Sigma. HPLC grade acetonitrile and HPLC grade water were purchased from Fisher. Hp used in this study was a kind gift from Bio Products Laboratory (BPL, Hertfordshire, UK). Hpx was purchased from Athens Research and

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4 Amino acids located close to the α/β interface of Hb subunits are consistently oxidized by hydrogen peroxide (18).

5 The heme refers to protoporphyrin IX bearing either FeII or FeVI for simplicity. Heme is used interchangeably with hemin, a protoporphyrin IX containing a FeVI with chloride.
Technology (Athens, GA). Hydrogen peroxide (H$_2$O$_2$) (30% w/w) was purchased from Sigma. Dilute solutions of H$_2$O$_2$ were prepared fresh daily from a stock by making appropriate dilution in deionized water and kept on ice. The concentration of H$_2$O$_2$ was determined spectrophotometrically at 240 nm using a molar extinction coefficient of 43.6 M$^{-1}$ cm$^{-1}$ (19).

**Protein Purification and Handling**—Normal blood for Hb preparation was obtained by patient consent from the Division of Transfusion Medicine, National Institutes of Health. Human HbA was isolated from whole blood using established methods (20). HbA was purified further by using an XK50/100 column containing Superdex 200 medium on an AKTA FPLC system to remove RBC catalase (21). In some cases, samples were passed through a DEAE column for catalase removal. Catalase activity assays were performed on all Hb samples to confirm the complete removal of catalase (22). The purity of both proteins (HbA and HbS) was verified by isoelectric focusing and reversed phase RP-HPLC. The molar extinction coefficients used to calculate Hb concentrations in heme equivalents were 14.6 M$^{-1}$ cm$^{-1}$ at 576 nm for HbO$_2$ and 4.4 M$^{-1}$ cm$^{-1}$ at 630 nm for metHb in 50 mM potassium phosphate buffer (pH 7.4) at 22 °C in both cases (23).

**Spectrophotometric and HPLC Analyses of Autoxidation**—Autoxidation experiments were carried out as described previously (24). HbO$_2$ (65 μM heme) was incubated in 50 mM potassium phosphate buffer (pH 7.4), and visible spectra (350–700 nm) were recorded during a 24-h incubation time at 37 °C. Another set of autoxidation experiments was performed in the presence of catalase (200 units/ml) (1 unit of catalase will decompose 1.0 μmol of H$_2$O$_2$ to oxygen and water/min (pH 7.0) at 25 °C at a substrate concentration of 10 mM H$_2$O$_2$) (22). The spectral changes over time were monitored in a temperature-controlled photodiode array spectrophotometer (Agilent 8453, Santa Clara, CA). The absorbance changes at 576 nm due to the spontaneous oxidation of oxyHb were plotted over time and analyzed by nonlinear least square curve fitting to single or double exponential equations using Microsoft Excel program to obtain autoxidation rate constants. Rate constants with standard deviations were obtained by averaging data from at least three different sets of experiments. To investigate autoxidative changes within each globin subunit, aliquots were taken from each Hb solution at longer incubation times (24 h) and then analyzed by RP-HPLC (see further details on the RP-HPLC method below).

**Kinetics of Heme Loss from Hemoglobins**—To assess the rate of heme transfer, we measured the absorbance changes when a heme acceptor, a double mutant (H64Y/V86F) apomyoglobin (ApoMb), binds the heme released from metHbA and metHbS to yield holomyoglobin, a green adduct (25). In these experiments, visible spectra between 350 and 700 nm were recorded every 2 min for 16 h at 37 °C using 300 mM potassium phosphate buffer, 600 mM sucrose at pH 7.0. Final concentration of Hb in heme equivalents was 2 μM, and the final concentration of H64Y/V86F apoSbMb was 20 μM in a 1-ml total reaction volume. Data collection started immediately after mixing the two solutions.

**Rapid Reaction Kinetics of Peroxide with Hemoglobins**—OxyHb (5 μM) (after mixing) reaction with excess H$_2$O$_2$ (500 μM) (after mixing) was measured in an Applied Photophysics SF-17 stopped-flow spectrophotometer (Leatherhead, UK) with a dead time of ~1.5 ms, in 50 mM phosphate buffer (pH 7.4) at room temperature. Several spectra were captured as a function of time using an Applied Photophysics photodiode array accessory. Global analysis of spectral data and curve fitting routines was carried out as reported earlier using Applied Photophysics software (26). In another set of experiments, the ferric form of both proteins was mixed with H$_2$O$_2$ in the stopped-flow under the same experimental conditions, and the reaction was monitored at 405 nm in 50 mM phosphate buffer (pH 7.4) at room temperature. The measured rate constants were plotted against H$_2$O$_2$ concentration to obtain the second order rate constants (Table 2).

**Hydrogen Peroxide-mediated Oxidation of Hemoglobins**—Spectral changes due to H$_2$O$_2$-mediated oxidation of HbA and HbS (65 μM, heme) were monitored by photodiode array spectrophotometer using different heme to H$_2$O$_2$ ratios (i.e., 1:0, 1:1, 1:2.5, 1:5, and 1:10, and in some experiments 1:30 and 1:50 heme to H$_2$O$_2$ ratios were used), in a total of 1-ml solutions at room temperature for 1-h incubation times. Ferryl Hb formation as a result of ferrous Hb (ferrous Hb, heme groups contain positively charged iron (Fe$^{2+}$), which can reversibly bind to oxygen molecules) or ferric Hb oxidation was followed by monitoring characteristic absorbance changes over time using previously reported extinction coefficients in the visible region, specifically at the newly formed peaks (27). For the verification of the ferryl intermediate, 2 mM sodium sulfide (Na$_2$S) was added to transform ferryl Hb to sulfhemoglobin (sulfHb). Upon the addition of sulfide, the iron in ferryl Hb is reduced to the ferric state, and at the same time, sulfur is incorporated into the porphyrin ring, which can be monitored by the appearance of an absorbance band at 620 nm (18). A stock solution of 2 mM Na$_2$S in a buffer solution was prepared for this purpose by dry weight using a molar mass of 240.18 g/mol for Na$_2$S. The concentration of sulfHb was calculated using the extinction coefficient of sulfhem, ε$_{620}$ nm = 24.0 M$^{-1}$ cm$^{-1}$ (28).

**Kinetics of Ferryl Hemoglobin Formation and Decay**—The formation and reactivity of the ferryl heme, which occurs on addition of H$_2$O$_2$, were tested in HbA and HbS solutions, respectively. Ferryl Hb was prepared by adding 10 eq H$_2$O$_2$ to 50 μM HbO$_2$ solution at room temperature. After a reaction time of 1 min, catalase was added to remove excess H$_2$O$_2$. The reaction mixtures were then left at room temperature for the following time intervals: 1, 5, 10, 20, 30, and 60 min. At the end of each of these periods, 2 mM Na$_2$S was added to capture the residual portion of ferryl Hb as sulfhem. Visible light optical absorbance spectra were recorded between 500 and 700 nm. Spectra for each sample were obtained after the completion of the experiment, and sulfHb concentrations were calculated.

In another set of experiments, ferryl Hb was generated by the addition of 5, 10, 30, or 50 eq of H$_2$O$_2$ to metHb (65 μM) to ensure maximum formation of the ferryl heme in 50 mM phosphate buffer (pH 7.4). After 2 min of reaction time, excess catalase (250 units) was added to stop the reaction and to prevent heme bleaching. Decay of the resulting ferryl species back to methHb (k$_{f}$) was followed at room temperature. Spectra were recorded between 500 and 700 nm for 1 h, scanning every 30 s. The optical changes were monitored, and the time courses for...
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reduction of ferryl Hb were fitted to a single exponential function using Microsoft Excel.

**RP-HPLC Analyses of Hb Treated with Hydrogen Peroxide**—HbO₂ (250 μM, heme) was incubated in 1 ml of 50 mM phosphate buffer (pH 7.4) with or without H₂O₂ (250, 500, 750, 1000, and 1250 μM) at 25 °C. 2600 units of catalase in 50 mM phosphate buffer was added into the reaction mixture after 1 h to terminate the oxidation reactions. RP-HPLC analyses were performed with a Zorbax 300 SB C3 column (4.6 × 250 mm) using Waters HPLC system consisting of a Waters 626 pump, 2487 dual-wavelength detector, and a 600-s controller installed with Empower 2 software (Waters). Hb (20 μg) in 25 μl of water was loaded onto the C3 column equilibrated with 35% acetonitrile containing 0.1% TFA. Globin chains were eluted with a gradient of 35–50% acetonitrile within 100 min at a flow rate of 1 ml/min. The eluent was monitored at 280 nm for globin chains and at 405 nm for the heme components (29).

To monitor oxidative changes in heme and in heme-to-protein modifications (i.e. altered heme products (AHPS)) after H₂O₂ treatment, we incubated 250 μM HbO₂ with or without H₂O₂ (250, 500, 750, 1000, and 1250 μM) in 1 ml of 50 mM sodium acetate buffer (pH 5.0) at 25 °C. Catalase (2600 units) in 50 mM phosphate buffer was added to the reaction mixture after 1 h to terminate the oxidation reactions. Oxidized Hb (20 μg) in 25 μl of water was loaded onto the C3 column equilibrated with 35% acetonitrile containing 0.1% TFA. The gradient was initially 35% acetonitrile for 10 min and then increased to 37% acetonitrile over 5 min. The gradient was then increased to 40% acetonitrile over 1 min and then to 43% acetonitrile over 10 min. The flow rate was 1 ml/min at 25 °C. The eluent was monitored at 280 nm and 405 nm (29).

**Quantitative Mass Spectrometric Analysis of Hemoglobin Oxidation Reactions**—Quantitative mass spectrometric experiments performed with 178 μM (heme), HbSO₂, and HbAO₂ were carried out after incubation overnight in 20 mM phosphate buffer (pH 7.4) at 22 °C. To study the effect of increasing H₂O₂ concentrations, the following experimental conditions were utilized: experiment 1, control incubations with HbSO₂ and HbAO₂ in air-equilibrated phosphate buffer; experiments 2–4, HbSO₂ and HbAO₂ were incubated with 2.5, 5.0, and 10.0 mM excess of H₂O₂ per heme.

**Spin Trapping Reaction Conditions**—40 μM (per heme) HbSO₂ and HbAO₂ (in 100 mM ammonium bicarbonate (pH 8.0)) were subjected to both a 5-fold and 10-M excess of H₂O₂ per heme. All samples were prepared for LC-MS/MS analysis as described below.

**LC-MS/MS Analysis**—All Hb samples were tryptically digested, desalted, and analyzed by mass spectrometry using a previously described method (18). Briefly, tryptic peptides were analyzed by reverse phase-liquid chromatography mass spectrometry (RP LC/MS/MS) using an Easy nLC II Proxeon nano-flow HPLC system coupled on line to a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific). Data were acquired using a 10ppm method (for 60 min) dynamically choosing the most abundant precursors (scanned at 400–2000 m/z) from the survey scans for HCD fragmentation.

Data were searched against the Swiss-Prot human database (release 2014_03; contains 542,782 sequence entries) supplemented with HbS and porcine trypsin using the Mascot (version 2.4) search engine (Matrix Sciences, London, UK) as described previously (18) with the following amendments to Mascot searches: variable modifications, including cysteine tri-oxidation (+48 Da), cysteine dioxidation (+32 Da), tryptophan oxidation (+16), tyrosine oxidation (+16 Da), and methionine oxidation (+16), were included for identifying hot spot oxidation; DMPO-labeled cysteine and tyrosine (+111 Da) were included as variable modifications for all DMPO-treated samples. Mascot output files were analyzed using the software Scaffold 4.2.0 (Proteome Software Inc.). Peptide identifications were accepted if they could be established at greater than 99.9% (probability). Protein probabilities were assigned by the Protein Prophet Algorithm (30).

**Quantitative Mass Spectrometric Analysis**—Peptides listed in Table 3 from LC-MS/MS data were analyzed to quantify changes in HbS and HbA under the oxidative conditions listed for experiments 1–4. Each peptide was further validated by retention time reproducibility. All quantitative experiments were performed in triplicate, and standard deviations were obtained by averaging relative abundance data from three different experiments. Briefly, extracted ion chromatograms (XICs) were generated from the most abundant monoisotopic peak of each isotopic profile (representing charged states of each peptide). To construct XICs, Xcalibur (version 2.2) software was used with a designated mass tolerance of 0.01 Da, and mass precision was set to three decimals. For relative quantification, the ratio of each isoform was calculated based on the sum of the XIC peak area from all forms (including all charge states and versions that result from different cleavage sites), which was normalized to 100%.

**Hemoglobin Oxidation Reactions in a Lung Epithelial Cell Culture**—Non-malignant mouse lung epithelial E10 cells were cultured in CMRL-1066 media supplemented with 10% fetal bovine serum, 1% l-glutamine and 1% penicillin-streptomycin in 5% CO₂ atmosphere at 37 °C. The media were changed every 48 h. The cells were grown to 80–90% confluence in complete media. For all mitochondrial functional assays, only E10 cells from passages 5 to 10 were used to eliminate any variability in mitochondrial function. Before exposures, cells were serum-starved overnight. All Hb samples were passed through a high capacity endotoxin removal column (Thermo Fisher Scientific, Waltham, MA) prior to incubation with cells. Endotoxin levels were confirmed to be lower than 5 EU/ml by an LAL (Limulus amebocyte lysate) chromogenic endotoxin quantitation kit (Thermo Fisher Scientific).

Cells were exposed to either hemin (8 h) or various forms of endotoxin-free HbA or HbS (12 h) in a serum-free media. For experiments with Hp, an equimolar concentration of human plasma-derived unfractionated Hp was added to the culture media 5 min prior to the addition of Hb species. In a similar set of experiments, the heme scavenger protein Hpx was added to the culture media at a 2:1 molar ratio to heme. Following exposure, cells were rinsed with PBS three times. For immunoblotting experiments, immediately after the incubation, cells were washed with ice-cold phosphate-buff-
ered saline 4 to 5 times and then lysed using ice-cold RIPA buffer (Thermo Fisher Scientific) supplemented with protease and phosphatase inhibitors. Cell lysates were stored at −80 °C for further use.

**Mitochondrial Oxygen Consumption Rate and Bioenergetic Measurements**—Mitochondrial oxygen consumption was monitored in intact E10 cells in real time by an XF24 Analyzer (Seahorse Bioscience, Billerica, MA). Briefly, E10 cells were seeded overnight in a specialized 24-well V7 culture plate (Seahorse Bioscience) at a density of 20,000 cells/well and incubated with various HbA and HbS redox species for up to 12 h. After incubation, the media were replaced with unbuffered XF-assay media (Dulbecco’s modified Eagle’s medium supplemented with 10 mM glucose, 5 mM sodium pyruvate, 2 mM L-glutamine (pH 7.4)) to perform the XF assay according to the manufacturer’s guidelines. Following measurement of initial oxygen consumption rate (OCR), mitochondrial complex inhibitors were sequentially injected into each well. Three OCR readings were taken after addition of each inhibitor and before automated injection of the subsequent inhibitor. Mitochondrial complex inhibitors, in order of injection, included oligomycin (1 μM) to inhibit complex V (i.e. ATP synthase), FCCP (0.75 μM) to uncouple the proton gradient, and a mixture of antimycin A (1.0 μM) to inhibit complex III, and rotenone (1.0 μM) to inhibit complex I. Optimization of cell density and working concentration titers for each individual inhibitor were done prior to the actual experiments according to the manufacturer’s guidelines. The OCR values from individual wells were automatically recorded and plotted by XF24 software version 1.8 (Seahorse Bioscience). After each run, total protein in each well was measured by bicinchoninic acid (BCA) protein assay reagent (Thermo Fisher Scientific) to normalize the OCR values. OCR values were also normalized with total cell number in each well to rule out any Hb interference in the total protein. Hb species in these experiments did not show any significant interference with the OCR measurements. Various cellular bioenergetic parameters were calculated from the OCR plot, e.g. basal OCR, ATP-linked respiration, maximal OCR, and reserve respiratory capacity (31, 32). OCR values obtained after rotenone/antimycin A treatment represents the residual non-mitochondrial respiration. The non-mitochondrial OCR for each treatment was subtracted from initial OCR, prior to the addition of inhibitors to achieve the basal OCR. Likewise, the difference between the OCR induced by antimycin A and FCCP addition was considered as maximal OCR, and the difference between maximal and basal OCR was considered as the mitochondrial reserve capacity (26, 32). OCR values from different time points for each individual well were used to calculate values of different bioenergetic parameters according to XF Cell Mito Stress Test protocol (Seahorse Bioscience). A simultaneous recording of extracellular acidification rate (ECAR) indicative of cellular glycolytic lactate generation was also monitored in real time along with the OCR.

**Mitochondrial Membrane Potential Measurements**—Loss of mitochondrial trans-membrane potential in cultured E10 cells was assessed by using a cationic lipophilic dye 5,6,6′,5″-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1), which accumulates as J-aggregates within mitochondria as a result of a negative mitochondrial membrane potential (33). When excited at 490 nm, the monomeric dye emits at 527 nm (green), whereas the J-aggregates emit at 590 nm (red). The ratio of fluorescence intensities at 590 and 530 nm measured within intact cells is considered indicative of mitochondrial membrane potential (34). Briefly, cells were washed twice with pre-warmed PBS and were incubated with serum-free media containing JC-1 (8 μM) for 30 min at 37 °C in the dark. The dye-loaded cells were washed twice with pre-warmed PBS to remove the excess dye and then either suspended in PBS followed by the measurement of red (λex 530 nm, λem 590 nm) and green (λex 490 nm, λem 530 nm) fluorescence intensities in a Jasco FP8500 fluorescence spectrophotometer.

**Immunoblot Analysis of Heme Oxygenase**—Cell lysate proteins were separated by PAGE using Novex 4–12% bis-tris precast gel and transferred to nitrocellulose membranes according to the manufacturer’s protocol (Life Technologies, Inc.). Equal protein amounts were loaded following measurement of protein by BCA protein assay reagent (Thermo Fisher Scientific). Membranes were probed overnight with monoclonal anti-mouse HO-1 primary antibody at 4 °C at the manufacturer’s recommended concentrations (Abcam, Cambridge, MA). Blots were developed with Amersham Biosciences ECL Prime Western blotting detection reagent (GE Healthcare) after incubation with the HRP-conjugated secondary antibody (1:5000 dilutions) for 1 h. Blots were then further probed with monoclonal anti-β-actin rabbit primary antibody (Sigma) to ensure equal protein loading.

**Immunocytochemistry and Confocal Microscopy**—For immunocytochemistry, E10 cells were grown on coverslips up to 40–50% confluency. Following incubations with various HbA and HbS redox forms, E10 cells were fixed in 4% paraformaldehyde for 30 min, permeabilized with PBS containing 0.05% Triton X-100 for 15 min, and blocked with PBS with 5% BSA for 1 h. Immunostaining was performed with mouse anti-HO-1 primary antibody (Abcam, Cambridge, MA) or rabbit COX IV primary antibody (Cell Signaling Technology, Inc., Danvers, MA) according to the manufacturer’s recommended concentration at 4 °C. Finally, the cells were probed with secondary goat anti-mouse Alexa Fluor 594-conjugated antibody for HO-1 or with secondary goat anti-rabbit Alexa Fluor 488-conjugated antibody for COX IV and visualized under Zeiss LSM710 meta confocal microscope (Zeiss, Thornwood, NY) after mounting with Prolong-Gold with DAPI from Life Technologies, Inc.

**Measurement of Cellular Oxidative Stress Parameters and Viability**—Following treatment with various Hb species, protein carbonyl content and lipid hydroperoxide levels were measured in E10 cells using available commercial kits (Cayman Chemical Co., Ann Arbor, MI). Mitochondrial superoxide generation was monitored in live cells using Synergy HTX multimode plate reader (Biotek Instruments, Inc., Winoski, VT) at 580 nm using MitoSOX red dye (Thermo Fisher Scientific). Cell viability was assessed by trypan blue method (33) using an automated cell counter (Nexcelom Bioscience LLC, Lawrence, MA).
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**Results**

**Autoxidation and Oxidative Reactions of Hemoglobins**—HbO₂ readily undergoes oxidation of its heme iron that becomes a source of ROS, namely anionic superoxide (O₂⁻), which subsequently dismutates to H₂O₂. In this experiment (using a UV-visible spectrophotometric method), we followed the formation of metHb and/or the decay of the ferrous iron of both proteins to the ferric (metHb) form. Fig. 1, A and B, shows representative spectral intermediates taken at 0, 8, 16, and 24 h during the autoxidation of HbAO₂ and HbSO₂ at 37 °C and under physiological pH (pH 7.4). In the case of HbAO₂, it is clear that spectral changes during the spontaneous oxidation process maintained two reaction components with clear isosbestic points. The HbSO₂ spectrum at 8 h began to show deviation from these points with a buildup of considerable metHb and increased light scattering effects as indicated by absorbance increase at 700 nm (see below).

Estimation of the autoxidation rate constants were obtained by fitting the initial phase of the absorbance decreases at 576 nm to a single exponential expression. The autoxidation rate constants (over 8 h) for the two proteins are reported in Table 1. The k_auto (where auto is autoxidation) for HbAO₂ is around 0.065 h⁻¹, in agreement with previously reported rates by our group under similar conditions (18), and the rate for HbSO₂ was 0.177 h⁻¹, ~2-fold faster than that of HBA in agreement with early and more recent reports (3, 35). Autoxidation rates for both proteins were reduced by more than 50% with the use of 200 units of catalase in each sample. This decrease in the autoxidation rate confirms the buildup of ROS (such as H₂O₂) and the subsequent removal by the antioxidant catalase. Oxidative changes within Hb subunits were also evaluated using the RP-HPLC method such that the absorbances at 280 and 405 nm were recorded to monitor changes in the globin chains. As can be seen (insets for Fig. 1), the α-globin chains and β-globin chains of untreated HbAO₂ were separated into two distinct peaks (heme peak is not shown). Under our chromatographic conditions, the β and α chains of HbAO₂ eluted at 42 and 46 min, respectively, and the β and α chains of HbSO₂ eluted at 43 and 46 min, respectively. The slight right shift of HbSO₂ (43 min) versus (42 min) for HbAO₂ is due possibly to the increase in hydrophobicity as a result of the replacement of a hydrophilic Glu by a hydrophobic Val at the β₆ position. By integrating and comparing the areas of the β₆ and α peaks before and after 24 h for both proteins, we observed a 14% reduction in the β₆ peak as opposed to 9% in the β₆ of HbAO₂.

**Kinetics of Heme Loss from Hemoglobins**—Since HbSO₂ showed an accelerated rate of autoxidation and oxidative changes as the protein autoxidizes, we measured the rates of heme loss from both metHbS and its HbA counterpart during aerobic incubation. Heme loss leads to apoglobin precipitation under physiological conditions, and the released heme induces inflammatory responses and generates ROS (2, 11). Spectral changes were measured during the autoxidation of 65 μM (heme) HbS in 50 mM phosphate buffer (pH 7.4) at 37 °C and monitored in an Agilent 8453 spectrophotometer. Spectra measured at time 0 (black line), after 8 h of incubation (blue line), after 16 h of incubation (red line), and after 24 h of incubation (cyan line). The insets are the HPLC analyses of HbA and HbS (time = 0, blue traces) and after 24 h autoxidation (red traces). RP-HPLC analysis was performed using a Zorbax 300 SB C3 column (4.6 × 250 mm). Hb (20 μg) in 25 μl water was loaded on a C3 column equilibrated with 35% acetonitrile containing 0.1% TFA. The globin chains were eluted with a 35–50% acetonitrile gradient for 100 min at a flow rate of 1 ml/min, and the elution was monitored at 280 nm.

**TABLE 1**

| Sample | k_auto | k_auto + catalase |
|--------|--------|------------------|
| HbA    | 0.065 ± 0.014 | 0.027 ± 0.022 |
| HbS    | 0.177 ± 0.042 | 0.073 ± 0.016 |

**FIGURE 1.** Autoxidation kinetics of HbA and HbS. A and B, typical time-dependent spectra recorded during the autoxidation of HbA and HbS. Spectral changes were measured during the autoxidation of 65 μM (heme) HbS in 50 mM phosphate buffer (pH 7.4) at 37 °C and monitored in an Agilent 8453 spectrophotometer. Spectra measured at time 0 (black line), after 8 h of incubation (blue line), after 16 h of incubation (red line), and after 24 h of incubation (cyan line). The insets are the HPLC analyses of HbA and HbS (time = 0, blue traces) and after 24 h autoxidation (red traces). RP-HPLC analysis was performed using a Zorbax 300 SB C3 column (4.6 × 250 mm). Hb (20 μg) in 25 μl water was loaded on a C3 column equilibrated with 35% acetonitrile containing 0.1% TFA. The globin chains were eluted with a 35–50% acetonitrile gradient for 100 min at a flow rate of 1 ml/min, and the elution was monitored at 280 nm.
changes during heme exchange between metHbA and metHbS and H64Y/V86F, the receptor apomyoglobin, are shown in Fig. 2A. Heme transfer is confirmed by the shift in the Soret peak and the appearance of a new peak at 600 nm (Fig. 2A). Time courses for heme loss measuring the decrease in absorbance at 410 nm (as heme is transferred to the H64Y/V86F apoMb) are shown in Fig. 2B. In agreement with early reports, time courses for HbA and HbS were biphasic with fast components representing heme loss from β-subunits of both proteins (25). However, in the case of metHbS, it is very clear that longer times result in aggregation of the apoglobin and an increase in turbidity. Estimates of averaged absorbance changes at the Soret peak at 410 nm were normalized, and the initial phases of absorbance were represented from Fig. 2, A, absorbance spectra of metHbS (---) and holoMb (H64Y/V86F) (· · ·), B, absorbance changes at 410 nm are plotted as a function of time for over a 6-h time frame. Time courses for metHbA and metHbS were fitted to a double exponential expression (open and closed circles for HbA and HbS, respectively, and the fit is the solid line). C, absorbance spectra obtained at intervals of 30 s for 1 h upon treatment of ferrous HbSO2 (65 μM) with 10 eq of H2O2. Ferryl Hb spectrum (---) is characterized by new emerging peaks at 544 and 576 nm and the subsequent formation of the stable sulfHb that has a strong peak at 620 nm. D, overlayed sulfHb spectra of 65 μM (heme) of both HbA and HbS. Calculated values for sulfHb using extinction coefficient are reported in the text.

Oxidative Stability of Ferryl HbS

The reaction of HbO2 with excess H2O2 results in the formation of a transient oxyferryl Hb. The ferryl species autoreduces to ferric iron, and in the presence of additional H2O2, ferryl is regenerated back in a classic pseudoperoxidase cycle reported for both Hb and myoglobin (Mb) (Scheme 1) (36). The autoreduction pathway represented by k1 is a step that involves the transfer of electrons to the ferryl heme iron via tyrosine residues in Hb and Mb (37, 38).

Spectral changes accompanying the rapid mixing of HbO2 with excess H2O2 were studied using a stopped-flow apparatus. Spectral time courses were analyzed by singular value decomposition with subsequent nonlinear least square fitting to the model in Scheme 1 (data not shown). The estimates for k1 (oxidation of the ferrous to the ferryl species) for HbA and HbS were very close and are reported in Table 2. The rates for HbA are close to those reported earlier by our group (39). The initial oxidation of the ferrous heme was also captured at a longer time course in a diode spectrophotometer. The loss of typical α and β bands at 544 and 576 nm and the subsequent formation of the ferryl spectrum characterized by peaks at 541 and 585 nm are represented in Fig. 2C. The ferryl spectrum reverts back with time to a high spin ferric Hb with peaks at 510, 550, and 630 nm (this spectrum is not shown for simplicity). The ferryl intermediate can be captured by derivatization with Na2S to generate a stable sulfHb that has a strong peak at 620 nm (Fig. 2C).

The levels of sulfHb in HbA and HbS solutions were estimated from Fig. 2, C and D, to be 12.3 and 28.4 μM, respectively. These data confirm the accumulation of a considerable ferryl intermediate in HbS, which is almost 2.3-fold higher than that of HbA solutions. This led us to explore further the basis of differences in the ferryl stability among these two proteins.

Kinetics of Ferryl Hemoglobin Decay—To explore possible differences in autoreduction rates (k2 in Scheme 1), we followed...
**Oxidative Stability of Ferryl HbS**

| Sample | $k_1$ $\times 10^3$ s$^{-1}$ | $k_2$ $\times 10^3$ s$^{-1}$ | $k_3$ $\times 10^3$ s$^{-1}$ |
|--------|----------------|----------------|----------------|
| HbA    | 14.4 ± 0.013 | 46.3 ± 1.7 | 1.7 ± 0.1 |
| HbS    | 15.0 ± 0.015 | 46.9 ± 4.0 |

The ferryl decay of both Hb proteins to their respective ferric states (see Fig. 3A for a typical transition for HbA). Decay of a freshly prepared ferryl species for both proteins was followed at room temperature by monitoring changes in absorbance at 541 nm. In the presence of catalase, no heme damage was observed as reflected by the clear isosbestic points during the spectral transition of ferryl to ferric heme (data not shown). Under these conditions, any differences in the rates between HbA and HbS, monitored through the decrease of ferryl and increase in ferric heme, reflect differences in autoreduction rate constants ($k_3$). The decay of the ferryl, monitored at 541 nm, is clearly slower for HbS than HbA indicating that the former has a lower autoreduction rate constant (Fig. 3B). The time courses for ferryl HbS decay were fitted to a single exponential expression with a rate constant equal to 3.6 ± 0.06 h$^{-1}$, compared with 5.5 ± 0.21 h$^{-1}$, for ferryl HbA, almost 1.6 times slower than the rate of decay of ferryl HbS. We have also monitored ferryl decay at 545 nm and found similar rate constants to those derived at 541 nm. Fig. 3C shows a plot in which time courses of metHb formation were measured as increases at the 630 nm wavelength in both solutions.

**Hydrogen Peroxide-induced Oxidative Changes in Hemoglobins**—One of the hallmarks of oxidation triggered by the ferric/ferryl redox cycle is Hb subunit oxidative changes that can be detected by HPLC (14). The effects of H$_2$O$_2$ treatment on HbAO$_2$ and HbSO$_2$ were evaluated using the RP-HPLC method (Fig. 4). As can be seen from Fig. 4A, there was a corresponding decrease in peak height of $\alpha$ and $\beta$ subunits with increasing H$_2$O$_2$. Based on peak integration and the retention fraction of each subunit, we were able to plot retention fraction of each subunit as a function of the heme/H$_2$O$_2$ ratio. As shown in Fig. 4B, the integrated area of $\beta$ subunit retention fractions are much lower than $\alpha$ subunit fractions (for both proteins), indicating the $\beta$ subunit to be more prone to oxidative damage. The data in Fig. 4, A and B, also indicate that HbSO$_2$ is more susceptible to oxidative changes than HbAO$_2$, upon treatment with higher concentrations of H$_2$O$_2$. These results are consistent with our previous observations that confirm H$_2$O$_2$ induces extensive decomposition of $\beta$-globin chains of Hb (14). Other internal oxidative changes (initiated by the ferryl heme) that can be monitored by RP-HPLC are heme and the AHPs that result from covalent attachment to the protein (40, 41). As shown in Fig. 5, changes in both heme and AHPs occur as a function of pH and H$_2$O$_2$ concentration. Treatment of HbAO$_2$ with increasing H$_2$O$_2$ concentration at pH 5.0 led to a small loss of heme (20 min) and a concomitant increase in AHPs (34 min) (Fig. 5, A and C). The same treatment with HbS led to a greater loss of heme and a greater increase in protein-bound heme (Fig. 5, B and D).

**Quantitative Mass Spectrometric Analysis of the Hot Spot Amino Acid Oxidation in Hemoglobins**—To explore the consequential post-translational oxidation of $\beta$ subunit hot spot residues (listed in Table 3) for both Hbs under increasing H$_2$O$_2$ conditions, we employed quantitative mass spectrometry to target all hot spot peptide charge states. Amino acid residues identified by LC-MS/MS analysis in this study correlated well with previously published data (42). XICs were generated from the most abundant monoisotopic peak of each peptide isotopic...
Oxidative Stability of Ferryl HbS

profile (listed in Table 3), and the resulting ratio differences were compared. For example, the most abundant monoisotopic peak (860.061 m/z) represented in Fig. 6A for the βCys-93-containing peptide, GTFATSELHCDKLHVDPENFR, was used to construct the XIC in Fig. 6B. Because the βCys-93 residue exists in either the oxidized or unoxidized form, the percentage of both isoforms was calculated based on the sum of the XIC peak area from all charged forms of βCys-93 peptides.

Of the hot spot residues, the most prevalent oxidative changes between HbS and HbA were found to be restricted to peptides containing βCys-93. Indeed, this surface amino acid has previously been shown to be an important end point for free radical-induced protein oxidation with Hb (14). We confirmed the oxidized Cys-93 moiety did not impact trypsin digestion by comparing the averaged total ion current ratio of the internal peptide (β subunit tryptic peptide VNVDEVGGEALGR does not get oxidized) and the Cys-93-containing peptide (oxidized and unoxidized) for HbA and HbS; the ratio changed very little for both Hbs regardless of the H₂O₂ condition (data not shown). With the exception of marginally higher Trp-15 and Trp-37 oxidation for HbS, the difference in oxidation between all other α and β residues listed in Table 3 (regardless of H₂O₂ amount) was negligible. The quantitative mass spectrometric analysis results listed in Table 4 indicate that the relative level of HbS βCys-93 is substantially more oxidized in the presence of excess H₂O₂ compared with HbA and is therefore less stable. In the absence of H₂O₂, Cys-93 oxidation levels were below the detection limit of the instrument. However, treatment with 5.0 m excess H₂O₂ resulted in a 2.0-fold higher level of Cys-93 oxidation for the HbS β subunit compared with HbA. This dose-dependent increase correlates with the oxidation kinetic data indicating that HbS oxidizes and undergoes oxidative changes more readily than HbA.

DMPO labeling confirms that globin-centered radicals and ferryl ions are more abundant in HbS. The exposure of Hbs to H₂O₂ has previously been shown to initially produce a porphyrin cation radical (ferroin ion) that oxidizes cysteinyl and tyrosyl amino acids to form globin-centered radicals (43–47). The spin trap DMPO reacts with these modified amino acids to form a nitrooxide radical that is further oxidized to the corresponding globin radical-derived nitrone adduct by the ferryl moiety. These DMPO-derived adducts are stable under fragmentation conditions used in LC-MS/MS analysis (44, 48). We therefore utilized DMPO to characterize the Hb radicals in HbA and HbS in the presence of H₂O₂ (5- and 10-fold excess relative to heme). Analyses of LC-MS/MS and full MS data identified DMPO-labeled Cys-112, Cys-93, and αTyr-42 peptides with the addition of H₂O₂ (Fig. 6C). Among the three DMPO-labeled amino acids, only Cys-93 was substantially different between HbA and HbS, DMPO Cys-93 labeling in the absence of H₂O₂ was at negligible levels (~1% for both HbA and HbS). As shown in Table 5, the relative level of DMPO labeling was considerably more abundant for HbS than HbA. These labeling results further substantiate the observation that ferryl levels are more prevalent in HbS and likely account for the observed accelerated oxidation and oxidative changes within the protein.

Cellular Oxygen Consumption and Mitochondrial Membrane Potential in the Presence of Different Oxidation States of Hemoglobins—Using an XF-extracellular flux analyzer, we measured the effects of ferryl HbA and HbS on cellular OCR as an indicator of mitochondrial respiration. The mitochondrial bioenergetic profile was obtained by sequential addition of oligomycin, FCCP, and rotenone to cells treated with different Hb
redox forms. Administration of oligomycin (1 μM), which inhibits mitochondrial complex V, resulted in a drop in initial OCR. This allowed us to measure the portion of OCR that is only linked to ATP synthesis, i.e., ATP-linked respiration. The maximal respiratory OCR was achieved by adding the protonophore FCCP (1 μM), which resulted in dissipation of the proton gradient through the inner mitochondrial membrane leading to rapid uncoupling of oxidative phosphorylation. Finally, a combination of mitochondrial electron transport chain inhibitors (1 μM rotenone and 1 μM antimycin A) was administered to determine the non-mitochondrial OCR in E10 cells. Both ferryl HbA and HbS (at a 50 μM concentration) caused a significant inhibition in basal OCR following a 12-h exposure (Fig. 7A). Likewise, ferryl forms of both proteins also caused a significant impairment of ATP-linked respiration (Fig. 7B). However, ferryl HbS (but not HbA) induced a significant loss in maximal OCR and mitochondrial reserve capacity (Fig. 7B). Ferryl HbS (50 μM) also induced minor changes in the glycolytic flux as indicated by the apparent rise in ECAR over the control, although this observed difference was not statistically significant (Fig. 7C).

Functional mitochondrial status (after treatment of E10 cells with either ferryl HbA or HbS) was further assessed by moni-

**TABLE 3**

All hot spot peptides, including charge state and cleavage variants derived from hemoglobin A and hemoglobin S

| Peptides Modified residue | Charge state | m/z |
|---------------------------|--------------|-----|
| 82GTFATSELHCDK26          | βCys-93      | 2   | 735.33 |
| 82GTFATSELHCDKLVDPENF28   | βCys-93      | 3   | 860.06 |
| 105LLGNVLCVLAVHGF120      | βCys-112     | 4   | 645.31 |
| 31MFLSFPTTK9             | aMet-32      | 2   | 589.99 |
| 41FFGDLSTPDAMGMPK29        | βMet-55      | 3   | 692.32 |
| 9SAVTALWDGK37             | βTrp-15      | 2   | 676.76 |
| 31LVVYPWTQR40             | βTrp-37      | 2   | 645.86 |
| 17VGAHEYGAELER31          | αTyr-24      | 3   | 525.58 |
| 41TFPFDLQGSAQVK56         | αTyr-42      | 3   | 626.97 |
| 62VADALTNAHVDMPNALSADLHAK280 | αMet-76  | 4   | 628.92 |
| 10LHSCLVTLAAHPAETPAPHASLD127 | αCys-104 | 4   | 524.26 |
| 92GTVSLPAHETPAPHASLD126  | βCys-93      | 2   | 754.66 |

**FIGURE 5.** RP-HPLC analyses of HbA and HbS proteins, their relative hemes, and altered heme products after treatment with hydrogen peroxide. A and B, Hbs (250 μM in heme) were incubated in 1 ml of 50 mM sodium acetate buffer (pH 5.0) in the presence or absence of 250, 500, 750, 1000, and 1250 μM of H₂O₂ at 25 °C for 1 h. 1 ml of catalase (2600 units/ml) in 50 mM phosphate buffer (pH 7.4) was added to 1 ml of reaction solution to terminate the oxidation reactions. RP-HPLC was performed using a Zorbax 300 SB C3 column (4.6 × 250 mm). 20 μg of oxidized Hb in 25 μl of water was loaded on the C3 column equilibrated with 35% acetonitrile containing 0.1% TFA. The gradient was initially 35% acetonitrile, stable for 10 min, then increased to 37% acetonitrile over 5 min. The gradient was increased to 40% acetonitrile over 1 min and then to 43% acetonitrile over 10 min. The flow rate was 1 ml/min at 25 °C. The eluent was monitored at 280 nm (data not shown) and 405 nm. C, heme retention fractions of HbA and HbS were calculated by dividing the area of heme peak (at 20 min) subjected to H₂O₂ oxidation with the area of heme peak without treatment with H₂O₂ (1 protein, 0 peroxide) for both HbA and HbS. D, newly generated AHP fractions of HbA and HbS were calculated by comparing the area of AHP peak (at 34 min) with the original area of heme peak before treatment with H₂O₂ (1 protein, 0 peroxide) for both HbA and HbS. The area of each peak was integrated with Origin 6.0 software.
FIGURE 6. Isotopic profile and XIC of HbS βCys-93 tryptic peptide and LC-MS/MS spectrum of βCys-93 tryptic peptide with DMPO adducts. The most abundant monoisotopic peak of each isotopic profile was used to generate XICs. To construct XICs, Xcalibur (version 2.2) software was used with a designated mass tolerance of 0.01 Da, and mass precision was set to three decimals. For relative quantification, the ratio of each isoform was calculated based on the sum of the XIC peak area from all forms (including all charge states and versions that result from different cleavage sites), which was normalized to 100%. The reaction solution of HbS with H₂O₂ in the presence of DMPO was digested with trypsin and analyzed by LC-MS/MS. A, representative isotopic profile of the triply charged βCys-93 tryptic peptide GTFATLSELHCDKLHVDPENFR. B, XIC of βCys-93 tryptic peptide (residues 83–104). C, fragmentation spectra of the triply charged DMPO-labeled βCys-93 tryptic peptide GTFATLSELHCDKLHVDPENFR is shown. These spectra show singly charged y and b fragment ions. * corresponds to y and b ions shifted in mass by 111 Da (from expected mass) confirming the location of the DMPO adducts to be Cys-93.

### TABLE 4
Quantitative mass spectrometric analysis data representing oxidative reactions of hemoglobin A and hemoglobin S with hydrogen peroxide

| Reaction conditions | Cys-93 oxidation % HbA | Cys-93 oxidation % HbS |
|---------------------|-------------------------|------------------------|
| Control air equilibrated buffer | Below detection | Below detection |
| (H₂O₂) 2.5:1 (heme) | 21.1 ± 4.2% | 32.9 ± 2.7% |
| (H₂O₂) 5:1 (heme) | 31.3 ± 2.8% | 64.8 ± 5.0% |
| (H₂O₂) 10:1 (heme) | 58.4 ± 3.4% | 67.6 ± 6.8% |

### TABLE 5
Quantitative mass spectrometric data representing DMPO-labeled Cys-93

| Reaction conditions | Cys-93 DMPO-labeled % HbA | Cys-93 DMPO-labeled % HbS |
|--------------------|---------------------------|---------------------------|
| DMPO no H₂O₂       | 1.09 ± 0.06%              | 1.10 ± 0.05%              |
| DMPO (H₂O₂) 5:1 (heme) | 6.29 ± 0.36%           | 16.04 ± 0.32%            |
| DMPO (H₂O₂) 10:1 (heme) | 32.34 ± 3.6%        | 45.74 ± 4.99%            |
monitoring changes in mitochondrial transmembrane potential ($\Delta\psi_m$) using the JC-1 dye. The $\Delta\psi_m$ can be used as a sensitive marker of cell death and apoptosis (17). As shown in Fig. 7D, ferryl HbS (50 $\mu$M) resulted in a significantly greater $\Delta\psi_m$ loss over ferryl HbA as indicated by the decrease in 590:530 nm emission fluorescence ratio. We also compared the effects of ferrous and ferric forms of both the Hb proteins on cellular bioenergetics. E10 cells when incubated with ferrous forms (50 $\mu$M) of HbA and HbS for 12 h did not show any loss of basal or maximal OCR compared with untreated control (Fig. 7E).

However, at lower time points (8 h) ferrous HbA showed an increase in cellular respiration that may be due to its slower rate of autoxidation that can maintain higher oxygen supply to cells (data not shown). Incubation of these cells with ferric HbS (50 $\mu$M) but not HbAO2 for 12 h caused a significant drop in the basal and maximal OCR (Fig. 7E).

In a separate set of experiments, ferryl HbS-mediated impairment of mitochondrial respiration was further studied in the presence of the Hb scavenger protein Hp (Fig. 8A). When complexed (at equimolar concentration) with ferryl HbS (50 $\mu$M),
Hp effectively prevented a ferryl HbS-induced fall in basal OCR and maximal OCR.

Previous reports show that heme is highly toxic to cellular metabolism and respiration (31, 49, 50). Because we have seen a significant difference in heme release between HbA and HbS, we assessed the role of potential heme toxicity on the mitochondrial OCR (i.e., cellular metabolism) in E10 cells. Hemin (25 μM) caused a drastic inhibition of basal OCR and maximal respiration in E10 cells within a short period of time (8 h) (Fig. 8B). Hemin also showed a similar degree of inhibition on other bioenergetic parameters in E10 cells (data not shown). Hpx, a strong heme scavenger (when incubated with hemin at 2:1 molar ratio), significantly attenuated the hemin toxicity on E10 mitochondrial functional parameters (Fig. 8B).

Oxidative Changes in E10 Cells Induced by Ferryl Hemoglobins—We measured various oxidative markers in E10 cells following ferryl HbA or HbS treatment. Hb induces various cytoprotective mechanisms through the induction of heme oxygenase-1 expression (50). To assess HO-1 levels in E10 cells, we employed both immunoblotting and immunocytochemical staining following exposure to ferryl forms of HbA or HbS (50 μM) for 12 h. No significant HO-1 protein induction levels were observed in untreated cells. However, significant HO-1 expression was induced by both ferryl HbA and ferryl HbS (Fig. 9A).
Densitometric analysis of band intensity (normalized to β-actin internal standard) indicated significantly higher induced HO-1 expression by ferryl HbS compared with ferryl HbA (Fig. 9B). To support this finding, we also used laser confocal microscopy to visually observe the distribution of HO-1 levels in Hb-treated cells. The red fluorescence emitted by the Alexa Fluor 594-conjugated secondary antibody (against HO-1 primary antibody) revealed a greater expression of HO-1 in E10 cells treated with ferryl HbS (Fig. 9C). Because HO-1 induction is primarily in response to heme, we further explored cellular oxidation markers in the ferryl Hb-treated E10 cells. Both heme and ferryl Hb species have the capability of causing extensive intracellular protein and lipid oxidation (49–52). We further explored other important cell oxidative markers (e.g. protein carbonyl formation and lipid hydroperoxide formation) in our model system. Similar to HO-1 expression, ferryl HbS treatment resulted in protein carbonylation and lipid peroxidation in a greater degree than corresponding ferryl HbA (Fig. 9, D and E). We also identified significant HO-1 (in ferryl HbS treated E10 cells) colocalized to the mitochondrial compartment as visualized by the immunocytochemical staining of both HO-1 and mitochondrial electron transport chain complex IV (Fig. 10A). The appearance of bright yellow spots indicates the HO-1 protein translocation from either the cytosol or endoplasmic reticulum.
to the mitochondria. Furthermore, ferryl HbS also caused significantly more mitochondrial superoxide radical generation compared with ferryl HbA (Fig. 10B). Heme, in contrast, caused considerably higher ROS production over both ferryl proteins (Fig. 10B). However, in our experimental setup we did not observe any loss of cell viability by either ferryl species, even up to 18 h of incubation, which rules out the contribution of apoptotic cell death to the observed changes.

**Discussion**

Single amino acid mutation (β6Glu → Val) in HbS triggers a cascade of molecular and cellular events (leading to chronic...
Oxidative Stability of Ferryl HbS

It has been shown that both ferryl formation as well as ferryl autoreduction step is pH-dependent and that autoreduction of the protonated species occurs by abstraction of an electron, probably from the protein (through either intra- or inter-electron transfer) (37, 56). Thus, slower autoreduction means greater distribution of the ferryl form and its final reduction, whether through autoreduction itself or through reduction by a cellular component that leads to the wider distribution of damage. However when the transfers are rapid, lesser damage may occur to the protein and exogenous species.

Our data show that a persistent ferryl heme and its radical creates an oxidative milieu leading to the following: (a) heme attachment to nearby amino acids; (b) irreversible oxidation of hot spot amino acids particularly the βCys-93 side chain; (c) structural instability that leads to heme loss, and finally (d) oxidative damage to other biological entities such as the mitochondria.

In the case of HbS, the question remains how does a mutation (β6Glu → Val) at the A helix affect the F-helix where Cys-93 is located (Fig. 11)? As noted here, this mutation leads to rapid oxidation, heme loss, and the unfolding of the apoglobin. A likely explanation is the proximity of the hot spot βCys-93 to the heme and βTyr-145; this residue is known to stabilize ferryl globin radicals and is solvent-accessible in the R state (oxygen-bound conformation). βCys-93 oxidation possibly disturbs the extensive network of hydrogen bonding and salt bridges at the interface between the β2 FG corner and α1 C helix, where αTyr-42 is located (57, 58). It is also possible that the (βE6V) substitution results in conformational changes that position the distal His in closer proximity to the heme iron (59). For example, in an earlier crystal structural study where HbA was complexed with a CO ligand, shortening of the hydrogen bond interaction between CO and the distal histidine (β8His-63) imidazole was identified for the R structure HbA (2.8 Å versus 3.0Å for the T state). These “proximity” effects may potentially
lead to the following: (a) occlusion of the site thus encouraging the dissociation of the superoxide anion and hence speeding of autoxidation process; (b) stabilizing the single (O) of ferryl heme (Fe(IV)=O), and (c) directing the radical, formed on ferryl reduction by the protein, to escape via a route that leads to βCys-93. A combination of a site-directed protein engineering as well as crystallographic studies are currently underway to verify the structural basis of these possible oxidative pathways in sickle cell Hb.

In this investigation a systematic approach was taken to explore the unique oxidative reactions of HbS and its oxidation products in a biologically relevant system. The role of oxidized Hb and/or its heme as damage-associated molecular pattern molecule in triggering inflammatory pathways in animal models or in endothelial cells has been well documented (11, 51, 60). Mitochondria are considered as the primary and most vulnerable target of oxidative insult; however, the role of a compromised metabolism originating from altered cellular bioenergetics and its contribution to hemolytic diseases, in particular SCD pathogenesis, remains elusive. Therefore, we sought to determine the impact of ferryl Hb on mitochondrial bioenergetic function and whether the longevity of ferryl HbS induces more damaging effects on the mitochondria than HbA. Our study, the first in its kind, provides evidence that the bioenergetic dysfunction can be produced in intact cells incubated with oxidized Hb. These effects can be magnified as a result of increased Hb concentrations as well the redox states of heme iron. Ferric HbS showed some loss in mitochondrial respiratory function, but this effect was not observed with its ferrous species. Ferric (and ferrous) HbA, in contrast, did not show any inhibitory effect on cellular respiration.

To investigate ferryl Hb contribution to mitochondrial dysfunction, we prepared solutions prior to each experiment and ensured that at least 90–95% of total heme content was in the ferryl state and free of H2O2 contamination. Both protein species produced significant changes in basal and ATP-linked respiration. Moreover, ferryl species of HbS caused significant inhibition of maximal OCR and respiratory reserve capacity; HbS effects had significantly more impact on these bioenergetic parameters than ferryl HbA. We also observed a more profound dose-dependent decrease in mitochondrial membrane potential for ferryl HbS compared with HbA. This loss of membrane potential and respiratory reserve capacity by ferryl HbS is indicative of a severe loss of mitochondrial functions and energy reserve (61, 62). Finally, an apparent increase in the ECAR values as a result of ferryl HbS reactivity over the untreated control is indicative of a compensatory ATP supply by the glycolytic pathway. We also confirmed that apoptotic cell death was not a contributory factor toward mitochondrial changes in E10 cells incubated with ferryl HbS within at least the first 12 h.

In order for us to assess the extent of heme-mediated oxidative burden on the mitochondria, HO-1 expression was investigated. Our Western blot as well as confocal images confirmed that incubation of HbS with E10 cells induced substantial HO-1 expression in the E10 cells than seen with HbA.

Higher HO-1 can also promote oxidative changes in cell and mitochondrial dysfunction (62, 63). High expression of HO-1 by ferryl HbS was also accompanied by higher intracellular protein carbonylation and formation of lipid hydroperoxides indicative of stronger oxidative burden over the ferryl HbA in our model system. Both of these oxidative markers have been shown as causative factors for mitochondrial dysfunction (64). We found a significant portion of HO-1 colocalized in the mitochondrial compartment indicating an interesting link between HO-1 overexpression and mitochondrial dysfunction. Some previous studies have shown HO-1 translocation to mitochondria under different stress conditions, and most of these studies postulated a compensatory protective effect of HO-1 by scavenging extra heme load (65, 66). It is also possible that HO-1 translocation to mitochondria can lead to localized generation of toxic levels of CO upon degradation of the heme (62). HO-1 has also been shown to promote mitochondrial biogenesis in cardiac myocytes through CO generation, thus preventing apoptosis (67).

We have recently shown that Hp attenuates Hb-induced HO-1 in renal proximal tubule cells and in kidneys of a mouse model of SCD (68). Our experiment with Hp, which binds equally to HbA and HbS (data not shown), is informative. Addition of Hp to HbS solutions restored normal mitochondrial parameters in E10 cells. Hp is known to avidly bind Hb dimers and defuses its damaging ferryl protein radicals (69). Similarly, Hp, a powerful scavenger of heme, inhibited heme toxicity on the mitochondrial function confirming that both ferryl and its degradation product, heme, contribute to the overall oxidative toxicity of free Hb in solutions.

We further looked into some possible oxidative pathway activated by either ferryl species of both HbA or HbS to link the mitochondrial defect with oxidation of Hb. We found a significant rise in ROS (superoxide production) in the mitochondria induced by both HbS and HbA ferryl species. Although we observed marginally higher ROS production with ferryl HbS compared with HbA, hemin caused a massive increase in superoxide generation suggesting that heme can act as a mitochondrial electron transport chain inhibitor. Protein oxidation and lipid peroxidation were also evident in our experimental model, because both ferryl HbS and HbA caused significant protein carbonylation and lipid hydroperoxide formation. Ferryl HbS, however, showed greater intracellular protein oxidation and lipid peroxidation contributing further to the underlying basis of mitochondrial dysfunction in this model system (64). Taken together, our experimental model establishes a complex link between oxidized HbS and mitochondrial dysregulation.

Our findings are consistent with a recent investigation that demonstrated for the first time that mitochondrial inhibition occurs in platelets of sickle cell patients leading to platelet activation in those patients (70). This study found bioenergetic alteration in sickle cell patients characterized by deficient complex V activity, leading to decreased mitochondrial respiration and augmented oxidant production compared with healthy subjects. Interestingly, this study also reported that this dysfunction correlated with platelet activation and hemolysis in vivo, and this effect was reproduced in vitro by exposing healthy platelets to Hb or the complex V inhibitor.

In summary, we set out to compare oxidative reactions of highly purified HbS and HbA. We unambiguously show differences in the pro-oxidative activities of these two proteins and...
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further show a linkage to longevity of their respective ferryl species. Moreover, we show that a persistent ferryl HbS not only induced self-meditated oxidative changes but was able to promote cell and bioenergetics changes in a medium of cultured epithelial lung cells. This suggests that mitochondrial dysfunction triggered by Hb oxidation, and in particular higher oxidation ferryl species, likely contribute to SCD-induced vascular pathogenesis and that anti-sickling therapeutic interventions must take into account the oxidative toxicity of long-lived ferryl HbS.

Author Contributions—T. K. and S. J. contributed equally. T. K., S. J., and A. I. A. designed the work. T. K., S. J., M. B. S., F. M., and Y. J. performed the experiments and data analysis. Conception and design was by A. I. A. T. K., S. J., M. B. S., M. T. W., and A. I. A. wrote and edited the manuscript.

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