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Exploring Oxidative Reactions in Hemoglobin Variants Using Mass Spectrometry: Lessons for Engineering Oxidatively Stable Oxygen Therapeutics

Michael Brad Strader and Abdu I. Alayash

Abstract

Significance: Worldwide demand has driven the development of hemoglobin (Hb)-based oxygen carriers (HBOCs) as potential acellular oxygen therapeutics. HBOCs have the potential to provide an oxygen bridge to patients and minimize current problems associated with supply and storage of donated blood. However, to date, safety and efficacy issues have hampered the approval of viable HBOCs in the United States. These previous efforts have underscored the need for a better molecular understanding of toxicity to design safe and oxidatively stable HBOCs.

Recent Advances: High-resolution accurate mass (HRAM) mass spectrometry (MS) has recently become a versatile tool in characterizing oxidative post-translational modifications that occur in Hb. When integrated with other analytical techniques, HRAM data have been invaluable in providing mechanistic insight into the extent of oxidative modification by quantifying oxidation in amino acids near the reactive heme or at specific “oxidative hotspots.”

Critical Issues: In addition to providing a deeper understanding of Hb oxidative toxicity, HRAM MS studies are currently being used toward developing suitable HBOCs using a “two-prong” strategy that involves (i) understanding the mechanism of Hb toxicity by evaluating mutant Hbs identified in patients with hemoglobinopathies and (ii) utilizing this information toward designing against (or for) these reactions in acellular oxygen therapeutics that will result in oxidatively stable protein.

Future Directions: Future HRAM studies are aimed at fully characterizing engineered candidate HBOCs to determine the most oxidatively stable protein while retaining oxygen carrying function *in vivo*. *Antioxid. Redox Signal.* 26, 777–793.

Keywords: hemoglobin mutants, oxidation reactions, mass spectrometry

Introduction

CIVILIAN (AND MILITARY) blood transfusions are dependent, worldwide, on an extraordinary number of donors (approximately 92 million annually) who provide whole blood and packed red blood cells to roughly 15% of the world’s population. While these transfusions are lifesaving, US hospitals consistently report blood shortages; this situation is much more severe in underdeveloped countries where blood donation rates are less than the United States (WHO summary report on Global Database on Blood Safety [GDBS] 2011) (102a). Another major issue related to this shortage is the imbalance between developing and developed

countries in the level of access to safe blood. For example, 65% of the 92 million donations are collected in the 10 highest income countries. On the contrary, 43 countries in the poorer African Region, where blood-borne illness is more prevalent, collect less than 5% of global donation; this region makes up approximately 12% of the global population (GDBS) (102a). Global blood safety concerns impacting blood supply (most profound in developing countries) include the potential for costly diagnostics when processing blood obtained from patients with infectious disease (*i.e.*, HIV) (6, 12, 29, 44).

The demand for blood donation alternatives has driven the commercial development of Hb-based oxygen carriers

Laboratory of Biochemistry and Vascular Biology, Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, Maryland.

(HBOCs) or “blood substitutes” over the past 30 years (43). Initially, there was a high level of optimism for HBOCs to replace blood transfusion requirements. The benefits of such an accomplishment would potentially eliminate the problems associated with blood availability, reduce the risk of disease transmission, and improve oxygen delivery and need for long-term blood storage (34, 66, 88).

Despite these advantages, no viable HBOCs have been approved in the United States. The primary reason for this stems from multiple reports of adverse cardiovascular and cerebrovascular events associated with hypertension in individuals transfused with these products (79, 86). In hindsight, this should not be surprising since HBOCs (studied to date) are specifically acellular Hb-derived proteins; because they are not “actual blood” they do not perform other blood functions such as nutrient transport, immune response, and coagulation. Preliminary evaluation using animal models suggests that some HBOCs do oxygenate tissues shortly after infusion (3, 15, 86). Current-generation HBOCs are intended therefore to complement standard blood transfusions by acting as an “oxygen bridge” in extreme life-threatening situations, such as battlefield trauma and in surgical settings and/or where patients refuse blood transfusions due to religious reasons (3).

Historical Overview of HbOC Design Strategies

The first attempt to produce the so-called blood substitutes involved transfusion of stroma-free, unmodified acellular Hb

into humans, which resulted in renal dysfunction, abdominal pain, and hypertension (64, 66, 81). Additional analysis of these early studies indicated that diluting Hb into blood plasma resulted in the accumulation of Hb dimers due to rapid tetrameric dissociation; dimeric Hb readily passed through the kidneys resulting in rapid clearance, which can be damaging to kidneys. In addition to this pathology, Hb dimers were characterized to be unstable and therefore susceptible to oxidation, heme loss, and precipitation; these events ultimately led to oxidative stress, blockage of kidney tubules, and ultimately renal failure. Finally, it was also observed that these acellular Hbs scavenge endothelial NO (a vessel dilator) thus negatively impacting vasoregulation and possess oxygen binding behavior vastly different than what occurs in the protective environment of red blood cells (5, 21, 45, 64).

To address these drawbacks, several different biochemical and recombinant strategies (Fig. 1) have been attempted with varying success. Among these included chemically cross-linking human- or bovine-derived Hb using a site-specific bifunctional crosslinker such as di-bromo bis-fumarate (DBBF/also known as diaspirin crosslinked Hb [DCLHb]), polymerization/conjugation with polyethylene glycol polymers (PEGylation), and genetically engineering α -crosslinked recombinant Hb (expressed in bacteria or yeast host systems) (3, 16, 36, 40, 47, 73, 97, 98). These modifications aim at either covalently linking dimers in a stable tetrameric form or in the case of polymerization/conjugation (such as PEGylation) to increase the surface area or hydrodynamic volume; all three modification strategies have been shown to increase

FIG. 1. Recombinant and biochemical strategies for the production of Hb-based oxygen carriers as blood substitutes are illustrated. (A) Tetrameric stabilization accomplished by joining two $\alpha\beta$ heterodimers through intramolecular crosslinking between two α or two β subunits by either genetically engineering crosslinked recombinant Hb or a site-specific bifunctional crosslinker such as DBBF/DCLHb or NFPLP. (B) Effective molecular weight and surface area of Hb are increased by conjugating to polyethylene glycol (PEG-2000 or 5000). (C) Hb polymerization to create molecular weights significantly greater than native tetrameric (64 kDa) is produced when Hbs are reacted with polyfunctional crosslinking agents (e.g., glutaraldehyde, *O*-raffinose, *O*-adenosine). NFPLP, 2-nor-2-formyl pyridoxal-5'-phosphate; DBBF, di-bromo bis-fumarate; DCLHb, diaspirin crosslinked Hb. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

intravascular retention and substantially reduce renal filtration of Hb dimers (4, 17, 25, 80).

To date, several different commercially developed HBOCs (using anyone of the methods in Fig. 1) have been tested in animal and human subjects. These Hbs, in general, demonstrated reasonable preclinical safety and efficacy in healthy volunteers in Phase 1 trials. However, as clinical development advanced into later stages, an undesirable safety and efficacy profile became clearer in the patient population studied leading to the termination of the clinical development of many first-generation HBOCs (8, 35, 52, 59, 73, 75, 83, 87). These HBOCs failed clinically, in large part, due to serious adverse events associated with their infusion in humans attributed to nitric oxide (NO) scavenging by extracellular Hb, heme-mediated oxidative reactions, and oxygen oversupply (100).

The Basis of HBOC Toxicity

Several recent *in vitro* and animal studies showed that Hb oxidation plays a key role in the genesis of many adverse events associated with Hb (chemically and nonchemically modified) (16, 64). Outside the RBC, acellular HBOCs are oxidatively toxic due to heme redox reactivity, which is ironically essential for the oxygen (O_2) carrying function. On O_2 binding, ferrous heme iron ($HbFe^{2+}-O_2$) spontaneously auto-oxidizes to form a non- O_2 carrying ferric heme iron ($HbFe^{3+}$ aka metHb). During this naturally occurring process, reactive oxygen species such as superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are formed, which (in the case of H_2O_2) drives a catalytic pseudoperoxidase cycle that includes the transformation of $HbFe^{2+}-O_2$ to a transient reactive oxyferryl Hb ($HbFe^{4+}=O$) intermediate. The $HbFe^{4+}=O$ species autoreduces back to $HbFe^{3+}$ and in the presence of additional H_2O_2 , the ferryl Fe^{4+} iron is regenerated. However, if the reaction with H_2O_2 begins with the $HbFe^{3+}$, a ferryl protein cation radical ($\cdot HbFe^{4+}=O$) is formed (Equation 1–3) (76). These oxidative processes are well controlled within the RBCs where Hb is continuously reduced to the functional ferrous form by several reductive enzymes (catalase, superoxide dismutase, methemoglobin reductase, *etc.*). However, when left unchecked (*i.e.*, in noncompartmentalized environment), acellular Hb becomes oxidants that damage themselves and nearby biological molecules. These reactions are summarized by the following equations:

This oxidative toxicity is primarily due to the high mid-point redox potentials ($E^\circ_{1/2} \sim 1.0$ V) of the ferryl heme and its associated protein cation radical (76). These internal reactions result in irreversible oxidation of amino acids, areas in the protein known as the “oxidative hotspots” (particularly the β Cys93 side chain) that cause the collapse of β subunits, Hb degradation, and the ultimate release of heme (39). Recent animal studies from sickle cell mice suggest that this resulting heme moiety gives rise to serious consequences, including endothelial cell activation and vaso-

occlusion (9, 14). These studies have also shown that heme released from oxidizing Hb binds to Toll-like receptors (TLR4) that trigger a cascade of inflammatory reactions (9). Consequently, heme has been recognized as a “damage-associated molecular pattern” (DAMP) molecule on par with molecules such as lipopolysaccharides and other inflammatory stimulants (28). Clearly, these internal oxidative pathways (leading to heme release) would have to be curtailed to minimize these inflammatory reactions.

Two-Prong Strategy Toward Engineering Oxidatively Stable HBOCs

The challenge for HBOC developers is to design stable Hbs with optimal biochemical properties to reduce these oxidative toxicities. These include (i) enhancing globin stability for storage by reducing the rate of auto-oxidation and heme loss and (ii) adjusting oxygen affinity; this optimization will also reduce the oxidant potency of HBOCs. To date, one of the most versatile approaches for making candidate HBOCs has been a recombinant strategy which involves (in one case) genetically linked in-frame tandem copies of the α subunit gene to produce a di- α gene with a single glycine linker between the C-terminal end of one α subunit and the N-terminal end of the other in a low oxygen affinity variant (Presbyterian β N108K). This di- α gene is then coexpressed with V1 M β -subunit genes in *Escherichia coli* to produce the recombinant Hb rHb1.1 (47). Crystal structural data of rHb1.1 have indicated that the glycine linker does not perturb the overall tertiary, quaternary, or even local structure of Hb (Fig. 2) (13). The major advantage of using this recombinant strategy is that one can potentially engineer HBOCs with

FIG. 2. Graphic representation of a typical genetically engineered Hb (Optro (rHb 1.1 deoxy)). This recombinantly expressed (in *Escherichia coli*) Hb-based oxygen carrier (Hb Presbyterian [β N108K], blue) is intramolecularly crosslinked via an $\alpha\alpha$ glycine linker (red color). Highlighted (blue) ribbon represents the two linked amino (Val1 and Arg141) acids. Distances were measured and the theoretical model was generated using the PyMOL Molecular Graphics System distance measuring utility and mutagenesis wizard, respectively. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

beneficial amino acid substitutions using mutagenesis strategies (98). To date, rational mutagenesis approaches developed from a “two-prong strategy” (as opposed to random mutagenesis where screening for multiple properties is not feasible) have provided some promising results (98). However, well-documented engineering problems associated with recombinant proteins, such as correct protein folding and heme orientation within the heme pocket, present additional hurdles that protein engineers have to overcome (25).

In the search for favorable amino acid substitutions, our laboratory has relied on studying Hb variants identified in patients with hemoglobinopathies. Naturally occurring human Hb mutants potentially result in protein resistance to oxidative challenges, while others develop into a full circulatory disorder; in this regard, these mutants can be viewed as “Experiments in Nature” (27, 95). Exploring subunit-specific oxidative stability within these mutants may therefore lead to re-engineering of oxidatively stable HBOCs based on a combination of evolutionary stable mutations and rationally designed prototypes (Table 1 for summary of Hb mutants reviewed here). Our “two-prong” strategy involves (i) understanding the mechanism of Hb toxicity by evaluating mutant Hbs identified in patients with hemoglobinopathies and (ii) utilizing this information toward engineering acellular blood substitutes that are more oxidatively stable than native HbA. The implementation of this two-prong strategy for studying Hb variants will be highlighted in the section describing quantitative mass spectrometry for characterizing oxidative post-translational modifications (PTMs).

Approaches to Characterizing HBOCs

Several analytical approaches are used to characterize Hb variants and candidate HBOCs when implementing this strategy. One example of the classic methods for assaying auto-oxidation and denaturation involves incubating rHb variants in an air equilibrated buffer and spectrophotometrically following auto-oxidation and precipitation over time and/or by chemically inducing oxidation (at pH 7 at 37°C)

(11, 39, 63, 84). This review will focus on the use of high-resolution accurate mass (HRAM) mass spectrometry (MS) for characterizing oxidative PTMs that occur in Hb. HRAM analysis is new to the field of characterizing Hb oxidative stability and when integrated with other more familiar methods (*i.e.*, spectrophotometric, stopped-flow spectrophotometric assays) is invaluable in providing mechanistic insight into the extent of oxidative toxicity by quantifying oxidation in amino acids near the reactive heme or at specific oxidative hotspots. A basic introduction to mass spectrometry will be presented followed by examples of HRAM characterization of Hb variants. This review will also discuss promising results for a candidate HBOC designed from principles learned by studying Hb variants.

Introduction to HRAM Mass Spectrometry for the Study of Oxidative Changes and PTMs in Hemoglobins

The postgenomic era has had a huge impact on how biologists and biochemists study proteins. Scientists are now using high-throughput strategies that are versatile for characterizing enriched proteins, protein complexes, and whole proteomes, which not only identify and quantify proteins but can also identify a wide variety of PTMs, under a variety of experimental conditions (54). The most commonly used and extremely powerful technique for the high-throughput characterization of proteins, peptides, and PTMs is liquid chromatography coupled online with mass spectrometry (LC-MS) (38, 54, 85). By combining the power of bioinformatics, chromatography, and HRAM mass spectrometry, LC-MS is a versatile tool capable of comprehensive protein identification and in-depth characterization of PTM changes from complex biological samples (55).

Major Components of Mass Spectrometer

Mass spectrometers (MS) are analytical instruments that measure ionized analytes in the gas phase. The basic setup for a mass spectrometer consists of an ion source, a mass

TABLE 1. SELECT HUMAN HEMOGLOBIN MUTANTS AND RELEVANCE TO HEMOGLOBIN-BASED OXYGEN CARRIERS

<i>Hb</i>	<i>Mutation position</i>	<i>Substitution</i>	<i>Clinical features</i>	<i>Biochemical properties</i>	<i>Relevance to HBOCs</i>
Providence (1)	β 82 (EF6)	Lys $\rightarrow$ Asn $\rightarrow$ Asp	Erythrocytosis	Oxidative stability	Hb Providence-based HBOC
Toms River (89)	γ 67 (E11)	Val $\rightarrow$ Met $\rightarrow$ Asp	Anemia; cyanosis	Oxidative instability; Met $\rightarrow$ Asp (PTM)	Comparison of α , β , and γ to probe oxidative stability
Bristol-Alesha (89)	β 67 (E11)	Val $\rightarrow$ Met $\rightarrow$ Asp	Hemolytic anemia; reticulocytosis	Oxidative instability; Met $\rightarrow$ Asp (PTM)	Comparison of α , β , and γ to probe oxidative and structural stability
Evans (89)	α 62 (E11)	Val $\rightarrow$ Met	Hemolytic anemia	No Met $\rightarrow$ Asp (PTM)	Investigate unusual α stability
HbS (42)	β 6 (E1)	Glu $\rightarrow$ Val	Sickle cell disease; vaso-occlusion hemolytic anemia; and so on	Oxidative instability; deoxy HbS polymerizes	Investigate oxidative instability
HbE (90)	B26 (E8)	Glu $\rightarrow$ Lys	Minimal microcytosis; anemia: severe when coupled to β -thalassemia	Oxidative instability accelerated heme loss?	Investigate oxidative instability and to understand impact on β -thalassemia

FIG. 3. Basic setup for a mass spectrometer and mass analyzers currently used with ESI-based mass spectrometers.

(A) The basic setup for a mass spectrometer consists of an ion source, a mass analyzer that measures the mass-to-charge ratio (m/z) of gas-phase ions, and a detector that registers the number of ions at each m/z value. Most MS configurations are generally coupled online to a UPLC or HPLC system. In this illustration, ESI is shown where the ESI source is located at the spray needle. (B) Commonly used mass spectrometer configurations (for protein/PTM identifications) with one or more of these analyzers are illustrated. (1) The ion-trap first captures ions (for a short time interval) and subjects them to either MS or fragmentation analysis (MS/MS). (2) The Q-TOF mass spectrometer typically involves ion selection via a quadrupole mass filter (Q) followed by fragmentation in a collisional cell (q_2); the fragment ions then travel through the TOF analyzer at a rate proportional to their m/z ratio. (3) The FT-ICR analyzer captures ions under high vacuum in a high magnetic field; the ions orbit within the cyclotron at a frequency proportional to their m/z ratio. (4) The Q-Exactive mass spectrometer involves ion selection with a quadrupole mass filter, ion capture in the Orbitrap analyzer followed by fragmentation in a HCD cell, and analysis in the Orbitrap. ESI, electrospray ionization; FT-ICR, Fourier transform ion cyclotron; HPLC, high-performance liquid chromatography; PTM, post-translational modification; TOF, time-of-flight; UPLC, ultra-performance liquid chromatography. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

analyzer that measures the mass-to-charge ratio (m/z) of gas-phase ions, and a detector that registers the number of ions at each m/z value (Fig. 3A). For electrospray ionization (ESI-MS), this MS configuration is generally coupled online to an ultra-performance or high-performance liquid chromatography system (UPLC and HPLC, respectively), which separates the peptide analyte; as peptides are eluted from the column (see section “HPLC Online Peptide Separation”), they are ionized by the electrospray source and then directly emitted into the mass spectrometer (at the spray needle or emitter tip).

Electrospray and Matrix-Assisted Laser Desorption/Ionization

The most common ion sources for peptide and protein identification utilize either ESI, which is displayed in Figure 3A, or matrix-assisted laser desorption/ionization (MALDI). These soft ionization methods (developed in the 90s and early 2000s) revolutionized the field of mass spectrometry as they allowed for the nondestructive ionization of macromolecular structures (*i.e.*, peptides or proteins) (23, 41). Before their development, hard ionization (*i.e.*, electron

and chemical ionization) limited the utility of mass spectrometry to the analysis of small volatile molecules stable at temperatures required for vaporization (99). However, ESI and MALDI ionization convert macromolecular structures (including peptides, proteins, lipids, carbohydrates, and nucleic acids) into ions with minimal chemical degradation (23, 41, 99).

Specifically, ESI involves applying high voltage, *via* ionization source at spray needle (Fig. 3A), to a liquid aqueous/organic/acid mixture to create an aerosol of droplets that are then emitted into the mass spectrometer through a heated capillary carrying a potential difference in atmospheric pressure (due to vacuum). Immediately after emission, the droplet surface area decreases due to solvent evaporation; as the droplet surface area decreases, the positively charged peptide ions eventually experience a columbic explosion (due to charge repulsion) resulting in desorption of solvated ions (67). MALDI, in contrast, desorbs (*via* sublimation) and ionizes the analyte from a crystalline matrix using a directed laser pulse. Traditionally, MALDI is used to analyze relatively simple peptide mixtures. The application of ESI for studying proteins far exceeds the use of MALDI because ESI ionizes analytes out of a solution and as a result is more amenable to liquid-based chromatographic separation tools. The primary focus of this review will, therefore, be on MS methods that utilize ESI coupled online with liquid chromatography (LC-MS).

Mass Analyzers

The mass analyzer is a central component for HRAM MS methods. In the context of peptide/protein and PTM identifications, its key parameters are sensitivity, dynamic range, duty cycle, resolution, mass accuracy, and the ability to generate sequence information by virtue of fragmenting peptides (tandem or MS/MS spectra) (2, 70). There are four major types of mass analyzers currently used with ESI-based mass spectrometers. These include the linear ion trap, time-of-flight (TOF), Fourier transform ion cyclotron (FT-ICR), and the Orbitrap analyzers. All four (uniquely different in design and performance) can be the principle analyzer but are generally featured in tandem as part of hybrid mass spectrometers that combine the strengths of each analyzer.

Commonly used mass spectrometer configurations (for protein/PTM identifications) with one or more of these analyzers are illustrated in Figure 3B. Briefly, the linear ion-trap (Fig. 3B-1) first captures ions (for a short time interval) and subjects them to either MS or fragmentation analysis (MS/MS) *via* collisional-induced dissociation (CID) (32). Linear ion traps are a common component of HRAM hybrid mass spectrometers such as the LTQ-Orbitrap (50, 51, 82). TOF analyzers (traditionally coupled with MALDI ion sources) are most often used with the HRAM hybrid Q-TOF (quadrupole-time-of-flight). A typical configuration for a Q-TOF mass spectrometer (Fig. 3B-2) involves ion selection *via* a quadrupole mass filter (Q) followed by fragmentation in a collisional cell (q2); the fragment ions then travel through the TOF analyzer at a rate proportional to their m/z ratio (19, 46). The FT-ICR analyzer captures ions under high vacuum in a high magnetic field (similar to an NMR instrument); the ions orbit within the cyclotron at a frequency proportional to their m/z ratio (56, 57). The FT-ICR mass spectrometer (Fig. 3B-3) has the best resolving power for analyzing intact proteins.

Finally, the Orbitrap analyzer (type of ion trap analyzer) consists of an outer barrel-like electrode and a coaxial inner spindle-like electrode that trap ions in an orbital motion around the spindle (49). Two major Orbitrap configurations include coupling the Orbitrap to a linear ion trap analyzer (LTQ-Orbitrap) or quadrupole mass filter (Q-Exactive) (50). A typical LTQ-Orbitrap configuration involves ion capture in the linear trap, analysis in the Orbitrap analyzer, and then ion fragmentation (MS/MS) *via* CID in the linear trap (50). The Q-Exactive mass spectrometer (Fig. 3B-4) involves ion selection (and isolation) with a quadrupole mass filter, ion capture in the Orbitrap analyzer followed by fragmentation in an HCD cell, and analysis in the Orbitrap (26).

Step 1: LC/MS/MS Analysis: The Bottom-Up Approach (LC/MS/MS) for Identifying Proteins and PTMs

Two HRAM methods are the frequently applied “bottom-up” (also known as shotgun proteomics) and the less widely used “top-down” strategies. An essential component of the quantitative methods described in this review, the bottom-up strategy involves enzymatic digestion of intact proteins into peptides that are then analyzed by LC-MS. The resulting peptides are fragmented by CID or HCD (higher energy C-trap dissociation) to generate MS/MS spectra that are then analyzed by database search algorithms (such as Mascot/SEQUEST) for sequence information (22, 71, 103). The ion current from reproducibly matched peptides (or fragment ions) can then be used for label-free quantification of Hb oxidation (discussed in Step 2). Key features of the bottom-up strategy (Fig. 4) are discussed in the following sections. The alternative top-down strategy involves direct analysis and/or fragmentation of intact proteins by the mass spectrometer to potentially identify protein isoforms (from complex mixtures) resulting from translational start and stop sites, mRNA splice variants, and PTMs of expressed gene products.

A major advantage of the bottom-up method is that peptide MS/MS spectra are much more efficiently analyzed than top-down fragmentation spectra of intact proteins; to date, the bioinformatic software required to interpret top-down spectra lags far behind algorithms designed for interpreting peptide MS/MS spectra. The bottom-up strategy is therefore much more effective at systematically identifying a large number of proteins (*via* identified peptides) and their corresponding PTMs from complex biological samples. There are two different methods for conducting bottom-up MS proteomic measurement. The first implementation uses one-dimensional (1D) or two-dimensional (2D) gel electrophoresis followed by in-gel digestion and either peptide mass fingerprinting or tandem mass spectrometry (1D LC MS/MS) to identify peptides that are matched to their corresponding proteins. The second more high-throughput approach involves in solution direct proteolytic digestion of complex mixtures (enriched protein, protein complexes, or whole proteomes) followed by 1D or 2D liquid chromatography coupled online with ESI MS/MS. The latter 1D LC MS/MS implementation is more commonly used in the quantitative examples described later in this report.

Enzymatic Digestion of Intact Proteins into Peptides

As stated above, peptide MS/MS spectra are more efficiently analyzed (relative to fragmentation spectra of intact

proteins) for PTM identifications. Before LC-MS analysis, it is therefore critical to optimally digest protein samples toward maximizing the number of identifiable peptide isoforms (a peptide can exist unmodified or contain PTMs) in a given sample (Fig. 4). Important to MS/MS fragmentation is the use of site-specific proteases during the enzymatic digestion of a protein sample. Although a variety of different digestive enzymes are available for generating peptides, trypsin is the most commonly used protease because it cleaves C-terminally at lysine and arginine with a remarkable degree of specificity. The frequency of lysine and arginine in proteins generally results in a decent number of peptides that are the ideal size for gas-phase fragmentation (between 5 and 30 amino acids) (92). Furthermore, trypsin cleavage results in peptides with positive C-terminally protonated ends, beneficial for CID fragmentation; trypsin digestion results in a large frequency of +2 charged peptides (and +3 charge) that fragment well to produce product ion spectra that are very informative. These characteristics of trypsin generally increase the number of identifiable peptides (reflected by the primary sequence covered) per LC-MS analysis.

While LC-MS analysis of trypsin digestions most often reveal oxidative hotspots containing Hb peptides (see section "Database Searches of MS/MS Fragmentation Spectra"), alternative digestion strategies are commonly utilized for the purpose of mapping PTM identifications. One such strategy involves digestion with multiple enzymes; this approach increases the likelihood of generating additional peptides that are missed by one of the other proteases and increases the confidence of PTM identification when the same PTM is identified by overlapping peptides generating from more than one protease (48). In scenarios where the protein of interest has a low frequency of lysine or arginine residues (*i.e.*, the transmembrane spanning domains), other cleavage strategies can be used, including the use of cyanogen bromide cleavage or the use of other proteases such as chymotrypsin (cleaves C-

terminally at aromatic amino acid residues) or pepsin (cleaves nonspecifically) (92).

HPLC Online Peptide Separation

Due to the complex nature of biological samples, online chromatographic separation is important for improving sensitivity and dynamic range for bottom-up peptide identifications. Dynamic range here refers to the ability to detect low-abundance peptides/proteins in the presence of high-abundance peptides/proteins. The goal of HPLC separation (before MS and MS/MS analyses) is to therefore simplify the mixture of peptides presented to the mass spectrometer at any given point in time. Indeed, direct infusion (without online chromatography) of a peptide mixture into the mass spectrometer would result in limited detection of only the most abundant peptides and those with the highest ionization efficiency (53). The separation of this complex mixture of peptides *via* HPLC allows for the mass spectrometer to interrogate a much wider range of peptides and thus provide sequencing and PTM detection of low-abundance peptides (from low-abundance proteins or low-abundance PTM containing peptides).

In a typical LC/MS/MS experiment, the peptide mixture is loaded onto an HPLC column (typically a C18 reversed-phase column that separates peptides based on hydrophobicity) *via* an autosampler. Peptides are eluted from the column *via* gradient elution and subsequently ionized into the mass spectrometer *via* an electrospray emitter (directly connected to the HPLC column or part of the HPLC column to minimize dead volume). During the entire chromatographic separation, the mass spectrometer is acquiring full-scan intact peptide masses and subsequently isolating and fragmenting the peptides to produce MS/MS spectra. Because ESI efficiency is greatly enhanced at lower flow rates (low flow rates generate much smaller initial droplets), modern online HPLC

system components are scaled down to provide flow rates at the optimal nl/min range (61, 102). For single-dimension chromatography (1D), online separation columns most often consist of reversed-phase (RP) stationary phases. RP columns (typically C18) are hydrophobic; 1D peptide separations therefore involve the use of an organic mobile phase (acetonitrile) to elute peptides as a function of hydrophobicity. For 2D chromatographic separation, the 2nd dimension column generally consists of a strong cation exchange (SCX) column and RP in the 1st. A 2D configuration (generally only used for extremely complex samples) requires incremental ionic mobile-phase elution (off the SCX column) followed by organic mobile-phase elution for the 1D.

Database Searches of MS/MS Fragmentation Spectra

MS/MS fragmentation spectra provide sequence information that is searched against genomic databases (described in the next section). As stated, HRAM mass spectrometers utilize collision-induced dissociation CID (or HCD) to fragment peptides. CID fragmentation energizes peptide ions (precursor ion) *via* collisions with gas molecules (such as helium or nitrogen) to produce predominantly y and b ions (although fragmentation also results in some a/x and c/z ions) (69, 94). A typical MS/MS spectrum therefore represents y and b ion series (among other less populated ion types) of a fragmented peptide at various positions along the peptide backbone (103). Experimentally derived MS/MS fragmentation spectra (Fig. 4) are searched by database algorithms such as SEQUEST, Mascot, MyriMatch, and X-tandem (just to name a few) against predicted protein databases (generally created from genome annotation) to identify which peptide they represent (18, 22, 71, 91). The basic function of a database algorithm is to interrogate an MS/MS spectrum and identify the most likely peptide sequence matching (based on the y and b fragment ions) the spectrum from a provided translated genomic database.

There are four main processes involved in peptide identification from MS/MS spectra *via* database search algorithms: *in silico* peptide list generation from the database, candidate sequence generation, theoretical spectrum prediction, and scoring. Depending on the database and enzyme specificity, the above database algorithms first create a list of peptide sequences. For example, if the researchers used trypsin on a human blood sample (to generate a complex mixture of tryptic peptides), they would configure the algorithm to generate a complete list of all tryptic peptides from the predicted human proteome database (such databases can be easily downloaded from UniProt and other online genome databases). The algorithm next takes MS/MS spectra, extracts m/z values, and then compares these m/z values to calculated m/z values derived from peptides produced by the *in silico* list. A theoretical spectrum is then generated and compared to the experimentally derived spectrum and a score is assigned (18, 93). It is up to the researcher to decide what scores are considered probable matches. The researcher then utilizes additional filtering software (*i.e.*, Scaffold software) that groups all high-scoring peptide matches to their corresponding protein.

An important step before PTM quantification (discussed in the next sections) is to first reproducibly identify which peptides contain PTMs; this of course will reflect where on the protein sequence the modification occurs. Database

search algorithms are also used to identify peptides with PTMs; to do this the researcher specifies the change in mass associated with the modified form on a particular peptide residue (such as 48 Da shift in mass associated with tri-oxidation of cysteine residues). When the algorithm generates candidate sequences, it considers either of two masses possible for the specified residues. This allows the algorithm to match and score peptides that would otherwise be missed by looking for MS/MS that matched to peptides predicted by genomic sequence. Peptides identified with PTMs can then be further studied using quantitative methods.

Step 2: Quantitative Approach for Characterizing Hb Oxidative PTMs

This section illustrates how quantitative HRAM mass spectrometry has recently been used to provide mechanistic insight into the extent of oxidative changes within hemoglobin variants. First, we describe recent studies evaluating several mutants identified in patients with hemoglobinopathies and show how information gleaned from these studies was utilized toward the design of oxidatively stable HBOCs (blood substitutes). Table 1 provides a list of Hb variants recently investigated in our laboratory (1, 42, 62, 89). Information from several of these studies has been utilized toward the design of candidate acellular blood substitutes (discussed at the end of this section). To date, Hb mass spectrometry laboratories (with an interest in Hb oxidation studies) utilize a quantitative strategy similar to the one shown in Figure 5 (or some minor variation of it) (10, 74, 89, 96, 104). For example, our laboratory generates extracted ion chromatograms of oxidative hotspot containing peptides directly from full-scan data; however, a targeted approach using select ion monitoring can also be used to quantify peptides identified from the bottom-up approach (96). As previously stated, the first step in all Hb quantitative experiments involves implementation of the bottom-up strategy (LC/MS/MS analysis) to identify modified/oxidized Hb residues. After identifying oxidatively modified peptides, the second step involves a label-free method of quantifying the abundance of the oxidized peptide relative to the unmodified peptide; comparative differences in this ratio (relative to native HbA) provide insight into oxidative stability.

HRAM Analysis Provides Mechanistic Insight into Subunit-Specific Post-Translational Transformation of Methionine to Aspartate

In a recent report from our laboratory, Strader *et al.* (89) provide an example of how this quantitative mass spectrometric approach was used to characterize the impact of positionally equivalent E11 methionine (E-helix is adjacent to the reactive iron in the distal heme pocket) mutations in three recombinant Hb variants; Toms River (γ Val67Met) (γ V67M), Alisha Bristol (β Val67Met) (β V67M), and Hb Evans (α Val67Met) (α V62M) (89) (Table 1). The origin of this study began with a focus on characterizing the fetal Hb variant Hb Toms River (γ V67M) from an infant girl in Toms River, New Jersey; the patient was heterozygous for the V67M mutant allele. In addition to identifying the γ V67M isoform, 1D/LC/MS/MS bottom-up analysis (step one in Fig. 5) also confirmed the presence of an unexpected γ V67D variant in the patient hemolysate (Fig. 6A, B); based on DNA

FIG. 5. Schematic diagram of label-free quantitative mass spectrometry strategy. Schematic diagram illustrating the basic workflow for quantitative mass spectrometry utilized toward characterizing Hb-based oxygen. Peptides are chromatographically separated, ionized *via* electrospray, and analyzed (*top left corner*) during a gradient run. The total ion chromatogram represented in the *top right corner* consists of thousands of MS (full scan) and MS/MS spectra that are first analyzed by Step 1 (bottom-up strategy) to identify oxidized Hb peptides followed by Step 2, a label-free method of quantifying the abundance of oxidized peptides relative to the unmodified peptide. To see this illustration in color and view labels, the reader is referred to the web version of this article at www.liebertpub.com/ars

sequencing, the presence of this variant could only occur as a consequence of post-translational oxidation of γ M67.

We reasoned that Met transformation to Asp was driven by an oxidative event that emanated from the nearby heme group. As outlined in the second step of Figure 5, an integrated quantitative mass spectrometric approach was then utilized to explore the relationship between increasing H_2O_2 levels and γ Met-67 post-translational conversion to V67D; the results established a direct linkage between accelerated auto-oxidation (the rate was seven times faster than native HbA) and the Met(E11) modification. Figure 6C and D shows how HRAM data from a hybrid Q-Exactive Orbitrap mass spectrometer (illustrated in Fig. 3B-4) were utilized to generate extracted ion chromatograms (XICs) for quantifying the impact of increasing H_2O_2 on γ Met-67 (the example in Fig. 3C, D corresponds to the singly charge D67 containing peptide DLTSLGDAIK). Specifically, XICs were generated from the most abundant monoisotopic peak of all Met-67 (thioether, sulfoxide, and sulfone) and Asp-67 peptide isotopic profiles using a designated mass tolerance of 0.01 Da, and mass precision set to three decimals. For relative quantification, the ratio of each Asp-67 or Met-67 peptide isoform was calculated based on the sum of the XIC peak area from all forms, which was normalized to be 100%. The experimental conditions and results from these XIC studies show that increasing H_2O_2 concentrations resulted in higher V67D levels (Fig. 6E). It is important to note that precursor isotopic resolution (that HRAM instruments provide) is required for the type of XIC construction described above; generating XICs from the most abundant isotopic peak results in XICs with superb signal to noise ratio and eliminates the potential for ion current from coeluting ions to interfere with data analysis. High-resolution data were also required in $\text{H}_2^{18}\text{O}_2$ follow-up experiments aimed at further examining the direct role of H_2O_2 in V67D formation. Impressively, experimental

conditions with labeled $\text{H}_2^{18}\text{O}_2$ and HbF γ V67 M resulted in Met-67 and Asp-67 (peptide isotopic profile shown in Fig. 6F) containing peptides with incorporated ^{18}O , which correlates with the results shown in Figure 6E and confirms that the source of oxygen for the Asp modification comes from H_2O_2 (a proposed mechanism is discussed in the article (89)).

This study was expanded to characterize the impact of positionally equivalent E11 methionine mutations in recombinant β V67 M (Hb Bristol-Alesha) and α V62 M (Hb Evans) variants. Met $\rightarrow$ Asp conversion was identified in the β (E11) but not in the α (E11) variants under similar experimental conditions. The initial crystal structures of the three recombinant Met(E11) HbCO forms show similar active site thioether side-chain orientations (Fig. 7A, B), with the γ side-chain carbon atoms all being roughly 5.0–5.3 Å from the heme iron. However, in agreement with solution experiments, auto-oxidation of the crystals over a period of weeks revealed electron density maps indicative of Asp(E11) formation in the active sites of the HbA β V67 M mutant but not in the active site of the HbA α V62 M variant (Fig. 7C, D). These findings demonstrate the unique redox reactivity of the γ and β heme iron atoms, which leads to post-translational oxidative conversion of amino acid side chains within these subunits, but not within the α subunits of human Hb. We went on to show that Asp was formed by an oxidative mechanism involving the ferryl heme complexes. Interestingly, ferryl Hb complexes were seen in Hb Bristol-Alesha (Fig. 7C, D), however, in the case of Hb Evans, the internal electron pathway may have minimized the accumulation of sufficient quantities of ferryl heme iron to drive this transformation.

Comparative HRAM Analysis of Oxidation in HbS and HbA

As previously stated, internal oxidative pathway reactions result in irreversible oxidation of amino acid in oxidation

MGHFTTEEDKATITSLWGKVNVEDAGGETLGRLLVYPWTQRRFFDSFGNLSSAS
 AIMGNPKVKAHGKK**MLTSLGDAIK**HLDDLKGTFAQLSELHCDKLHVDPENFKLL
 GNVLTVLAIHFGKEFTPEVQASWQKMTGVSALSSRYH

FIG. 6. Mass spectrometric characterization of Toms River's patient blood and the recombinant Hb γ Val67Met counterpart. Displayed above the panels is the primary sequence of V67 M showing the tryptic peptide (highlighted in red) with position M67 (highlighted in blue). All charge states of this peptide sequence were the target of our quantitative proteomic approach. Fragmentation spectra of the singly charged +1 γ V67 M (**A**) and γ V67D tryptic peptides representing residues 67–76 from patient hemolysates are shown in (**B**). Both spectra show singly charged y and b fragment ions. (**C**) Isotopic profile of the singly charged γ V67D tryptic peptide listed above. (**D**) Extracted ion chromatogram (XIC) of γ V67D tryptic peptide (residues 67–76). XICs were generated from the most abundant monoisotopic peak represented in the isotopic profile of each targeted peptide. (**E**) Histogram representing the fold-change differences of V67D for experimental conditions 1–6 (1: CO ligand control, 2: control (air equilibrated buffer), 3: 1:1 H_2O_2 , 4: 2.5:1 H_2O_2 , 5: 5:1 H_2O_2 , 6: Ferric/5:1 H_2O_2). These data indicate M to D conversion increases with increasing H_2O_2 . (**F**) The isotopic profile represented by labeled isotopologous peptides after oxidation with $H_2^{18}O_2$. The most abundant isotopic peak (M+H) of 1036 m/z represents the γ V67D tryptic peptide with 2 ^{18}O labeled carboxyl oxygen molecules. The 1034.56 m/z peak represents the γ V67D tryptic peptide with a mixture of $^{18}O/^{16}O$ labeled carboxyl oxygen, and the lowest abundance 1032.56 m/z represents the unlabeled peptide (M + H). ^{18}O mono-substituted and unlabeled γ V67D tryptic peptide, respectively. This research was originally published in a study by Strader *et al.* (89) © the American Society for Biochemistry and Molecular Biology.

FIG. 7. Structures of the active sites of Hb Toms River γ V67M, Hb Bristol-Alesha β V67M, and Hb Evans α V62M. (A) Electron density of γ MetE11 in HbCO Toms River 2Fo-Fc, refined electron density (4MQK). (B) Comparisons of MetE11 side-chain orientations in refined models for the CO forms of Hb γ V67M (4MQK), Hb β V67M (4MQG), and Hb α V62M (4MQC). (C) Oxidation of Hb β V67M crystals (HbCO form, 4MQG; metHb form after 4 weeks, 4MQI). The electron density suggests the appearance of D at E11 position due to oxidation. The ligand electron density also suggests a ferryl complex with a single coordinated distal atom rather than aquomet complex with a spherical water O atom about 2 Å away from the central iron atom. (D) Oxidation of α V62M crystals (HbCO form, 4MQC; metHb form after 7 weeks, 4MQH). There was no change in the electron density for the α E11 side chain in the oxidized Hb indicating no modification of the M62 residue. However, the coordination in the wild-type met- β active site again suggests a significant fraction of ferryl complex. This research was originally published in a study by Strader *et al.* (89) © the American Society for Biochemistry and Molecular Biology.

hotspots, regions (particularly the β Cys93 side chain) that cause the collapse of β subunits, Hb degradation, and the ultimate release of heme (39, 72). The quantitative strategy outlined in Figure 5 has also been recently used to quantify the levels of irreversible oxidation of hotspot residues, including β Cys93 in the presence of H_2O_2 (10, 72, 74, 96, 104). This surface amino acid has previously been shown to be an important endpoint for free radical-induced protein oxidation (72).

HbS (β 6Glu $\rightarrow$ Val substitution, linked to sickle cell disease [SCD]) is known to be unstable *in vitro*, is less stable than HbA (on exposure to heat, oxidants), and autoxidizes at faster rates than HbA (7, 33, 58). In a recent study by our group, Kassa *et al.* (42) indicated that chemically induced oxidation of HbS resulted in higher accumulation of the highly reactive ferryl HbFe $^{4+}$ (due to slower autoreduction of ferryl to the ferric form) in HbS solutions (42). HRAM

quantitative MS analysis was also used in this HbS study to compare differences in the post-translational oxidation of β subunit hotspot residues for both HbS and HbA under increasing H_2O_2 conditions (42). Amino acid residues identified by LC-MS/MS analysis in this study correlated well with previously published data; the most prevalent oxidative changes were found to be restricted to peptides containing β Cys93 (10, 72, 74, 96, 104). XICs for the β Cys93 containing peptide GTFATLSELHCDKLHVDPENFR (Fig. 8A) were used to construct the XIC in Figure 8B. Because β Cys93 residue can either be oxidized or unoxidized, the percentages of both isoforms were calculated based on the sum of the XIC peak area from all charged forms of β Cys93 peptides. The HRAM quantitative results from this study indicated that HbS β Cys93 is substantially more oxidized (>2-fold difference when the ratio of H_2O_2 to heme equivalent is 5:1)

FIG. 8. Isotopic profile and XIC of β Cys93 tryptic peptide are illustrated. XICs were generated from the most abundant monoisotopic peaks of each isotopic profile. (A) Representative isotopic profile of the triply charged β C93 tryptic peptide GTFATLSELHCDKLHVDPENFR. (B) XIC of β C93 tryptic peptide (residues 83–104). (C) LC-MS/MS deconvoluted spectrum of β C93 tryptic peptide from HbS with DMPO adducts. The reaction solution of HbS with H_2O_2 in the presence of DMPO was digested with trypsin and analyzed by LC-MS/MS. The fragmentation spectra of the triply charged DMPO-labeled β C93 tryptic peptide GTFATLSELHCDKLHVDPENFR are 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 Cys93. This research was originally published in a study by Kassa *et al.* (42) © the American Society for Biochemistry and Molecular Biology. XIC, extracted ion chromatogram.

in the presence of excess H_2O_2 compared to HbA. This dose-dependent increase correlated (with previous reports) with the oxidation kinetic data providing further evidence that HbS oxidation results in a higher accumulation of the highly reactive ferryl HbFe^{4+} (and protein radical).

To further confirm that ferryl ions and globin-centered radicals are more abundant in HbS, HRAM quantitative experiments were performed using the spin trap label 5,5-dimethyl-1-pyrroline N-oxide (DMPO). The exposure of Hbs to H_2O_2 has previously been shown to initially produce a porphyrin cation radical (and ferryl ion) that oxidizes cysteinyl and tyrosyl amino acids to form globin-centered radicals (20,

31, 60, 96). The spin trap DMPO reacts with these modified amino acids to form a nitroxide radical that is further oxidized to the corresponding globin radical-derived nitron adduct by the ferryl moiety (20, 31, 60). These DMPO-derived adducts are stable under fragmentation conditions used in LC-MS/MS analysis. Analyses of LC/MS/MS and full MS data in the presence of H_2O_2 (5- and 10-fold excess relative to heme) identified DMPO-labeled Cys112 and Tyr42 α peptides with the addition of H_2O_2 . Among the three DMPO-labeled amino acids, only Cys93 was substantially different between HbA and HbS. DMPO Cys93 labeling (Fig. 8C) in the absence of H_2O_2 was at negligible levels ($\sim 1\%$ for both HbA and HbS).

The degree of DMPO labeling was considerably more abundant for HbS than HbA (>2-fold when the ratio of H_2O_2 to heme equivalent is 5:1). 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. We have postulated that dominant Cys93 oxidation in sickle cell Hb is due, in part, to the persistence of its ferryl and radical species. Thus, highly oxidizing ferryl Hb and heme may be central to the evolution of vasculopathy in SCD and suggest therapeutic modalities that interrupt heme-mediated inflammation (42).

The Role of Unpaired Alpha Subunits in Destabilizing HbE

β -thalassemia (caused by chromosome 11 β -globin gene alterations) is characterized by reduction or absence of β -globin chain synthesis (101). HbE ($\beta 26\text{Glu} \rightarrow \text{Lys}$) is an oxidatively unstable human Hb often coinherited together with a β -thalassemia allele; the resulting HbE/ β -thalassemia condition is often severe requiring multiple transfusions (24, 37). Because of HbE instability, a higher level of unpaired α subunits within HbE containing red cells has been proposed to cause the pathogenesis associated with HbE/ β thalassemia (77, 78). However, it is experimentally challenging to determine the effects of free uncomplexed α subunits in the presence of another Hb tetramer (*i.e.*, pairs of $\alpha\beta 2$ of either HbA or HbE). Because we have previously identified βCys93 , as the main target of heme radical chemistry, we recently performed a comparative study that monitors the effects of excess α subunits on βCys93 oxidation within both HbE and HbA (90). Using the same HRAM quantitative mass spectrometric strategies described for HbS characterization (including the spin trap, DMPO), we quantified irreversible oxidation of βCys93 to reflect oxidative instability of β subunits. In the presence of free α subunits and H_2O_2 , βCys93 oxidation increased with higher H_2O_2 concentrations for

both HbA and HbE. In the presence of alpha-hemoglobin stabilizing protein (AHSP), which stabilizes the α -subunit in a redox-inactive hexacoordinate conformation (thus the α subunit is unable to undergo the redox ferric/ferryl transition), Cys93 oxidation was substantially reduced in both proteins. These experiments establish two important features that may have relevance to the mechanistic understanding of these two inherited hemoglobinopathies, that is, HbE/ β thalassemia: First, a ferryl and ferryl protein radical target βCys93 of both proteins but results in the destabilization of HbE due to its persistent ferryl species. Second, in the presence of excess free α -subunit and under the same oxidative conditions, these events are substantially increased for HbE compared to HbA and may therefore create an oxidative milieu affecting the already unstable HbE.

Step 3: Engineering Oxidative Stability in Human Hemoglobin Based on Rationally Designed Proteins

As stated, the first part of the two-prong strategy is to utilize information from Hb variant studies toward the rational design of acellular blood substitutes that are more oxidatively stable than native HbA (and chemically modified Hbs). We have begun to engineer HBOC prototypes based on naturally occurring human mutant Hbs and on rational design (65, 98).

The first of these variant studies involves the variant Hb Providence (Table 1); a mutant identified in a patient at Providence hospital in Washington DC. The patient was heterozygous for the allele expressing the $\beta\text{K82 N}$ variant; due to spontaneous deamidation of asparagine to aspartic acid, two β -subunit variants with the single amino acid mutations $\beta\text{Lys82} \rightarrow \text{Asn}$ ($\beta\text{K82 N}$) and $\beta\text{Lys82} \rightarrow \text{Asp}$ (βK82D) were identified in the patient hemolysate. We have previously separated Hb Providence into its three component fractions and contrasted oxidative reactions of these β -mutant fractions with HbA. The Hb Prov. $\beta\text{K82 N}$ variant oxidation reaction kinetics and ligand binding properties were similar to HbA;

FIG. 9. Pseudoperoxidase catalytic of Hb. Spectral analysis H_2O_2 oxidation reactions: Absorbance spectra obtained at intervals of 2 min for 1 h on treatment of (A) HbA and (B) βK82D with 30:1 H_2O_2 /heme equivalents. Note that while HbA undergoes the transition from ferrous to ferryl heme, spectral changes indicative of oxidative modification and some denaturation were evident, whereas in the case of Hb Providence (βK82D), H_2O_2 was consumed more efficiently as ferryl reverted back to ferric with little or no oxidative changes. (A, B) Reprinted with permission from Abraham *et al.* (1).

however, the β K82D fraction was more effective in consuming H_2O_2 and appeared more oxidatively stable. The spectrophotometric data in Figure 9 show that ferryl β K82D, unlike native HbA, reverts back to the ferric state more readily (HbA shows oxidative damage), suggesting that β K82D safely internalizes radicals (through the ferric/ferryl pseudoperoxidase cycle) better than HbA (1). The β K82D amino acid substitution has recently been reported to improve expression levels of soluble recombinant Hb in *E. coli* (30). Taken together, the structural and oxidative stability of Hb Prov. β K82D may assist in reducing auto-oxidation, oxidative modifications, and heme loss; such features are important goals in the design of oxidatively stable HBOCs. In collaboration with Dr. John Olson at Rice University in Houston, TX (using the rational engineering approach discussed earlier in this review), a genetically crosslinked di- α gene (see section describing two-prong strategy) β K82D Hb (rHb0.1) has recently been engineered. Preliminary kinetic, crystal structure and HRAM quantitative mass spectrometry data of β K82D rHb0.1 indicate that this candidate HBOC is structurally similar to and autooxidizes at similar rates to native HbA yet maintains exceptionally higher oxidative stability in the face of higher levels of peroxide (Strader M, unpublished data). Additional studies are underway to determine how the β K82D substitution impacts oxygen carrying function and if the observed oxidative stability reduces the rate of heme loss relative to control HbA.

Conclusion

Blood substitutes have long been a dream for physicians, researchers, and industrial scientists alike, because they can be stored for a long time, potentially save lives without waiting for typing, and substantially reduce the risk of infection. Despite these advantages, no viable HBOCs have been approved in the United States due, in large part, to clinical trial reports of serious adverse events (79, 86). To address these toxicological responses, several different biochemical and recombinant strategies have been attempted with limited success. A major factor for this limited success is that outside the RBC, acellular HBOCs are oxidatively toxic due to the reactive heme, which is ironically essential for oxygen binding. The two-prong strategy that we have described here is an approach aimed at exploring ways of mitigating Hb oxidative toxicity. Studying oxidative pathways in select Hb variants (with diverse oxidative profiles) has enabled us to develop criteria toward the rational design of a new series of oxidatively stable HBOC candidates. Future HRAM studies will therefore be aimed at characterizing these new candidate HBOCs to determine which are the most oxidatively resistant toward losing heme, while retaining oxygen carrying function.

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Address correspondence to:

Dr. Abdu I. Alayash

Laboratory of Biochemistry and Vascular Biology

Center for Biologics Evaluation and Research

Food and Drug Administration

Building 52/72 Room 4106

10903 New Hampshire Avenue

Silver Spring, MD 20993

E-mail: abdu.alayash@fda.hhs.gov

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Abbreviations Used

CID = collisional-induced dissociation
 DCLHb = diaspirin crosslinked Hb
 DMPO = 5,5-dimethyl-1-pyrroline N-oxide
 ESI = electrospray ionization
 FT-ICR = Fourier transform ion cyclotron
 HBOCs = hemoglobin-based oxygen carriers
 HPLC = high-performance liquid chromatography
 HRAM = high-resolution accurate mass
 MALDI = matrix-assisted laser desorption/ionization
 NFPLP = 2-nor-2-formyl pyridoxal-5'-phosphate
 PTM = post-translational modification
 RP = reversed phase
 SCD = sickle cell disease
 SCX = strong cation exchange
 TOF = time-of-flight
 UPLC = ultra-performance liquid chromatography
 XIC = extracted ion chromatogram