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Isolated Hb Providence β82Asn and β82Asp Fractions Are More Stable than Native HbA_0 under Oxidative Stress Conditions

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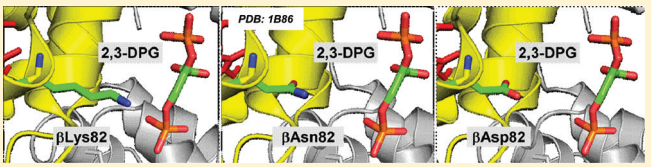
Supporting Information

ABSTRACT: We have previously shown that hydrogen peroxide (H_2O_2) triggers irreversible oxidation of amino acids exclusive to the β-chains of purified human hemoglobin (HbAo). However, it is not clear, whether α- or β-subunit Hb variants exhibit different oxidative resistance to H_2O_2 when compared to their native HbAo. Hb Providence contains two β-subunit variants with single amino acid mutations at βLys82→Asp (βK82D) and at βLys82→Asn (βK82N) positions and binds oxygen at lower affinity than wild type HbA. We have separated Hb Providence into its 3 component fractions, and contrasted oxidative reactions of its β-mutant fractions with HbAo. Relative to HbAo, both βK82N and βK82D fractions showed similar autoxidation kinetics and similar initial oxidation reaction rates with H_2O_2. However, a more profound pattern of changes was seen in HbAo than in the two Providence fractions. The structural changes in HbAo include a collapse of β-subunits, and α–α dimer formation in the presence of excess H_2O_2. Mass spectrometric and amino acid analysis revealed that βCys93 and βCys112 were oxidized in the HbAo fraction, consistent with oxidative pathways driven by a ferrylHb and its protein radical. These amino acids were oxidized at a lesser extent in βK82D fraction. While the 3 isolated components of Hb Providence exhibited similar ligand binding and oxidation reaction kinetics, the variant fractions were more effective in consuming H_2O_2 and safely internalizing radicals through the ferric/ferryl pseudoperoxidase cycle.



There are over 1,000 known variant human Hbs that are caused by single point mutations, while others are due to deletions, additions, or fusion of Hbs.[1] In some cases, these mutants are associated with hematological disorders such as anemia or polycythemia, while others are asymptomatic. Over the years, the investigations of these abnormal Hbs and their functional differences with the normal protein have provided considerable insights into our understanding of the structural–functional relationships in the Hb molecule.[2]

Less progress has been made on the oxidation reactions of these unique and diverse Hbs, and the potential contribution of these reactions to the pathophysiology associated with some of these mutations. It has long been known that the rate of oxidation of the ferrous Hb ($HbFe^{2+}$) to ferric "met" ($HbFe^{3+}$) increases with increasing partial pressure of oxygen and with increasing dimerization of the Hb tetramer.[3] Oxygen has an inherent ability to oxidize the ferrous heme iron producing superoxide anions ($O_2^{\bullet-}$), which dismutate further to hydrogen peroxide (H_2O_2) in a process known as autoxidation (eq 1).

$$HbFe^{2+} + O_2 \leftrightarrow HbFe^{3+} + O_2^{\bullet-} \rightarrow O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 \quad (1)$$

The reaction between the two-electron oxidant H_2O_2 and ferrous Hb leads to ferryl heme iron ($HbFe^{4+}$) formation. If, however, the reaction occurs with ferric iron, a cation radical species ($^{\bullet}HbFe^{4+}$) is formed in addition to the ferrylHb [4] (eqs 2–4)

$$HbFe^{2+} + H_2O_2 \rightarrow HbFe^{4+} = O_2^- + H_2O \quad (2)$$

$$HbFe^{2+} + HbFe^{4+} = O_2^- + H^+ \rightarrow 2HbFe^{3+} + OH^- \quad (3)$$

$$HbFe^{3+} + H_2O_2 \rightarrow {}^{\bullet}HbFe^{4+} = O_2^- + H_2O \quad (4)$$

Hb undergoes rapid oxidation in the presence of H_2O_2 in a manner analogous to peroxidase oxidation. However, unlike classic heme-containing peroxidases, radical leakage in the case of Hb is unavoidable. Therefore, the so-called pseudo-peroxidative reactions are invariably detrimental to the Hb protein itself and the surrounding tissue, particularly when Hb is found in an oxidative environment outside the red blood cells RBCs.[4] Both the ferryl heme and its associated protein radicals are capable of inducing a wide range of oxidative damaging reactions that can target other biological molecules in addition

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to self-destructive mechanisms which result in irreversibly oxidized amino acids on the protein.[5]

Recently, developed cell-free Hbs, in which the protein is site-specifically or randomly modified to produce a tetrameric and/or polymeric Hb as oxygen carrying blood substitutes, provided valuable insights into the interplay between oxygen delivery, autoxidation, and oxidative reactions and the impact of chemical and/or genetic modifications on these reactions.[6] Injuries as a consequence of these oxidative reactions have been documented in simple cell culture and organs such as kidneys or brain from animals infused with these proteins.[7−9]

The impact of single amino acid mutations on the overall oxygen affinity of Hb tetramers is well established. However, the contribution of such mutations on heme iron oxidation of the variant and nonvariant fractions to the overall stability of the protein is still unknown. The nature of amino acid substitution as well as the site and location of the mutation may also be critical in determining the stability of a given α- or β-mutant Hb in an oxidative environment.

Hb Bristol [β67(E11) Val→Asp] is a classic model of unstable Hb where a hydrophilic aspartate disrupts the hydrophobic heme pocket resulting in an unstable Hb. The slow conversion of the translated methionine via an unusual post-translational modification into an aspartate residue and its proximity to the heme and oxygen may be important in facilitating the reaction mediated by oxygen radicals involving the heme prosthetic groups.[10] Tyr145 is the penultimate C-terminal amino acid in the β-chain, which plays a critical role in Hb allosteric mechanism.[11] The substitution of this amino acid by His (Hb Bethesda) or Cys (Hb Rainier) dramatically alters oxygen binding, resulting in polycythemia.[12,13] It is still unknown whether these physiologically undesirable redox-active amino acids at this site (i.e., Cys/His) contribute to the overall oxidative instability of these mutant Hbs. In Hb E (βGlu→26Lys), recent structural and functional investigations showed that despite the limited impact of this mutation on its allosteric properties there was a clear difference in the redox reactions of this Hb when compared to that of normal human HbAo. Accordingly, these investigators suggest that changes observed in the redox properties of HbE may contribute to the pathophysiology in HbE/β-thalassemia individuals.[14]

Hb Providence was first discovered in the blood of a 25 year old female in Providence Hospital Washington, D.C.,[15] and the full structural and functional properties were subsequently reported by several investigators.[16,17] In this mutant, the variant residue β82 (EF6) lysine, one of the amino acids involved in the binding of 2,3-DPG and chloride is replaced by asparagine. This lysine to asparagine substitution reduces the number of cationic groups from seven to six. Partial in vivo deamidation of this residue forms additional aspartic acid.[16]

In this investigation, we have isolated Hb Providence into the 3 major fractions, normal HbAo and the two Providence variants, βK82D and βK82N, which have been subsequently digested, and peptides containing amino acid substitutions were verified. For each undigested fraction, autoxidation and H_2O_2 as well as the nitric oxide (NO) mediated oxidation reaction rates were determined. Our ligands binding kinetics carried out on the 3 isolated fractions confirmed earlier reports. In addition, we provide evidence to show that in spite of the similar autoxidation and H_2O_2 reaction kinetics there were differences in the oxidative stability of the mutant fractions when compared to that of the normal HbAo. In recent years, there have been extensive efforts in identifying the mechanisms that cause oxidative degradation, protein radical generation, and tissue oxidative damage due to the presence of free Hb in circulation. The oxidative stability of Hb Providence and its resistance to unfolding reported here are among important attributes that can be engineered in the design of new generation oxygen therapeutics in order to mitigate problems associated with its toxicity

EXPERIMENTAL PROCEDURES

Materials. Hb Providence was obtained by patient consent from the Clinical Center at the NIH. Serine proteases used in this work, chymotrypsin, trypsin, and Asp-N were purchased from Roche Diagnostics. Sodium acetate, ammonium bicarbonate, urea, dithiothreitol (DTT), iodoacetamide (IAA), trifluoroacetic acid (TFA), hydrogen peroxide, catalase, formic acid (FA), LC-MS grade water, MALDI matrices including sinapinic acid (SA), and α-cyano-4-hydroxycinnamic acid (CHCA) were supplied by Sigma Aldrich. Sodium dithionite was from British Drug House. HPLC grade acetonitrile and HPLC water was purchased from American Bioanalytical and LC-MS grade acetonitrile from Fisher Scientific. Tris-HCl was obtained from ICN Biomedicals. Hb antibodies (ab92492) against the αHb subunit were supplied by Abcam Chemicals. DMPO antibodies were from Enzo life sciences (ALX-210-530-R050).

Isolation of Hemoglobin Providence Variants. Hemolysate was prepared from a patient's whole blood and was used for separating the 3 fractions essentially as described.[18] Hb Providence fractions were separated according to the original protocol described by Moo Penn et al.[16] Briefly, a 200 μL hemolyzed blood sample of Hb Providence was introduced into a Waters AP-1 DEAE column and eluted with a 120 min gradient. Initial column conditions were 100% 0.05 M Tris-HCl at pH 8.6 at room temperature, followed by a gradient of 100% 0.05 M Tris-HCl at pH 6.9 over 120 min at a flow rate of 1.5 mL/min. The individual fractions were collected, buffer exchanged in water, and stored at −80 °C until further analysis. The purity of each fraction was confirmed by reverse phase HPLC and mass spectrometry methods. Each of the 3 Hb fractions obtained from the DEAE column was tested for catalase activity.[19] Briefly, to 1 μM (heme) Hb in 50 mM potassium phosphate buffer (pH 7.0), 15 μM H_2O_2 was added, and absorbance spectra were recorded for 2 min at 240 nm. Changes in the H_2O_2 concentration were determined using an extinction coefficient (ε = 240 nm) of 43.6 cm^{-1} M^{-1}. Highly purified HbAo (99%, a gift from Hemosol Inc., Canada) was used as a control. Little or no changes in the H_2O_2 concentration (<98%) comparable to the control indicated a negligible amount of catalase activity.

Identification of Hemoglobin Providence Variants by ESI Mass Spectrometry. The Hb fractions were analyzed by isolating the β-globin chains by HPLC fractionation on a reverse phase column according to the method described earlier. The globin chains were denatured in 50 μL of 6 M urea and 2 mM DTT in 50 mM Tris at pH 7.5, and incubated at 37 °C for 1 h. The samples were alkylated with 20 mM IAA for 20 min in the dark with gentle agitation. The samples were then diluted with 25 mM Tris at pH 7.5 to give a final urea concentration of 1 M followed by the addition of endoproteinase Asp-N reconstituted in 1% acetic acid. Proteolysis was stopped by the addition of 0.1% formic acid and dried under vacuum. The samples were resuspended in a minimum of 2% ACN in 0.1% TFA and desalted using ziptips before mass spectral analysis.

Trypsin digestion was carried out on the HPLC isolated globins; after denaturation with 6 M urea and alkylation with IAA, the samples were diluted to a final urea concentration of 1 M with 50 mM ammonium bicarbonate. Trypsin was added at a 1:25 enzyme to substrate ratio. Digestion was carried out overnight. Proteolysis was terminated by the addition of formic acid to a final concentration of 0.1%. The samples were desalted and prepared for LCMS analysis as described above.

The Asp-N digests of all fractions were analyzed on Waters Xevo QTOF coupled to a Waters nanoAcquity HPLC system. The instrument was used in nanospray mode with a flow rate of 300 nL/min. Reverse phase chromatography was carried out using a nanoAcquity BEH130 C18 75 μm × 100 mm column with a 1.7 μm bead size. Mass spectra were acquired using data-dependent acquisition with positive polarity and a normal dynamic range. The mass range was set to 100−1990 m/z, and data acquisition was carried out for 140 min. The collision energy was determined according to charge state vs collision energy profiles that ramped the collision energy upward from a low of 9 to a high of 35 with increasing charge and m/z. Helium was used as the collision gas. The instrument was programmed to select a maximum of 3 ions for MS/MS scans from a single MS survey scan. The scan rate for MS/MS was 1 s with continuum data. Charge state selection was set for +2, +3, and +4. Individual mass include lists were created for the βK82N and βK82D variants. The βK82D include list consisted of the following masses, 647.3, 675.8, 943.5, and 971.9. The βK82N include list consisted of the masses 1114.0 and 1142.5 with a 100 mDa window. A biphasic, 140 min gradient of 0.1% formic acid (buffer A) and 0.1% formic acid in ACN (buffer B) was used at a flow rate of 300 nL/min.

LCMS analysis of the trypsin digest was done using a ThermoFinnigan LTQ mass spectrometer coupled to a Surveyor HPLC pump. The instrument configuration was the same as that described for the mass spectrometry based analysis of the intact oxidized fractions. A biphasic gradient was used consisting of Solvent A 0.1% formic acid, and Solvent B, acetonitrile in 0.1% formic acid. The gradient was programmed as described above for the analysis of the Asp-N digest. The flow rate was 200 nL/min. The mass spectrometer was operated in a data-dependent mode and the top nine peaks from the full scan were isolated for MS/MS analysis. The minimum signal required was 100. The MS/MS isolation width was 2.0. The normalized collision energy was 35.0. The MSMS minimum signal required was 50. The MSMS Q activation was 0.25. AGC target values were 30,000 for full scan MS and 10,000 for MS/MS.

The RAW files from the QTOF data were analyzed with PLGS 3.2, using an IPI database that was appended with β-Hb primary sequences containing the βK82N and βK82D substitutions. Mascot version 2.2.03 was also used for database searches of the QTOF data. These files were searched using the IPI human database v.3.36 that was appended with β-Hb subunit primary sequences containing the βK82N and βK82D substitutions. The peptide mass tolerance was 100 ppm allowing for 1 missed cleavage. Cysteine trioxidation, cysteine carbamidomethylation, and methionine oxidation were searched as variable modifications.

Raw Files from the LTQ analyses were searched with Mascot as described above, except that peptide tolerance values were 1.0 for precursor mass and 0.6 for fragment ion spectra. The resulting Mascot files were submitted to Scaffold v 3.0. Minimum protein probability was set to 99.05, and minimum peptide probability was set to 90%. The minimum number of peptides was set to two. Selected LCMS data files from the Xevo QTOF analyses were also submitted to Scaffold for the MSMS spectra presented in Figure 2.

Ligands Binding, Autoxidation, and H_2O_2 Induced Oxidation Reactions of Isolated Fractions of Hemoglobin Providence. *Rapid Mixing Ligands Reactions.* The kinetics of oxygen dissociation from oxyHb and binding of CO to deoxy Hb of the three fractions were measured. Measurements were performed on an Applied Photophysics SF-17 microvolume stopped-flow apparatus with a dead time of about 1.5 ms. Typically, 30 μM (heme) Hb solutions were rapidly mixed with an equal volume of 1.5 mg/mL sodium dithionite, and the absorbance changes of the oxygen dissociation process were monitored at 437.5 nm in 0.05 M Bis-Tris buffer at pH 7.4 at room temperature. For each reaction, at least three kinetic traces were averaged and fit to exponential equations using the Marquardt−Levenberg fitting routines included in Applied Photophysics software.

The CO binding kinetics for the three fractions were measured at 437.5 nm in the Applied Photophysics stopped-flow instrument at room temperature in 50 mM Bis-Tris buffer, pH 7.4, containing freshly made 1.5 mg/mL sodium dithionite following a previously reported protocol.[20] The kinetics of NO oxidation of the oxy forms of the three fractions were carried out in the stopped-flow as previously described.[21] Briefly, 1 μM (heme) Hb solutions were mixed with the NO solutions (0−50 μM) in the stopped-flow instrument, and the reaction was followed by monitoring the absorbance changes at 420 nm.

Autoxidation and Hydrogen Peroxide Induced Oxidation. Autoxidation experiments for each fraction (20 μM) (heme) were carried out as previously described at 37 °C for 24 h.[20] The spectral changes over time between 450−700 nm were monitored in a temperature-controlled photodiode array spectrophotometer (Agilent 8453). Multiwavelength absorbance and specific extinction coefficients were used to determine the amount of oxyHb, ferric Hb (metHb), and hemichrome concentrations in each sample.[22] The oxyHb percentage changes due to the spontaneous oxidation of each fraction (oxy) were plotted over time and analyzed by nonlinear least-squares curve fitting to single or double exponential equations using SigmaPlot software to obtain the autoxidation rate constants.

Hydrogen peroxide mediated oxidation spectral changes for each Hb fraction (20 μM) (heme) were monitored upon the addition of 30 equivalents of H_2O_2 (30% (w/w)) in 50 mM potassium phosphate buffer, pH 7.4 at 37 °C over a period of 1 h. Ferryl Hb formation as a result of oxyHb oxidation was followed by monitoring characteristic absorbance changes over time using previously reported extinction coefficients in the Soret region for these species.[23] For the verification of the ferryl intermediate, 2 mM sodium sulfite (Na_2S) was added to transform ferryl Hb to sulf Hb. The concentration of sulf Hb was calculated using the extinction coefficient of sulfheme (ε 620−604 nm = 10.5 mM^{-1} cm^{-1}).[24]

Comparison of Oxidative Changes in Globin Chains. For the detection of oxidative changes in the globin chains, each fraction was treated with 30 molar excess H_2O_2. After the reaction, H_2O_2 was removed from the samples by buffer exchange five times with equal volumes of 50 mM potassium phosphate, pH 7.4, using 30-kDa cutoff centrifuge tubes (Amicon 30, Millipore Corp., Bedford, MA). For the detection of altered heme products (AHP), a typical oxidation experiment

was carried out in sodium acetate buffer at pH 4.7, and samples were kept on ice or stored at −80 °C until further analysis (see below).

Detection of Peroxide Induced Globin Chain Oxidation and Altered Heme Protein Formation Using Reverse Phase HPLC. Oxidized HbAo, βK82N, and βK82D were separated by analytical reverse phase (RP)-HPLC. A Zorbax StableBond 300 C3 250 × 4.6 mm column connected with a 12 × 4.6 mm guard column (Agilent Technologies, Palo Alto, CA) was used in a Waters HPLC system consisting of a Waters 626 pump, Waters 2487 dual-wavelength detector, and a Waters 600s controller installed with Millenium32 software (Waters Corp., Milford, MA). Acetonitrile and water (containing 0.1% TFA) were used as mobile phase solvents. The gradient conditions have been described earlier.[5] Absorbance of the eluents was monitored at 280 and 400 nm for protein, heme, and the AHP moieties, respectively, as described previously [5,25]

Mass Spectrometry Analysis of Oxidized Intact Fractions. Analysis of oxidized, intact globins was performed using a 4800 MALDI-TOF-TOF Analyzer (Applied Biosystems, Foster City, CA) by spotting protein samples mixed with sinapinic acid (10 mg/mL in 70:30 ACN water containing 0.1% TFA) in equal volumes. Analysis of oxidized, intact globins was also done using a Thermo Finnigan ESI-LTQ mass spectrometer coupled to a Thermo Surveyor HPLC pump and a Thermo Micro AS autosampler. The instrument was used with a nanoflow spray source and a 75 μm × 10.2 mm Picofrit (New Objective; Woburn, MA) column with a 15 ± 1 μm opening that was packed with Biobasic C18 resin. The autosampler was set to deliver a 10 μL injection. A biphasic, 140 min gradient of 0.01% TFA 0.05% acetic acid (buffer A), 0.01% TFA, and 0.05% acetic acid in ACN (buffer B) was used at a flow rate of 200 μL/min. Full scan MS was done with a mass range of m/z = 350 to 2,000. The gradient was programmed as follows: 0−40 min, 98% A and 2% B, 50 min 70% A and 30% B, 110 min 30% A and 70% B, 115 min 10% A and 90% B, 132 min 98% A and 2% B, and 140 min 98% A and 2% B. The resulting chromatograms of oxidized HbAo, βK82N, and βK82D were compared.

Mass Spectrometry Analysis of Trypsin Digested Peptides of Oxidized Fractions. Oxidized samples (50 μg) were suspended in 0.1 mg/mL surfactant (RapiGest SF), reduced, and then alkylated using DTT and IAA. Trypsin was added in a 1:50 enzyme−substrate ratio and incubated overnight at 37 °C. The reaction was quenched by adding 0.1% formic acid and desalted using C18 ziptips. The digests of the oxidized variants were analyzed on a Waters Xevo ESI-QTOF coupled to a Waters nanoAcquity UPLC system under the same conditions as those described above. The RAW files from the QTOF data were analyzed with Mascot v 2.2.03 using a database that included the β-Hb primary sequences containing the βK82N and βK82D substitutions using a 50 ppm mass tolerance window and allowing for at least 1 missed cleavage. The amino acid modifications that were included as variable modifications were trioxidation (C), oxidation (Y, W, H, M), dioxidation (Y, W, M), and kynurenin (W). For comparison of extent of oxidation, the ion chromatograms corresponding to the oxidized and nonoxidized cysteine containing peptides were extracted. The oxidized cysteine peptides eluted later (retention time Δ1 min). The peaks generated were summed to result in the spectra that contain both the oxidized and nonoxidized ions. The resulting spectra were subjected to Maxent process (Masslynx) to generate the deconvoluted spectra. The peak intensities corresponding to nonoxidized and oxidized peptides were compared to access the extent of oxidation.

Immunoblot Detection of Subunit Dimerization in Hemoglobin Fractions. The Hb fractions (150 μM (heme) were treated with 30 equivalents of H_2O_2 in the presence and absence of 100 mM DMPO. The oxidized samples were diluted to 20 μM (heme) and mixed with Laemmli sample buffer (Invitrogen, Carlsbad, CA) at a ratio of 4:1 and 200 μM of dithiothreitol. The samples were incubated at 90 °C for 5 min and then immediately loaded onto a 4−12% Tris-glycine polyacrylamide gel. Samples were run at room temperature for 1.5 h at 100 V. The protein bands were electrophoretically transferred to a nitrocellulose membrane in 1× transfer buffer (Novex, Invitrogen, Carlsbad, CA) containing 10% methanol for 2 h. The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h (TTBS buffer). The blots were incubated overnight with anti-DMPO antibody or anti-Hb α-antibody. The blots were then washed three times in TTBS and incubated for 1 h with horseradish peroxidase-conjugated antirabbit IgG in TTBS at room temperature for 2 h. The blots were washed three times in TTBS buffer and visualized using ECL Western Blotting detection reagents (Amersham Biosciences), and the image was captured using a Kodak gel detection system.

RESULTS

Isolation of Variants and Identification of Mutations by Mass Spectrometry. Hemolysate samples of Hb Providence were resolved into three individual fractions using DEAE chromatography (Figure 1). The chromatogram is consistent with previously published reports.[16] The peak with a retention time of 53.9 min corresponds to that of native HbAo (βK82). The second peak with a retention time of 75.3 min corresponded to the variant with the βK82N mutation, and the third peak at 96.4 min corresponded to the βK82D variant.[16]

The verification of the mutation at the βK82 position was confirmed by isolating β-globin chains of each of the Hb variants on a reverse phase (C3) column according to the protocol reported earlier.[5] The variant β-globin chains were proteolyzed using trypsin and Asp-N. Digestion with Asp-N produces peptides (β79−93) (79)DNL(82)KGTFATLESELHC(D94) from all of the variants, where the K82 is substituted by N in the βK82N variant and by D in the βK82D variant. However, in the βK82D fraction, an MH_2^{2+} peptide (β82−93) specific to Asp-N digestion of the βK82D variant is observed at m/z 675.80. The digests were analyzed using LC-ESI-MS/MS. The MS/MS spectrum of the corresponding MH_2^{2+} ions at m/z 846.33 (βK82N) (Figure 2B) and the MH_2^{2+} peptide (β82-93) specific to Asp-N digestion at m/z 675.80 (Figure 2C) confirm the presence of the mutations. Digestion of the βK82 variant with trypsin produces a unique peptide (Figure 2A). The βK82D and βK82N variants do not have a K residue at this position that can be cleaved by trypsin. By using a differential digestion approach, each variant peptide is identified by mass, sequence, and also by unique sites of proteolysis in the case of the βK82 variant and the βK82D variant. The use of Asp-N as a proteolytic agent increases the confidence of the identification of the βK82N. The βK82D and βK82N variants contain asparagine and aspartate, which have only a one unit mass difference.

Biochemical Characterization of Isolated Fractions. The kinetics of ligand binding and dissociation were measured on a stopped-flow instrument for all three fractions of Hb Providence. The kinetic parameters are summarized in Table 1.

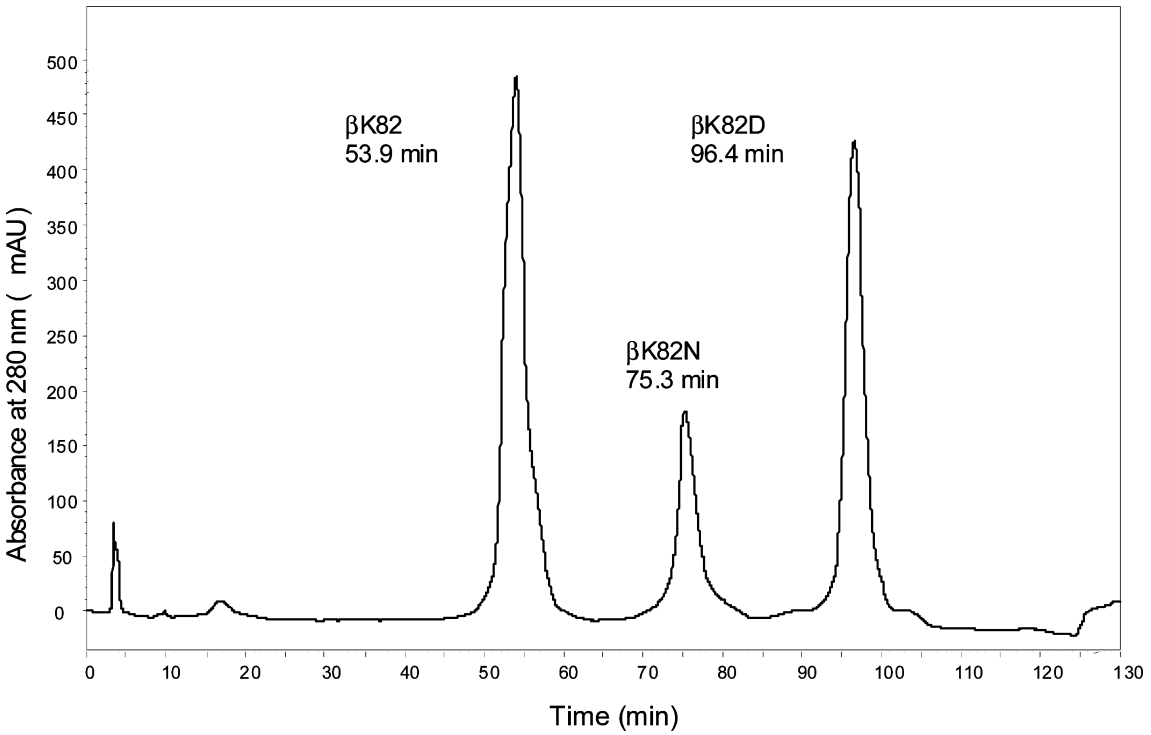

Figure 1. Separation of hemoglobin Providence on DEAE column chromatography. Chromatogram of Hb Providence showing resolution of the three β-variants on a Waters AP-1 DEAE column. Elution of HbAo (βK82) at 54 min, βK82N at 75 min, and βK82D at 96 min was observed.

The oxygen dissociation and the CO binding kinetics under our conditions measured by absorbance changes follow a single exponential process. The oxygen dissociation rate constant of the HbAo (βK82) fraction is similar to that of native HbAo, whereas the oxygen dissociation was about 20% faster for the βK82N and βK82D. The second order rate constant of CO binding to the deoxygenated HbAo (βK82) fraction was close to that of purified HbAo. Relative to HbAo, CO binds to both the βK82N and βK82D at a rate about 20% higher under similar experimental conditions, consistent with early reports.[15]

The NO binding to oxygenated Hb causes oxidation of the heme to form ferric (met) Hb and nitrate as final products. The reaction occurs very rapidly as measured on a stopped-flow instrument.[27] The pseudo-first-order reaction time course of Hb with NO in excess can be described by a single exponential equation. The apparent rate constants can be plotted versus increasing NO concentrations to derive the second order rate constants as reported in Table 1. The NO-induced Hb oxidation in HbAo (βK82) occurred at a rate of 19.4 μM^{-1} s^{-1}, close to that of purified native HbAo. The NO reaction rate constants of (βK82D) and (βK82N) are slightly higher than that of HbAo (see Table 1).

Autoxidation and Oxidative Reactions of Isolated Fractions. Figure 3 depicts a comparison in the spontaneous oxidation (autoxidation) of oxy forms of Hb Providence fractions over a 24 h time course in a temperature controlled spectrophotometer, at 37 °C in phosphate buffer at pH 7.4. The inset shows representative spectral intermediates taken at 0, 3, and 24 h during the autoxidation of fraction 1 (HbAo). The rate constants of autoxidation reported in Table 1 were derived from changes in oxyHb as a function of time and fitted to a nonlinear least-squares curve fitting with exponential equations. The autoxidation rate constants for the 3 fractions were at approximately 0.018 h^{-1} consistent with those reported for the highly purified HbAo.[23]

The reaction between H_2O_2 and ferrous or ferric Hb is considered a classic one, due to the slow kinetics compared with true peroxidases, which allowed the reaction to be followed spectrophotometrically (eqs 2−3). In the experiment illustrated in Figure 4 (left panel), Hb Providence fractions were treated with H_2O_2 in a ratio of 1:30 (heme/H_2O_2) over a 1 h time period. The oxy/ferrous Hb in the visible region with two peaks at 541 and 577 nm, respectively, decays very rapidly to give a typical ferryl spectrum with two major peaks at 545 and 580 nm with a flattened region between 500 and 600 nm. Closer inspection of spectra reveals very little differences in the progress of the reactions among the Hb fractions at this level of added H_2O_2. The ferryl spectrum then slowly reverts back to a ferric-like spectrum with absorption peaks at 510, 550, and at 630 nm. The autoreduction process is more evident in the variant fractions (left panel of Figure 4B,C) than is the case with HbAo (βK82) (left panel of Figure 4A), which may reflect more efficient pseudoperoxidase activities in these fractions. To capture the initial ferryl species in each solution, Na_2S was added, and the spectra of sulfHb (green Hb) formed are shown in Figure 4 (right panels). It is interesting to note that variant Hb fractions (right panel Figure 4B,C) generally formed more ferryl intermediate, which was readily converted to a final ferric species. The ferryl species formed in HbAo solution did not revert back to the ferric form but persisted much longer in solutions which ultimately self-destruct (right panel Figure 4A).

Hydrogen Peroxide Induced Structural Changes in Hemoglobin Providence Fractions. The structural integrity of Hb subunits in each fraction was next investigated. Figure 5A, shows the reverse phase (C3) HPLC chromatogram of HbAo fraction prior to treatment with H_2O_2. The β-globin chain elutes at 26.5 min, and the α-globin elutes at 28.5 min.

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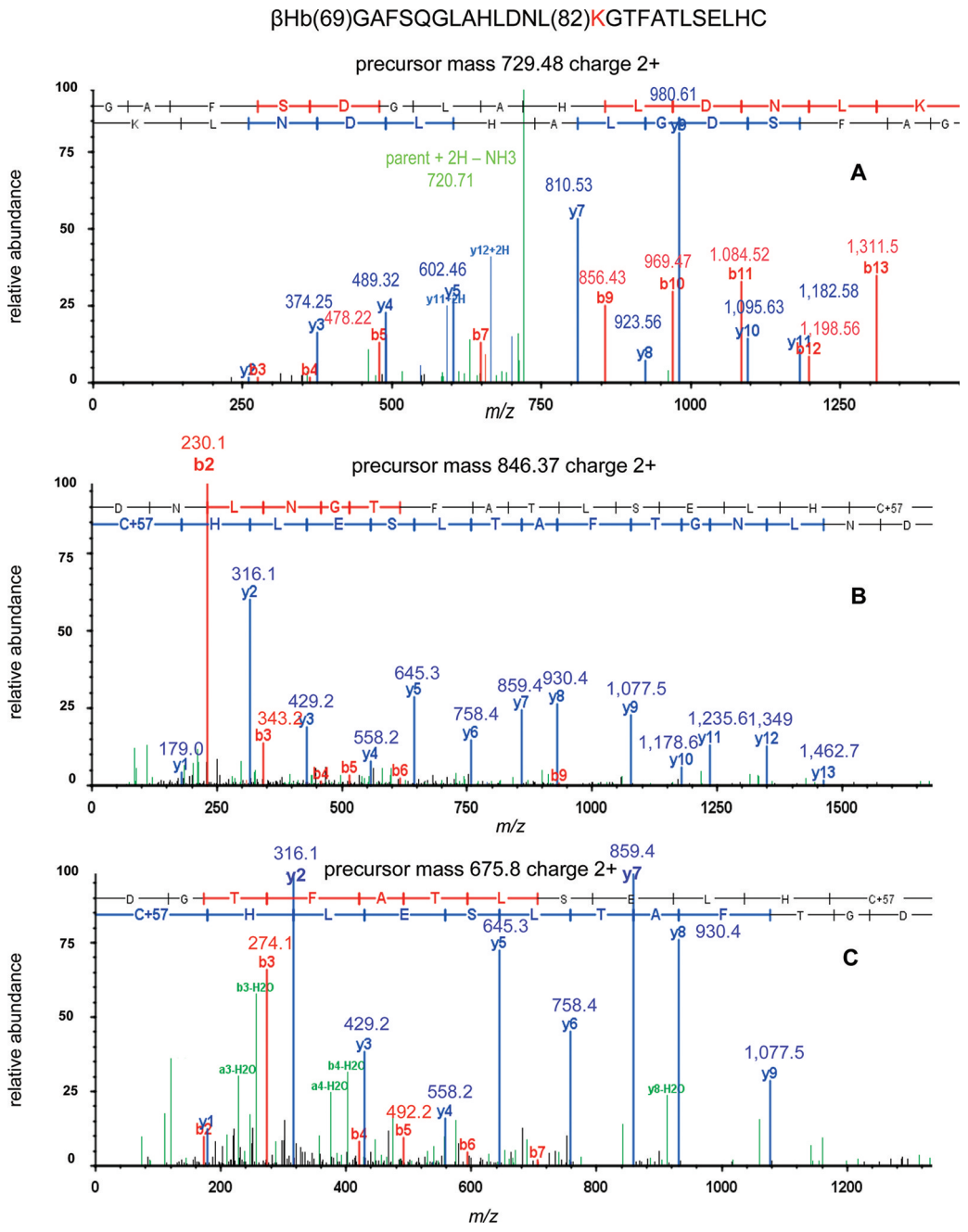

Figure 2. MS/MS spectra of the β-globin peptides encompassing β82 position for the variant fractions generated by Asp-N or trypsin proteolysis. At the top of the figure, the amino acid sequence of the β-Hb identified in the MS/MS spectra is shown. The K residue (in red) at position 82 is substituted by N (Asn) in the peptide that was identified by the MS/MS spectra (panel B), and by D (Asp) in (panel C). The amino acid sequence for the corresponding peptide appears at the top of each spectrum. The sequence in red arranged from the amino-terminus to the carboxy-terminus indicates the b-ion series. The sequence in blue arranged from the amino terminus to the carboxy-terminus indicates the y-ion series. The peaks in the spectra are also color coded with b-ions in red and y-ions in blue. (A) MS/MS spectrum from the semitryptic β69−82 peptide of the K82 variant. In this spectrum, the green peaks represent loss of NH_3. (B) MS/MS spectrum of peptide 79−93 resulting from digestion with Asp-N of Hb Providence containing βK82N. (C) MS/MS spectrum of unique Asp-N peptide 82−93 from the digestion of Hb Providence containing βK82D. The green colored peaks represent ions that have lost a molecule of water.

Upon treatment with 30 equivalents of H_2O_2 at pH 7.4, there was a considerable reduction in absorbance for the peaks corresponding to the β- and α-chains at the corresponding retention times (Figure 5B). The α- and β-globin chains of the (βK82N) and (βK82D) variants elute at similar retention times (data not shown). Treatment of the variants with H_2O_2 produces a decrease in the absorbance of the peaks corresponding to α- and β-chains; however, these peaks are still present at significant absorbances (Figure 5C,D). This stands in contrast to the HbA_o sample. The collapse of two subunits in HbAo observed in Figure 5B has been previously noted and is attributable to substantial oxidative changes in the HbAo molecule as a direct result of H_2O_2 treatment.[5]

Under acidic buffer conditions, the acid-catalyzed formation of heme to protein cross-links between heme and a distal histidine has been observed. This has been attributed to the

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Table 1. Ligand Binding and Oxidation Reaction Kinetics of Hb Providence Fractions[a]

Hb Providence fractions	oxygen off k_{off} (s^{-1})	CO on rate k'_{on} ($\mu M^{-1}s^{-1}$)	NO oxidation $k'_{ox,\ NO}$ ($\mu M^{-1}s^{-1}$)	autoxidation k_{auto} (h^{-1})
βK82	42.5 ± 0.19	0.229 ± 0.008	19.4 ± 4.7	0.018
βK82N	51.5 ± 0.24	0.279 ± 0.040	22.3 ± 3.1	0.017
βK82D	51.9 ± 0.19	0.285 ± 0.006	23.2 ± 3.6	0.018

[a]Comparison among Hb Providence fractions with regard to oxygenation and nitric oxide oxidation and autoxidation reactions: Ligand reactions with Hb fractions were carried out using rapid mixing stopped-flow apparatus (for details see Experimental Procedures). Oxygen off rate is the oxygen dissociation rate constant k_{off}. Carbon Monoxide (CO) on rate $k'_{on,\ CO}$ is the bimolecular rate constant of CO binding. NO-induced oxidation of oxyHb is $k'_{ox,\ NO}$ is the bimolecular rate constant (for details see Experimental Procedures). The values for autoxidation (k_{autox}) are the rate constants for the spontaneous oxidation of Hbs measured at 37 °C.

action of the ferryl iron, and its protein-based free radical.[28] The oxidized fractions were compared using a reverse phase (C3) HPLC column. Protein and heme contents were detected by their absorbance at 220 and 400 nm, respectively. Supporting Information, Figure S1, shows a typical HPLC profile for HbAo before and after treatment with H_2O_2 [1 heme/10 H_2O_2]. As shown in the figure, the major fractions with absorption at 220 nm at 25−28 min correspond to the α- and β-chains while the absorption at 400 nm at 16 min is due to the absorption of heme. Upon treatment with H_2O_2 under acidic conditions, there is a significant fraction with absorption at 400 that coelutes (25−28 min) with the now single, broad peak at a retention time of 25 − 28 min. This peak represents species referred to as altered heme products (AHP). We also investigated the H_2O_2-induced covalent modifications in oxidized variant fractions and observed similar heme-protein modifications. Because of the structural stability of these two variants slightly higher levels of AHP were found (data not shown).

α−α-Subunit Dimerization and its Inhibition by DMPO. The ferryl Hb, and the protein-based radical generated with this ferryl species, can oxidize susceptible residues including Tyr-42 of the human α-Hb subunit, which is involved in the formation of dimers and oligomers.[29] Hb Providence fractions were treated with H_2O_2 in the presence and absence of DMPO. DMPO has been shown to trap phenoxyl radicals that prevent tyrosyl radical initiated subunit dimerization.[30] The immunoblot of these oxidized fractions was also compared against an anti-α-hemoglobin antibody (Figure 6A) and DMPO antibody (Figure 6B). In a blot developed against the anti-α-antibody (Figure 6A), the oxidized fractions in the presence of DMPO alone cause negligible modification to the protein as seen in lanes 1, 4, and 7. In the presence of H_2O_2 alone, (lanes 2, 5, and 8), all fractions showed the formation of dimers and multimers, albeit to a lesser extent in the variants. This is reflected by the lower intensity of the dimeric and multimeric bands. In the presence of both H_2O_2 and DMPO (lanes 3, 6, and 9), the anti-α immunoblot does not show major differences when compared with lanes treated with H_2O_2 alone. However, the anti-DMPO treated immunoblot (Figure 6B) shows the presence of DMPO adducted globin chains. Figure 6B, lanes 3, 6, and 9, shows the presence of DMPO adducts of monomeric Hb. The presence of dimeric and multimeric Hb-DMPO adducts are highest in the HbAo (lane 3) followed by (βK82D) and (βK82N).

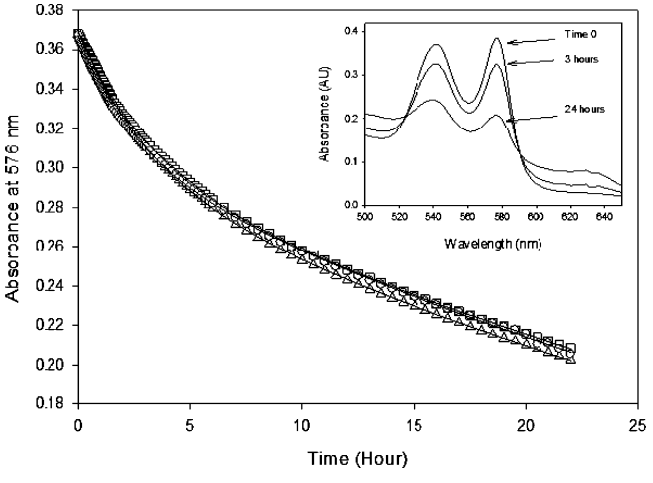

Figure 3. Spectral changes and oxyHb decay as a function of time during the autoxidation of the hemoglobin Providence fractions. The autoxidation of 50 μM Hb Providence fractions in air-equilibrated 50 mM phosphate buffer, pH 7.4, at 37 °C in a spectrophotometer. The normalized absorbance changes of oxyHb during the oxidation process are plotted versus time for all three β-Hb fractions. The insert shows representative primary time-dependent spectra recorded during the autoxidation of HbAo fraction at 0, 3, and 24 h time interval incubation.

Mass Spectrometric Analysis of Oxidative Changes in the Hemoglobin Providence Variants. We examined the nature of these structural changes in the three Hb Providence fractions after treatment with H_2O_2 in a 1:30 (heme/H_2O_2) ratio by MALDI and ESI-LC-MS. The MALDI mass spectra comparing the oxidized fractions are shown in Figure 7/ and the corresponding intact MALDI mass spectra of these fractions are shown as insets in Figure 7. As shown in the insets, the peaks at 15.1 kDa and 15.8 kDa correspond to the α- and β-globin chains, respectively. Upon comparison with the H_2O_2 treated fractions, the intensity of the peak corresponding to the α-globin chain at 15.1 kDa in HbAo (Figure 7A) appears lowest when compared to the variants (Figure 7B,C). The peak corresponding to the β-chain is observed in all three fractions; however, a broad peak was observed between 16−17 kDa largely only in HbAo βK82 (Figure 7A) when compared to the variants (Figure 7B,C). These results suggest extensive oxidative modifications occurring in the HbAo fraction compared to that of βK82N and βK82D variants.

Figure 8 shows base peak mass chromatograms obtained by ESI-LC-MS for the H_2O_2 treated Hb Providence fractions. At the expected retention times for the α- and β-subunits, the HbAo (βK82) fraction showed no distinct peaks (Figure 8A). This indicates that the α- and β-subunits have been oxidized to a heterogeneous mixture of byproducts (Figure 8A). In contrast, panels B and C, corresponding to βK82N and βK82D variants, respectively, show clearly resolved individual peaks that correspond to the α- and β-globin subunits, in addition to several discrete oxidation byproducts. Inspection of these deconvoluted peaks and their masses revealed the formation of discrete units of oxygen as indexed by mass shifts of multiples of 16 amu, including the possible formation of kynurenine from tryptophan

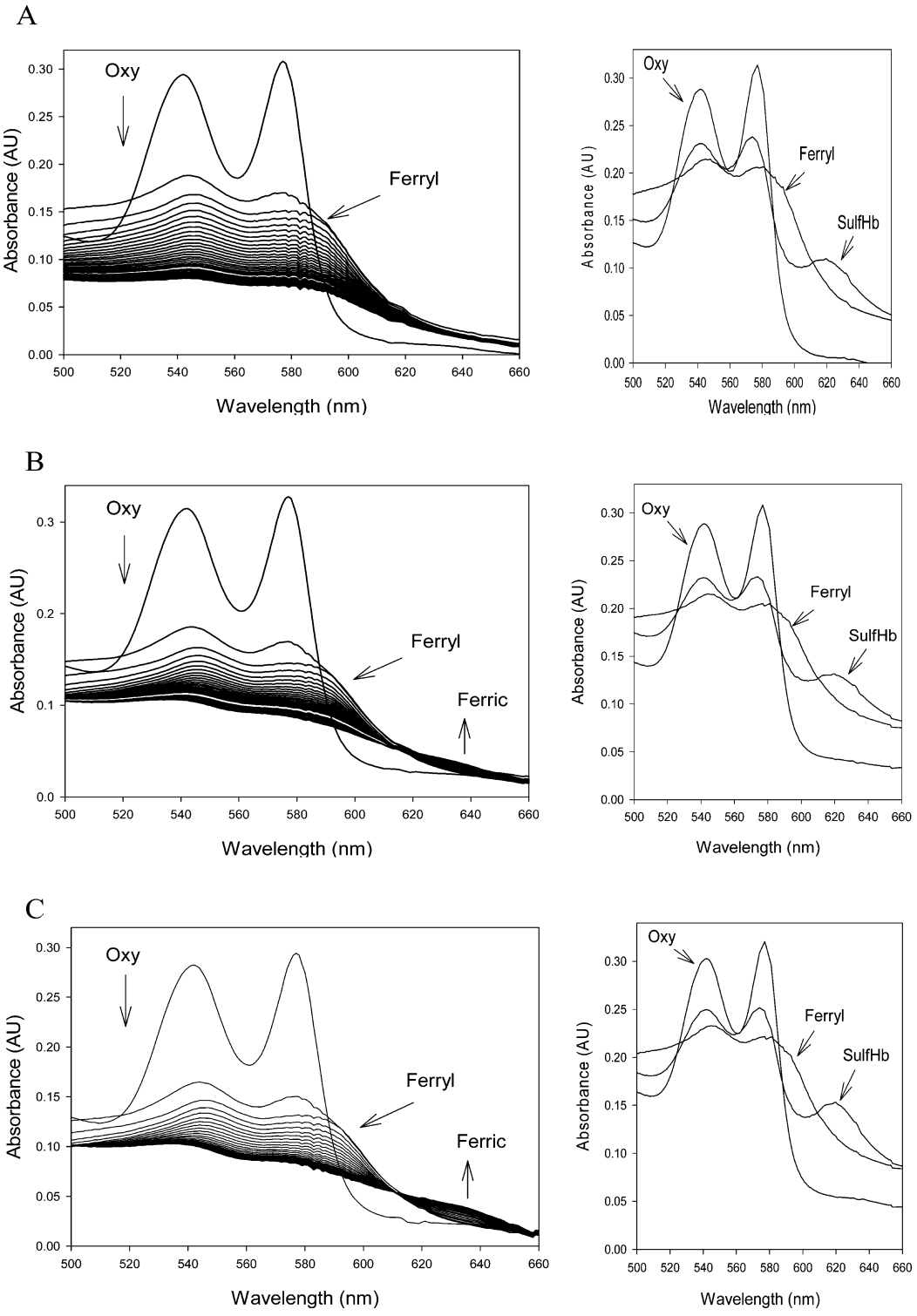

Figure 4. Spectral analysis of hydrogen peroxide oxidation reaction with hemoglobin Providence fractions. (Left panel) Absorbance spectra obtained at intervals of 2 min for 1 h upon treatment of the (A) HbAo (βK82), (B) (βK82N), and (C) (βK82D) with 30:1 H_2O_2/heme. (Right panel) Absorbance spectra obtained at 2 min after the addition of Na_2S (2 mM) in the presence of 30:1 H_2O_2/heme of (A) HbAo (βK82), (B) (βK82N), and (C) (βK82D).

(14 amu). Figure 8D,E,F shows the charged peak clusters corresponding to the three commonly occurring oxidized byproducts of the α-subunit. In these figures the βK82N variant is used as an example; however these same discrete oxidized products are present in all of the variants. The calculated uncertainty and the calculated average molecular weight of the measurement is shown at the top of each of the three panels. Peaks are also seen in both of these chromatograms that correspond to unaltered β-globin at a mass of 15,854 in the (βK82N) variant, and 15,855 in (βK82D). Taken together, the peak information and the deconvoluted masses obtained from these chromatograms indicate a pattern of discrete oxidation

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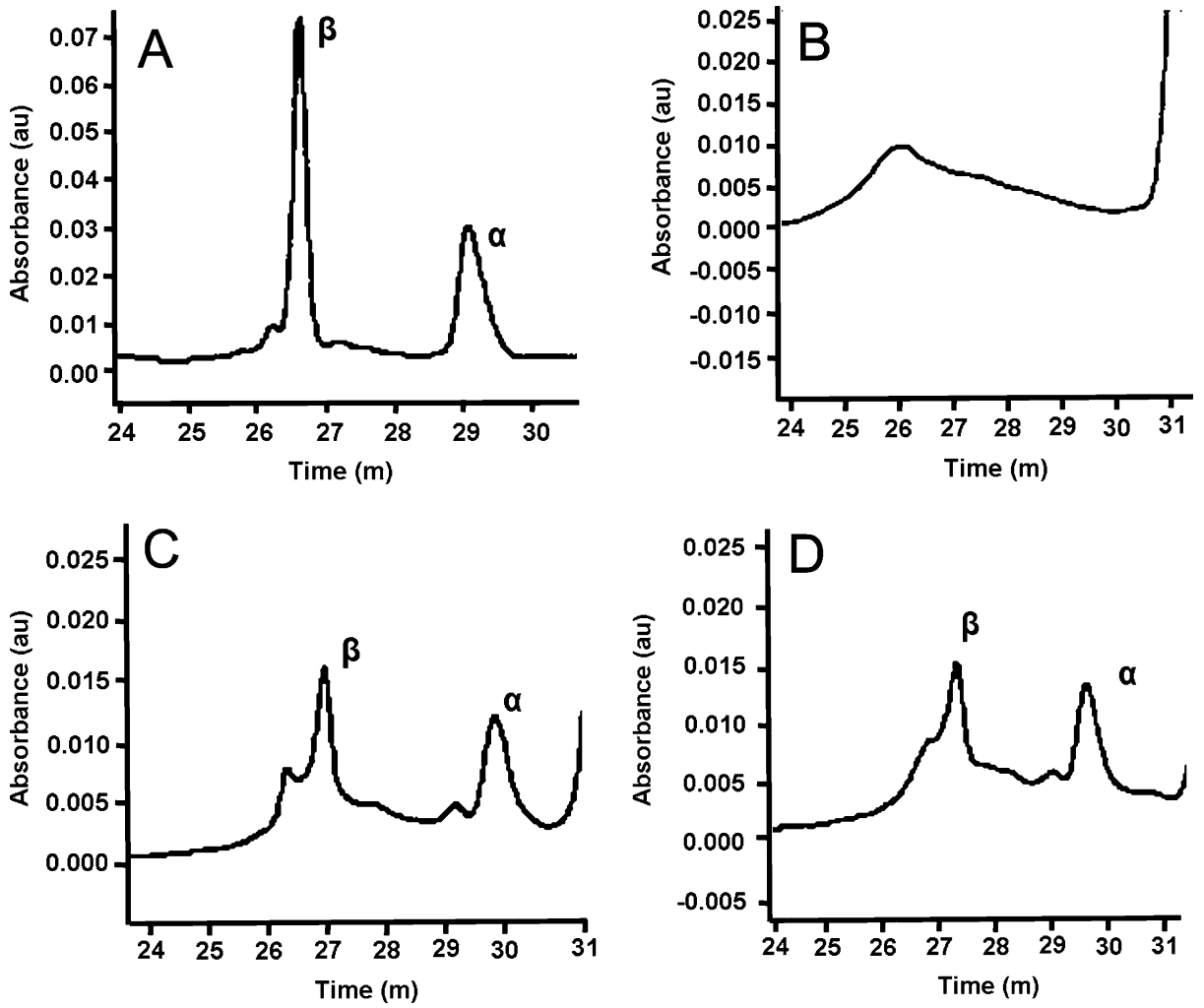

Figure 5. Reverse HPLC profiles of untreated and H_2O_2 treated hemoglobin Providence fractions. Panels A and B are reverse phase HPLC chromatograms of the purified hemoglobin Providence HbAo (βK82) fraction before and after oxidation with H_2O_2 at 30:1 H_2O_2/heme ratio for 1.5 h, respectively. Panels C and D are chromatograms of hemoglobin Providence fractions (βK82N) and (βK82D) after oxidation by H_2O_2, respectively.

occurring in the H_2O_2 treated variants. This can be contrasted with the extensive oxidative changes of the globin chains that occur in H_2O_2 treated HbAo.

Cysteic Acid Formation at βCys93 and βCys112. To identify and compare the extent of amino acid oxidation within the globin chain peptides, the oxidized fractions were subjected to reduction, alkylation, and trypsin digestion for analysis by ESI-LC-MS. Typical Mascot searches resulted in β-globin sequence coverage of ~93%, while 55% sequence coverage was obtained in the case of α-globin chains. The nonoxidized and oxidized tryptic peptides are reported in the Supporting Information, Table S1. Previous work by our group and others has implicated the irreversible oxidation of cysteine residues to cysteic acid, especially βCys93 and βCys112, when Hb is treated with bolus H_2O_2.[5,31] Specifically, βCys93 was shown to be among the key amino acids that undergo extensive oxidation. Hence, we focused our efforts on these cysteine residues to compare the extent of oxidation at a peptide level in the three variants.

The base peak chromatogram of the nonoxidized (m/z 739.809) and trioxidized (m/z 735.317) MH_2^{+2} ion of the β(83−95) peptide of HbAo is shown in Figure 9A. The mass of the nonoxidized peptide corresponds to the reduced alkylated cysteine residue (CH_2CONH_2 57.0 mass units) generated prior to trypsin digestion. The mass of the trioxidized peptide corresponds to the mass of three oxygen atoms on the sulfhydryl group of the cysteine residue. Figure 9B shows the base peak chromatogram of the nonoxidized (m/z 1039.460) and trioxidized (m/z 1036.480) MH_3^{+3} ion of the β(67−95) peptide of the (βK82D) variant. Although, the tryptic peptide containing the oxidized βCys93 occurs at different masses due to the βK82 mutation and generates peptides that differ in m/z, a qualitative comparison of the trioxidized peptides to their nonoxidized counterparts suggests that higher βCys93 oxidation occurs in the HbAo fraction than in βK82D. The MSMS spectra of the nonoxidized and oxidized β(83−95) peptides of the βK82 variant are shown in Figure S2A and B (Supporting Information), respectively. The MSMS spectra of the nonoxidized and oxidized β(67−95) peptide of the βK82D variant are shown in Figure S2C and D (Supporting Information), respectively.

Similarly, a comparison of βCys112 oxidation among the two fractions was carried out. Figure 9C,D shows the base peak chromatogram of the nonoxidized (m/z 592.982) and trioxidized (m/z 589.977) MH_3^{+3} ion of the β(105−120) peptide of the normal HbAo and the (βK82D) variant. Comparison of the trioxidized peptide at m/z 589.977 of the

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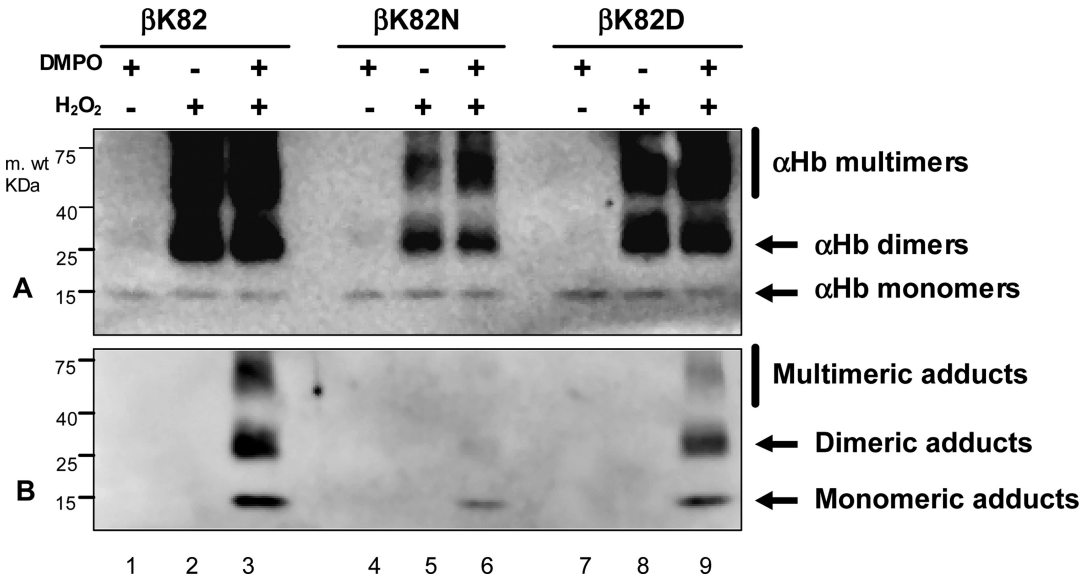

Figure 6. Peroxide-mediated α-subunit dimerization and inhibition by DMPO. Western blot analysis using antibodies against (A) hemoglobin α-subunit and (B) DMPO for HbAo (βK82), βK82N and βK82D fractions that were treated with 30:1 peroxide/heme ratio in the presence of 100 mM DMPO for 1.5 h at room temperature. Lanes 1, 4, and 7 are untreated HbAo, βK82N, and βK82D, respectively. Lanes 2, 5, and 8 are H_2O_2 treated HbAo, βK82N, and βK82D fractions, respectively. Lanes 3, 6, and 9 are H_2O_2 treated HbAo, βK82N, and βK82D in the presence of DMPO, respectively.

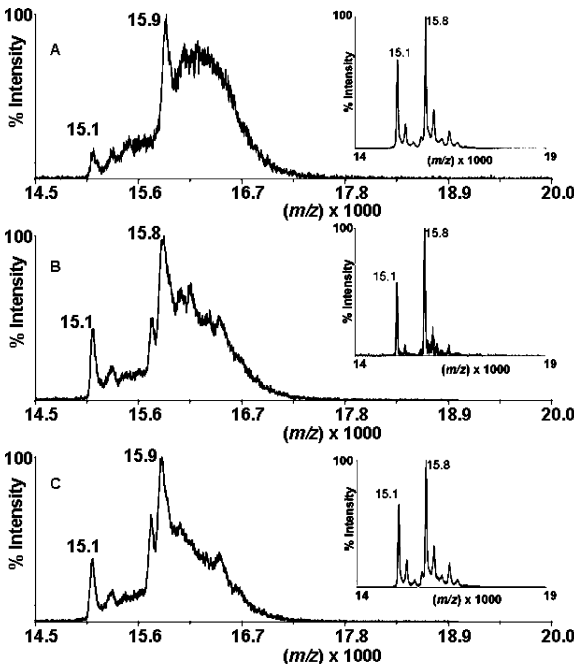

Figure 7. MALDI-MS analysis of H_2O_2-treated hemoglobin Providence fractions. (A) HbAo, (B) βK82N, and (C) βK82D treated with a 30:1 H_2O_2/heme ratio. Insets show corresponding intact α- and β-globin chains at 15.1 and 15.8 kDa, respectively. The α-chain at 15.1 kDa has the lowest intensity in treated HbAo (βK82) compared to that of βK82N and βK82D. The appearance of a broad band between 16 and 17 kDa indicates the formation of extensive oxidation products in HbAo compared to βK82N and βK82D.

two variants again suggests that βCys112 is oxidized to a greater extent in the HbAo (βK82) fraction when compared to the (βK82D) variant. The corresponding MSMS spectra of nonoxidized and oxidized β(105−120) peptides are shown in Figure S2E and F (Supporting Information), respectively.

The (βK82N) variant fraction was not included in the comparison mainly because these samples showed the presence of the βK82D peptides. On the basis of trends in retention times, these peptides were consistent with deamidation products corresponding to iso-aspartic acid and aspartic acid.[26] We did not perform further experiments to deconvolute the occurrence of deamidation that occurs at the peptide level to the extent of oxidation observed. Instead, independent assessment of oxidation was performed on the βCys112 peptide β(105−120) generated by the βK82N fraction. Interestingly, we did not observe the formation of the trioxidized βCys112 peptide in the treated βK82N variant. This result is consistent with the result observed in the immunoblot that suggests that the βK82N variant shows enhanced resistance to the formation of oxidative adducts. These results are summarized in tabular form in Table S2 (Supporting Information).

DISCUSSION

Site-specific modifications of free Hb have been observed under mild oxidative conditions and when the protein is challenged with excess H_2O_2 in vitro. First, the heme may become oxidatively modified and covalently linked to the protein, possibly via a histidine residue under acidic conditions and a serine residue under more neutral pH.[4] Second, extensive globin chain cross-links and irreversible modifications of key amino acids have also been observed in human Hb. The sulfur-containing amino acids including βMet55, βCys93, and βCys112 were observed in the oxidized forms of methionine sulfoxide and cysteic acid, respectively. We also found βTrp15, which irreversibly oxidizes to oxyindolyl and kynureninyl products.[5]

Little is known, however, about the oxidative reactions of variant Hbs and their stability in the face of oxidative challenges, particularly when mutation leads to a compromised affinity of the carrier RBCs to oxygen. More than 29% of the more than 200 human Hb β-chain variants investigated previously were unstable.[32] Others exhibit higher or lower oxygen affinities than normal Hb without a change in stability.[33]

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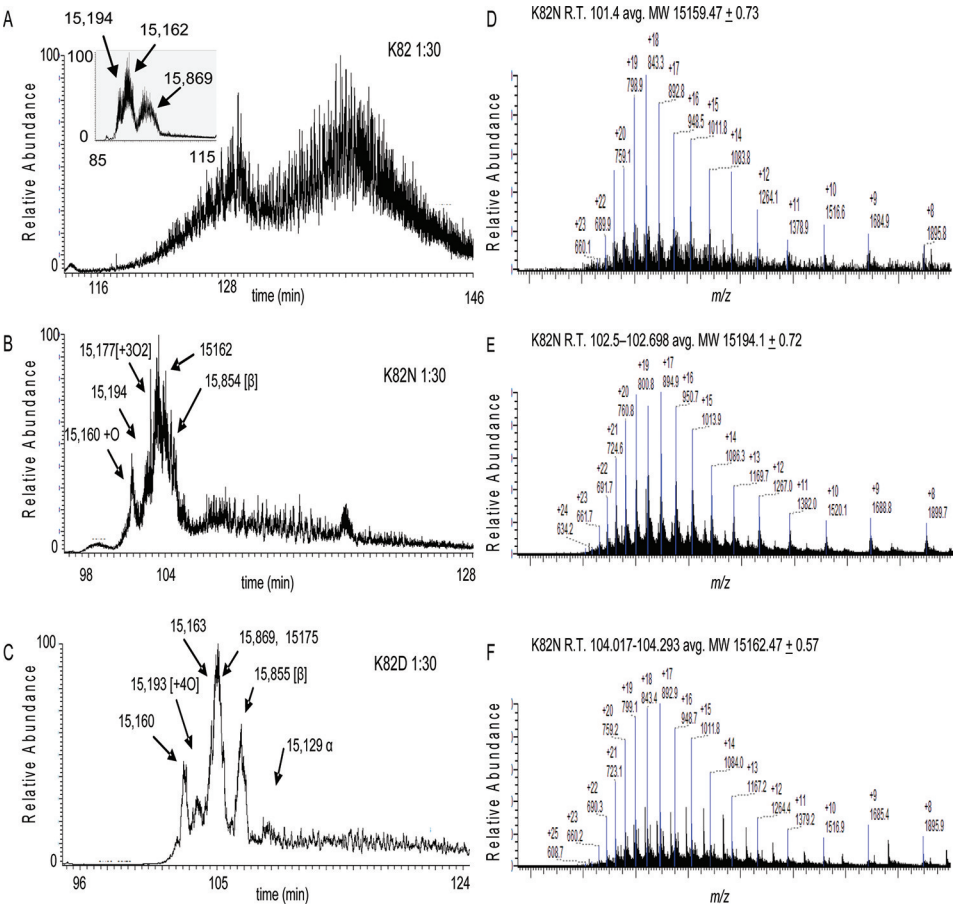

Figure 8. Comparison of LC-MS base peak chromatograms of H_2O_2-treated hemoglobin Providence fractions. (A) HbAo, (B) βK82N, and (C) βK82D treated with a 30:1 H_2O_2/heme ratio. The numbers indicate individual deconvoluted masses obtained from the charge state clusters of various oxidative states of α- and β-globin. Panels D, E, and F illustrate selected charge state envelopes taken from the K82N variant. The retention time, deconvoluted mass, and associated measurement error are shown at the top of each spectrum. The insert in A corresponds to the base peak chromatogram of intact HbAo.

In addition, it is unclear whether evolutionary pressures over time had any bearing on the oxidative stability of a given variant Hb when compared to its HbA counterpart within the same RBC.

Hb Providence provides a unique model system, in which oxidative reactions of the individual Hb fraction can be investigated in isolation of other fractions, and the contribution of each mutation if any to the overall stability of the protein can be determined. In this investigation, we have fractionated Hb Providence into its 3 component fractions, and the purity and site-specific mutation in each fraction has been confirmed by mass spectrometry. Particularly relevant to this investigation, we have ensured the removal of catalase (H_2O_2 scavenger) from all Hb fraction solutions. We have also determined the oxygen off and CO on rate kinetics for each fraction using rapid mixing techniques. These rates were found to be in complete agreement with those kinetic parameters reported earlier;[15] see Table 1. We have also reported for the first time the NO dioxygenation kinetics for each fraction, and these values were also close to those reported for HbA.[34]

Three ingredients are required for Hb mediated oxidative changes to occur, and these are (a) a protein-based radical, (b) a ferryl heme iron, and (c) a protonation event, likely the protonation of the oxoferryl. While it is possible to form 100% of the ferryl Hb species,[35] the globin free radical has been very difficult to detect using conventional techniques presumably due to rapid and uncontrolled side reactions. As well as oxidizing nearby proteins and lipids, these reactive intermediates also oxidize Hb's own amino acids in a self-destructive process as described earlier.[5]

Using sperm whale (SW) recombinant myglobins (Mb) as a model molecule, we reported that Gln(E7) SW metMb variants and Asian elephant double mutant metMb Gln(E7)/Phe(B10) have high pseudoperoxidase activity, rapidly removing the H_2O_2 produced by the process of autoxidation or when H_2O_2 is added to the protein in a catalase-like fashion.[36] The newly formed or added H_2O_2 rapidly reacts with the remaining oxyMb (MbO_2) to speed up subsequent oxidation. These mutants remove H_2O_2 catalytically, even though it degrades after multiple turnovers. The net result is marked protection of the remaining MbO_2 and increased resistance to autoxidation in the absence of catalase. The naturally occurring or engineered peroxidatic activity of Hb and Mb can function as a protective mechanism, consuming H_2O_2 and safely removing the radicals formed through internalization of the radical burden, preventing the depletion of important intracellular antioxidant molecules such as glutathione and ATP [4,37] Some naturally occurring acellular Hbs such as monomeric Hb of the clam *Lucina pectinata* was also shown by our group to exhibit a considerable resistance to autoxidation and oxidative modification when reacted with excess H_2O_2.[38]

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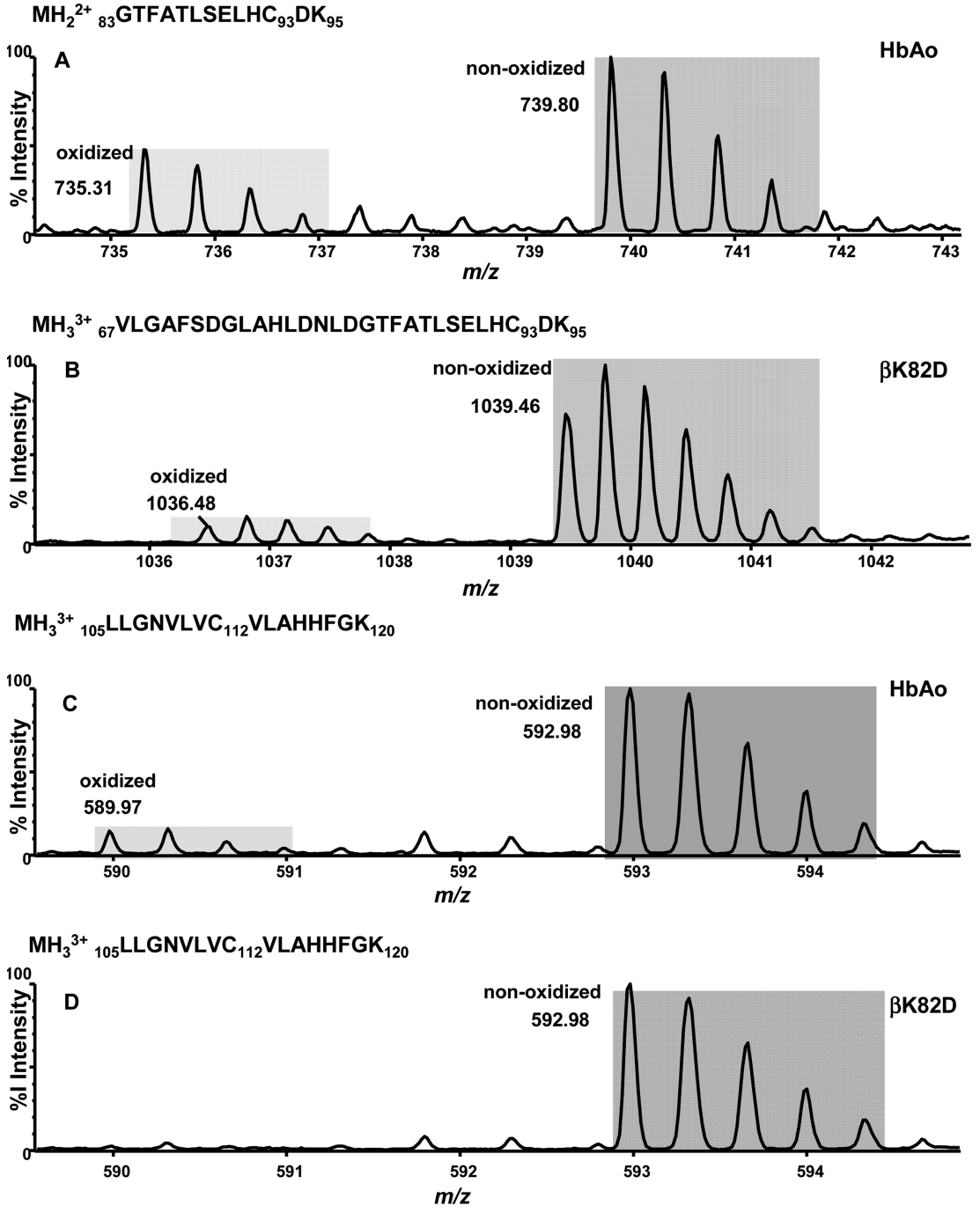

Figure 9. Mass spectral analysis of β-globin chain peptides that contain nonoxidized and oxidized cysteine (βCys112 and βCys93) in H_2O_2 treated hemoglobin Providence fractions. (A) Nonoxidized βCys93 peptide ($_{83}$GTFATLSELHC$_{93}$(CH$_2$CONH$_2$)DK$_{95}$) corresponding to m/z 739.80 $[MH_2]^{2+}$ and trioxidized βCys93 peptide $_{83}$GTFATLSELHC$_{93}$(O3)DK$_{95}$) corresponding to m/z 735.31 $[MH_2]^{2+}$ in H_2O_2 treated HbAo (βK82) fraction. (B) Nonoxidized βCys93 peptide ($_{67}$VLGAFSDGLAHLDNLD$_{83}$GTFATLSELHC$_{93}$(CH$_2$CONH$_2$)DK$_{95}$) corresponding to m/z 1039.46 $[MH_3]^{3+}$ and trioxidized βCys93 peptide ($_{67}$VLGAFSDGLAHLDNLD$_{83}$GTFATLSELHC$_{93}$(O3)DK$_{95}$) corresponding to m/z 1036.48 $[MH_3]^{3+}$ in H_2O_2 treated βK82D fraction. Panels C and D show the nonoxidized βCys112 peptide (L_{105}LGNVLVC$_{112}$(CH$_2$CONH$_2$)VLAHHFGK$_{120}$) corresponding to m/z 592.97 $[MH_3]^{3+}$ and trioxidized βCys112 peptide (L_{105}LGNVLVC$_{112}$(O_3)VLAHHFGK$_{120}$) corresponding to m/z 589.97 $[MH_3]^{3+}$ in H_2O_2 treated HbAo (βK82) and βK82D, respectively.

Our data on isolated Hb Providence fractions summarized in Figure 4 clearly demonstrate that these fractions are better peroxidases than their native HbAo. The latter undergoes rapid oxidative changes after the addition of molar excess H_2O_2 and after the complete transformation of the starting oxyHb to a ferryl Hb intermediate, as verified by the derivatization with Na_2S. However, in the case of Hb Providence (βK82N and (βK82D), the ferryl intermediate formed initially was reduced back to the ferric intermediate as oxyHb in these fractions and was able to deplete added H_2O_2 more effectively than native HbA_0. it is interesting to note that in the case of the βK82N fraction the turnover to a met intermediate was accomplished more readily in spite of the fact that there was more ferryl intermediate present at the start of the reaction.

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Our extensive structural analysis of the three different fractions after treatment with H_2O_2 confirms the extraordinary stability that variant fractions exhibited over their native counterpart when challenged with excess H_2O_2. There are several levels of structural changes whose accompanying Hb redox reactions have been used as hallmarks of emerging and damaging reactive intermediates.[4,5]

First, the addition of H_2O_2 to Hb or Mb and subsequent formation of highly redox active intermediates have been shown to induce covalent dimerization and/or aggregation of the proteins. The reaction of the ferric forms of these proteins with H_2O_2 leads to parallel production in ferryl and porphyrin radicals.[39] The tyrosyl radicals of residue Tyr-103 and Tyr-151 have been shown to participate in the covalent bond formation between Mb monomers.[40] In Hb, both Tyr-24 and Tyr-42 have been implicated in α-Hb subunit dimerization.[29] DMPO has been shown to prevent Mb dimer formation as it effectively traps the protein radicals.[41] Using immune-spin trapping, we observed the same effect with Hb fractions. As shown in Figure 6, extensive dimerization and adduct formation were very evident in HbAo (βK82) fraction. Lesser band intensities that correspond to fractions βK82D and βK82N were observed, particularly so in the case of the βK82N variant. Conversely, the degree of inhibition of covalent adduct formation by DMPO was more evident in the variant fractions and in the following order βK82N > βK82D > HbAo.

Second, as we have reported previously, when Hb is challenged with H_2O_2 a predictable pattern of oxidation and programmed oxidative changes appears, which results in deformation and the collapse of the β-chains.[5] Our HPLC data together with the mass spectrometric analysis of isolated chains confirms these oxidative pathways in all fractions. Again, the variant fractions showed a consistent pattern of resistance to the oxidative insult by H_2O_2. Generally, we observed that both α- and β-subunits in the variant Hbs showed better stability and integrity profiles in the presence of excess H_2O_2 than those counterpart subunits in HbAo.

Finally, as these structural changes and protein unfolding occur in both α- and β-subunits, specific amino acids on the protein can become more accessible consequent to oxidation over time.[5] Indeed, according to our previous assessments of the accessible surface areas (ASA), we found that sulfur containing amino acids were particularly more exposed and thus more susceptible to H_2O_2 radical chemistry attacks emanating from the heme. Our mass spectrometric and amino acid analysis show that βCys93 (0.5 Å) and βCys112 (0.1 Å) in the intact protein are more frequently oxidized in HbAo and to a lesser frequency in the βK82D and βK82N variants.

In summary, we fractionated Hb Providence into its three major components and found that these fractions react with ligands and oxidants at similar rates. However, in the presence of excess H_2O_2, Hb Providence fractions were found to be more resistant to oxidative changes and modifications than native Hb. We present evidence to show that these differences can be attributed to the enhanced catalase-like activity of the variant fractions, which enabled the efficient removal of the oxidant with less structural damage. Structural stability in the variant Hbs may stem also from a better folding of β-chain proteins and/or in the heme pocket region, which could result in less solvent accessible heme and a more stable protein.[42]

Biotechnological and Clinical Relevance. There is a considerable interest in producing recombinant human Hb (rHb) as an oxygen carrying therapeutic, but the production of such a protein is limited by the yield of holoprotein in *E. coli*. The major problems appear to be aggregation and degradation of apoglobin at the nominal expression temperatures, in addition to the limited amount of free heme that is available for holoHb assembly. One approach to solve the aggregation problem is to inhibit apoglobin precipitation by constructing mutants of Hb with increased stability of the helices of both subunits of HbA_0. Fetal Hb, for example, was shown to be more stable than human HbAo, and sequence comparisons between human β- and γ (fetal Hb)-chains indicate several substitutions that stabilize the $\alpha1\beta1$ interface, one of which, βHis116 to Ile, increases resistance to denaturation and enhances expression in *E. coli*.[43] Alternatively, Hb Providence (Asp) was recently shown to improve high levels of expression of soluble recombinant Hbs expressed in *E. coli* far more than the poor yields of another β-subunit mutant, Presbyterian (Asn108Lys). Globin resistance to unfolding discovered in Hb Providence and its unusual oxidative stability as we have shown in this work are among important attributes that should be considered in the design of a safe and effective oxygen carrying therapeutics, such assistance to autoxidation, oxidative modifications and heme loss.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Formation of peroxide-induced heme to protein cross-linking in Hb Providence fractions. SMS spectra of nonoxidized and oxidized βCys93 and βCys112 peptides in βK82 and βK82D variants. A table listing the nonoxidized and oxidized tryptic peptides obtained in the variants after treatment with H_2O_2 treated variants are also shown in the supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

Asp-N, endoprotease that specifically cleaves N-terminal to Asp; AHP, altered heme products; CHCA, alpha-cyano-4-hydroxycinnamic acid; CO, carbon monoxide; DEAE, diethylaminoethyl cellulose; DTT, dithiothreitol; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; 2,3-DPG, 2,3-diphosphoglycerate; ESI, electrospray ionization; FA, formic acid; HPLC, high pressure liquid chromatography; LC-MS, liquid chromatography−mass spectrometry; MALDI, matrix assisted laser desorption ionization; NO, nitric oxide; LTQ, linear ion trap mass spectrometer (Thermo Scientific); TFA, trifluoroacetic acid; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; TOF, time of flight; TTBS, Tris HCl buffer at pH 7.4 containing 0.1% Tween (surfactant); RBCs, red blood cells

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