Acute and Long-Term Stability Studies of Deoxy Hemoglobin and Characterization of Ascorbate-Induced Modifications

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Abstract □ The reaction of ascorbate with recombinant hemoglobin (rHb1.1) in the presence of differing partial pressures of oxygen was studied. In the presence of 15 000 ppm (1.5%) residual oxygen, ascorbate/oxygen-mediated reactions resulted in an increased rate of autoxidation, modification of the β-globin, increased oxygen affinity and decreased maximum Hill coefficient. One of the observed modifications to the β-globin was a 72 Da addition to its N-terminus. Detailed characterization indicates the modification was an imidazolidinone type structure. Thorough deoxygenation of the hemoglobin solution to <150 ppm of oxygen prior to addition of ascorbate was required to prevent these modifications. Addition of ascorbate to the deoxy hemoglobin (deoxyHb) at pH 8 induced aggregation, eventually leading to precipitation. No such precipitation was observed at pH 7. Long-term storage of the hemoglobin was carried out by addition of ascorbate to deoxyHb at pH 7. The level of methemoglobin remained at <2% for up to 1 year at 4 °C, with no detectable precipitation of the protein. Modifications similar to those observed by the acute studies were observed over the 1-year period and correlated with disappearance of the added ascorbate.

Introduction

Recombinant human hemoglobin (rHb1.1) is a pseudo-tetramer composed of two β-globins and genetically fused α-globins (di-α-globin). Each of the four subunits contains a heme prosthetic group. The Presbyterian mutation in the β-globin (Asn-108β→Lys) was introduced to decrease the oxygen affinity. Genetic fusion of the two α-globins prevents dissociation of the hemoglobin into αβ-dimers,[1] such that rHb1.1 can be used as an oxygen-carrying therapeutic. The ability to package and store this type of product as a ready-to-use large volume parenteral (LVP) liquid has many potential advantages during situations such as trauma and surgery when use of frozen formulations would not be as practical.

One of the major obstacles to long-term storage of hemoglobin is its propensity to autooxidize. Upon binding oxygen, the reduced form of the heme iron (ferrous, Fe^{2+}) can react with the oxygen to form ferric (Fe^{3+}) iron and superoxide:[2−4]

$$HbFe^{2+} + O_2 \leftrightarrow HbFe^{2+} - O_2 \rightarrow HbFe^{3+} + O_2^-$$

When the iron in heme is oxidized to the ferric form, oxygen can no longer bind to the heme iron and the hemoglobin is nonfunctional. Oxidation of the heme also leads to an approximately 1000-fold increase in the rate of heme loss,[5] which can result in denaturation and precipitation of the globin.[6] The activated oxygen species produced during the autoxidation reaction can damage the hemoglobin causing polymerization,[7,8] oxidation of the amino acid side chains,[9] and hemichrome formation.[10,11] These modifications can lead to changes in the tertiary and quaternary structure, resulting in decreased oxygen equilibrium binding parameters and eventually precipitation of the protein. In contrast, ferrous deoxy hemoglobin (deoxyHb) does not undergo autoxidation and is known to be intrinsically more stable than oxy hemoglobin (oxyHb) against thermal and chemical denaturation and precipitation of the protein.[12−14]

The nonenzymatic reduction of methemoglobin (metHb) has been achieved using reducing agents such as dithionite and ascorbate. In solution under deoxy conditions, dithionite ($S_2O_4^{2-}$) dissociates into $2SO_2^{\bullet-}$,[15] which can quickly react with MetHb, reducing it back to the ferrous form.[16,17] The byproduct of the reaction, bisulfite (HSO_3^-), can generate severe or fatal adverse reactions in humans,[18] making it unsuitable as a component of a LVP formulation. Ascorbate has been known since the 1940s to reduce deoxygenated metHb to the ferrous form[19,20] and can safely be given intravenously.[18] The literature indicates that ascorbate reduces metHb by first ionizing to the anionic form, ascorbate^{2-}, which reacts with the ferric iron to form ferrous hemoglobin and the anionic ascorbate radical, which eventually rearranges to form dehydroascorbate.[21]

To overcome the problems associated with storage of oxyHb solutions, we examined methods to store deoxygenated hemoglobin solutions at 4 °C. Ascorbate was added to reduce residual methemoglobin to the more stable ferrous form. Here we present studies that determined the effects of pH, oxygen tension, and ascorbate levels on rHb1.1 stability.

Materials and Methods

Materials—Recombinant hemoglobin (rHb1.1) was produced at Somatogen as previously described.[1] Ascorbic acid (sodium salt), sodium phosphate (monosodium and disodium salts), sodium chloride, ethylenediaminetetraacetic acid (EDTA, disodium salt), (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) (HEPES), potassium cyanide, and tris(hydroxymethyl)aminomethane (Tris hydrochloride and sodium salt) were all purchased from Sigma Chemical Company (St. Louis, MO). Polysorbate 80 was purchased from either Calbiochem Corporation (San Diego, CA) or ICN Chemicals (Los Angeles, CA). Glass vials and gray butyl

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rubber stoppers were purchased from Wheaton Corporation (Charlotte, NC). Stedim-5 bags were purchased from Stedim Corporation (Pleasant Hill, CA), aluminum foil overwraps were purchased from Kegan Corporation (Auburn, IN), and ZPT 100 "Ageless" oxygen scavengers were purchased from Mitubiabi Gas and Chemical Company (Japan).

Methods—*Dynamic Light Scattering*— Dynamic light scattering (DLS) was performed using a Nicomp 370 Submicron Particle Sizer equipped with a 40mW HeNe laser. Data were collected and averaged over 10 min and analyzed using the C370 v.12 software program provided by PSS (Particle Sizing Systems, Santa Barbara, CA).

Equilibrium Oxygen Binding—The parameters P_{50} and n_{max} were determined using a hemox analyzer as previously described[22] at 37 °C and pH 7.40 in 50 mM HEPES (free acid) and 150 mM NaCl.

Methemoglobin Concentration—Percent metHb was measured by determining the fractions of reduced and oxidized hemes using difference spectroscopy as described by Kerwin et al.[43]

Ascorbic Acid Analysis—Ascorbic acid analyses were performed by diluting samples 1:1 into 10 mM homocysteine (sparged with helium) to stabilize the ascorbate. The diluted samples were centrifuged in Centricon-30 microconcentrators (Amicon Inc., Beverly, MA), and the filtrate was analyzed by size exclusion chromatography (SEC) on a TosoHaas G2500W_{XL} (7.6 × 300 mm) column using an isocratic elution with 5 mM KH_2PO_4, pH 3, as the mobile phase. Flow rate was 1 mL/min. Analyte peaks were detected at 255 nm, and their concentration was determined by comparison with standards of known concentration.

Reversed-Phase HPLC (RP-HPLC)—Samples were prepared by precipitation with ice-cold acid/acetone[23] and solubilization of the pellet in 0.1% trifluoroacetic acid (TFA)/20% acetonitrile. The RP-HPLC analyses were performed using a Zorbax C3 analytical column (0.46 × 25 cm) mounted on an HP1090 HPLC system (Hewlett-Packard, Wilmington, DE). The oven temperature was maintained at 40 °C. Solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile. The flow rate was 1 mL/min. The column was equilibrated in 65% solvent A/35% solvent B. Following sample injection, the column was maintained at the starting conditions for 5 min then ramped to 51% solvent A/49% solvent B over a period of 45 min.

Size Exclusion Chromatography—The molecular weight distribution of the hemoglobin was monitored by high-performance SEC (HPSEC) as described by Kerwin et al.[43]

Liquid Chromatography-Mass Spectrometry (LC-MS)—Mass spectrometry was performed using a Finnigan Mat LCQ as the end detector with an HP1090 HPLC on the front end to run reversed-phase separation. Analysis by MS/MS was performed as previously described by Lippincott et al.[24]

Tryptic Mapping—Tryptic mapping was performed as previously described by Lippincott et al.[24]

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)—SDS-PAGE was performed based on the method of Laemmli.[25] Aliquots (5 μg) were diluted with 2 volumes of SDS sample buffer (Novex Corp., San Diego, CA) containing 0.1 M dithiothreitol (DTT) and heated at 65 °C for 5 min. Samples were electrophoresed on an 8−16% polyacrylamide gradient Tris-glycine gel for 2.5 h at 120 V. The gel was stained with Coomassie blue and then destained with a solution of 40% methanol, 10% glacial acetic acid, and 50% water. Destained gels were digitized using an IS-1000 digital imaging system (Alpha Innotech Corp., San Leandro, CA).

Pepsin Mapping of Multimeric rHb1.1—The multimeric fraction of rHb1.1 was isolated using SEC as previously described. The globins were precipitated with 20 volumes of cold 0.6% HCl in acetone, solubilized in 0.1% TFA, and separated by C3 RP-HPLC as previously described. Fractions containing β-globin and di-β-globin were collected, lyophilized, then resuspended in 8 M urea. Following resuspension, the samples were heated for 10 min at 60 °C, then diluted with 0.1% TFA to a final urea concentration of 2 M. The final globin concentration was 1 mg/mL. Pepsin (Pierce, Rockford, IL) was added to the sample at an enzyme: substrate ratio of 1:50 (w/w). After 2 h, the peptides were separated by RP-HPLC using a C18 column (Zorbax SB-300, 0.46 × 25 cm) on an HP1090 HPLC. Solvent A was 0.1% TFA(v/v) in water and solvent B was 0.1% TFA (v/v) in acetonitrile. The column was equilibrated in 5% solvent B at 1 mL/min. Following injection, the starting conditions were maintained for 5 min. The gradient was then developed by increasing to 70% solvent B over a period of 70 min. The absorbance was monitored at 215 nm.

Preparation of rHb1.1 Solutions Equilibrated with Headspace Oxygen Concentrations of 15 000 or 150 ppm—For oxygen and ascorbate modification experiments, solutions of rHb1.1 (50 mg/mL in 150 mM sodium chloride, 5 mM sodium phosphate, pH 7.2) were equilibrated with either a gas mixture of 15 000 ppm oxygen in nitrogen or purified nitrogen containing <10 ppm of oxygen. Equilibration was achieved by passing the gas mixture through water for humidification then over the hemoglobin solution, which was mixed using a Rotovap (Brinkman Instruments). The equilibration procedure was typically performed for 4−6 h, and the hemoglobin solution was maintained at ~10 °C in an ice-water bath. Following equilibration, the flask containing the hemoglobin solution was capped and transferred into a glovebag (Aldrich Chemical Company, Milwaukee, WI) along with all necessary equipment and solutions. The bag was sealed and equilibrated with either the gas mixture containing 15 000 ppm of oxygen or the mixture containing <10 ppm of oxygen. The oxygen content in the bag equilibrated with the <10 ppm oxygen mixture measured at 150 ppm of oxygen when monitored with a Mocon HS-750 analyzer (Modern Controls Inc.). Ascorbate (0.5 M in deoxygenated water) was added to aliquots of the hemoglobin solution to give final concentrations of 0.5, 1, 2, and 5 mM. Solutions were filtered through 0.2-μm syringe filters. Aliquots (0.5 mL) were transferred into 2-mL Wheaton glass vials, capped with gray butyl rubber stoppers, and sealed with crimp rings. All solutions were incubated at 4 °C. For pH-induced aggregation studies, hemoglobin solutions were diafiltered into buffer (150 mM sodium chloride, 5 mM sodium phosphate) at pH 7 or pH 8 at 4 °C. Solutions were equilibrated with nitrogen containing <10 ppm oxygen, reduced with 2 mM ascorbate, filtered through a 0.2-μm filter, and aliquoted into 2-mL glass vials as already described. The oxygen tension in the glovebag was maintained at ≪200 ppm oxygen.

Shaking-Induced Aggregation Studies—All manipulations of material were done in a nitrogen flushed glovebag (Aldrich) kept at <300 ppm of oxygen. Tween 80 was added to aliquots of deoxy-rHb1.1 (80 mg/mL in 150 mM sodium chloride, 5 mM sodium phosphate, pH 7.2, and <5 μM EDTA) to approximate final concentrations of 0, 0.025, 0.05, 0.075, and 0.1% (w/v). The solutions were then realiquoted (1.5 mL) into 3.5-mL Wheaton vials and sealed with gray butyl rubber stoppers, and the Tween 80 concentrations were measured as described below. The final Tween 80 concentrations were 0, 0.019, 0.045, 0.071, and 0.095%. All prepared samples were used within 1week of preparation. The deoxyHb aliquots (1.5 mL) were placed on their sides on a Hoeffer rotary shaker and shaken at either 100, 170, or 225 rpm for 1 h at room temperature. The samples were then assayed for protein aggregation by light obscuration.

Measurements of Protein Aggregates by Light Obscuration—Aggregates ≥2 μm were measured using a HIAC/Royco light particle counter equipped with a HIAC HRLD400HC sensor and a model 3000A sampler. Data were corrected for background counts and reported as the average from separate samples.

Measurement of Tween 80 Concentration in Hemoglobin Solutions—Samples were analyzed for Tween 80 in the following fashion: To a 1-mL aliquot of sample in a 15-mL polypropylene Falcon tube was added 1 mL of 6 M guanidine hydrochloride and 4 mL of dichloromethane. The mixture was then vortexed for 1 min to form a thick emulsion. The emulsion was separated by centrifugation at 4000 rpm for 20 min. The lower organic layer was isolated and placed into a clean 15-mL polypropylene Falcon tube. To that organic layer was added 1 mL of a cobalt ammonium thiocyanate test solution (250 g of ammonium thiocyanate, 110 g of cobalt nitrate, and 200 g of sodium chloride in 1 L of purified water). The mixture was then vortexed for 30 s and allowed to separate. The organic layer was then analyzed spectrophotometrically at 320 nm. The concentration of Tween 80 in the original sample, which is proportional to the absorption at 320 nm, was then calculated from a standard curve of know Tween 80 concentrations.

Preparation of deoxyrHb1.1 Solutions for Long-Term Stability Studies in Vials—Recombinant hemoglobin from our manufacturing plant (~50 mg/mL), in 150 mM sodium chloride, 5 mM sodium phosphate (pH 6.8−7.2), ~0.03% polysorbate 80, and <5 μM EDTA, was deoxygenated by stripping the oxyHb solution with nitrogen gas. Following deoxygenation, the hemoglobin was

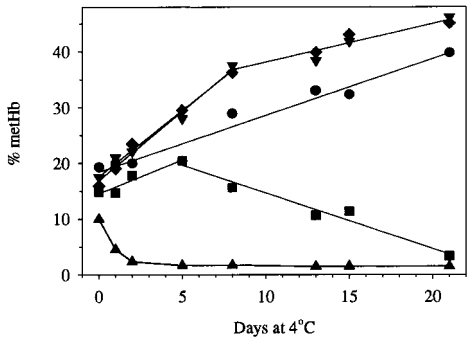


Figure 1—MetHb formation of rHb1.1-containing ascorbate in solution and equilibrated with 15 000 ppm of oxygen in the headspace. Recombinant hemoglobin (rHb1.1) was equilibrated with 15 000 ppm of oxygen and reacted with increasing concentrations of ascorbate. Symbols represent the following ascorbate concentrations in each sample: (circles) 0 mM ascorbate; (octagons) 0.5 mM ascorbate; (diamonds) 1 mM ascorbate; (squares) 2 mM ascorbate; (triangles) 5 mM ascorbate. Data from samples containing 0, 0.5, 1, and 2 mM ascorbate were fit using linear regression to generate rates of metHb formation.

concentrated to 80 mg/mL and ascorbate was added to a final concentration of 2 mM. The solution was then packaged into the glass vials as already described and incubated at 4 °C for up to 1 year.

Preparation of DeoxyrHb1.1 Solutions for Long-Term Stability Studies in Bags—Recombinant hemoglobin from our manufacturing plant (<50 mg/mL), in 150 mM sodium chloride, 5 mM sodium phosphate (pH 6.9−7.1), ~0.03% polysorbate 80, and <5 μM EDTA, was deoxygenated by stripping the oxyHb solution with nitrogen gas. Following deoxygenation, the hemoglobin was concentrated to 80 mg/mL and ascorbate was added to a final concentration of 2 mM. The solution was transferred to a nitrogen-purged glovebox and packaged as 30-mL aliquots in 60-mL flexible Stedim-5 containers then overwrapped with aluminum foil pouches. ZPT 100 "Ageless" oxygen scavengers were placed between the foil overwrap and the flexible containers prior to heat sealing the overwrap containers. The foil pouches were heat sealed and incubated at 4 °C for up to 1 year.

Results

Acute Studies—*Effects of Ascorbate and Oxygen Concentrations on Methemoglobin Formation*—The ability of ascorbate to reduce recombinant methemoglobin (met-rHb) in the presence of differing partial pressures of oxygen in the vial headspace was studied. Equilibration of the rHb solution with a gas mixture containing 15 000 ppm oxygen caused an increase in the rate of met-rHb formation above that observed for recombinant oxyhemoglobin (oxy-rHb) of 1% per day versus 0.7% per day, respectively (see Figure 1, oxy-rHb data not shown). Addition of ascorbate to 0.5 and 1 mM produced a two-stage autoxidation profile with an initial autoxidation rate of 2.4% per day through 8 days of storage, followed by a slower rate of 0.7% per day. Increasing the ascorbate concentration to 2 mM decreased the autoxidation rate to ~1.1% per day for the first 5 days, followed by a reduction in the met-rHb content at a rate of 1% per day for the remainder of the study period (15 days). In contrast, the hemoglobin solution containing 5 mM ascorbate demonstrated no overall increase in the level of met-rHb and showed a complete reduction by approximately 2 days following addition of the ascorbate. Hemoglobin equilibrated with 150 ppm of oxygen in the headspace oxidized at the rate of approximately 0.2% per day (see Figure 2). Addition of ascorbate (0.5−5 mM) decreased the concentration of met-rHb. The rate of reduction was dependent upon the concentration of ascorbate.

Effects of Ascorbate and Oxygen Concentrations on P_{50} and n_{max}—Recombinant hemoglobin typically has a P_{50} of

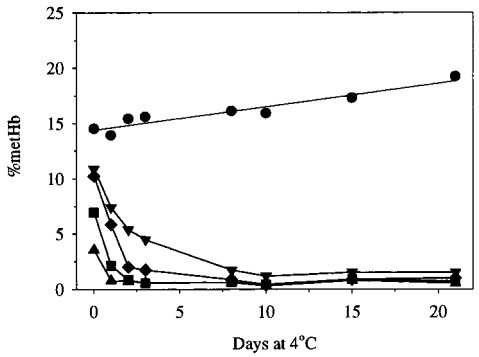


Figure 2—MetHb formation of rHb1.1-containing ascorbate in solution and equilibrated with 150 ppm of oxygen in the headspace. Recombinant hemoglobin (rHb1.1) was equilibrated with 150 ppm of oxygen and reacted with increasing concentrations of ascorbate. Symbols are the same as in Figure 1. The data for the sample containing 0 mM ascorbate were fit by linear regression to generate the rate of metHb formation.

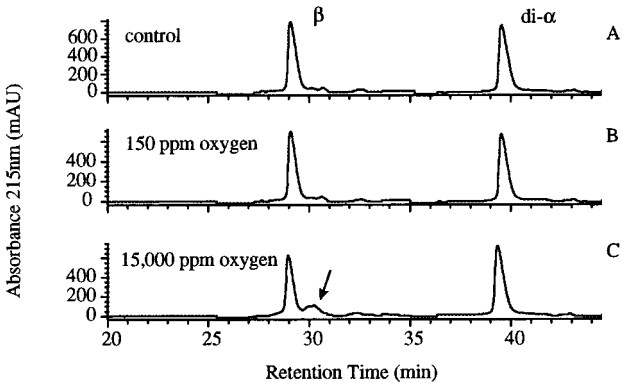


Figure 3—Reversed phase HPLC analysis of hemoglobin after incubation with 5 mM ascorbate at differing partial pressures of oxygen. Samples were the same as in Figure 1 and were analyzed following 15–16 days of storage at 4 °C. The control hemoglobin represents a sample of rHb incubated with 15 000 ppm of oxygen and 0 mM ascorbate. The arrow indicates the difference peak of the β-globin shoulder containing the M+72 addition.

32 mmHg and an n_{max} coefficient of 2.3. By 21 days, the hemoglobin sample equilibrated with 15 000 ppm of oxygen and containing 5 mM ascorbate demonstrated a P_{50} of 37.3 mmHg and an n_{max} coefficient of 1.5. The hemoglobin equilibrated with 150 ppm of oxygen and 5 mM ascorbate demonstrated a P_{50} of 33.8 mmHg and an n_{max} coefficient of 2.11 at 21 days.

Characterization of Ascorbate-Mediated Modification of Hemoglobin—Reversed-phase HPLC was used to evaluate potential ascorbate-mediated modifications of the globins. The sample equilibrated with 15 000 ppm oxygen and containing 5 mM ascorbate showed a decrease in the height of the β-globin peak compared with the control along with the appearance of an increased area on the trailing shoulder of the β-globin peak (Figure 3). In contrast, the chromatographic profile of the sample equilibrated with 150 ppm oxygen and containing 5 mM ascorbate was not significantly different from a control sample with no added ascorbate. The sample equilibrated with 15 000 ppm oxygen and 5 mM ascorbate was further analyzed by LC-MS. The analyses of the shoulders on the β-globin peak revealed the presence of three significant β-globin adducts with mass additions of 16, 42, and 72 Da. With the exception of the 42 Da mass gain on the β-globin, these mass additions were not observed in the hemoglobin solution prior to addition of the ascorbate. Tryptic mapping of the starting hemoglobin revealed a small peak assigned to acetylation of the N-terminus of the β chain with a characteristic 42 Da mass gain. Additionally, using single ion chromatograms, we searched for modifications on

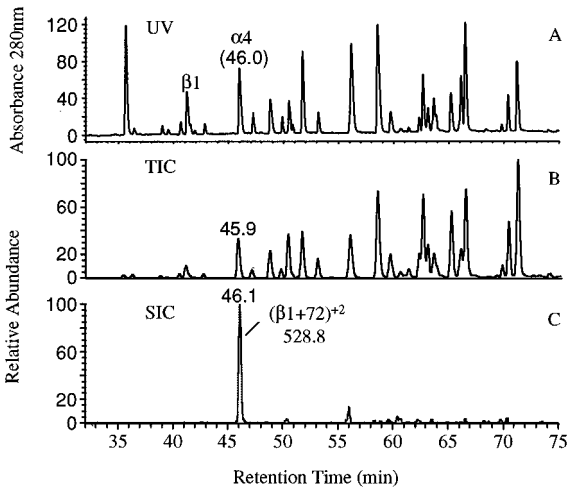


Figure 4—Tryptic map of ascorbate modified rHb1.1. Tryptic peptides of rHb1.1 incubated with 2 mM ascorbate were separated by C18 RP-HPLC and analyzed by mass spectrometry: panel A, UV trace at 280 nm; panel B, total ion chromatogram; panel C, specific ion chromatogram of the double-charged β1+72 ion.

tryptic peptides. Only one significant difference peak was found at 45 min; exhibiting a mass of 1055.4 amu and eluting as a trailing shoulder on the α4 peptide (see Figure 4). The peak was previously identified as the M+72 modification of the N-terminus of the β-globin affected by ascorbate and oxygen.[26] In agreement with the tryptic mapping, V8 mapping of this hemoglobin confirmed the existence of the M+72 at the N-terminus of the β-globin and did not show additional difference peaks.

The fraction containing the M+72 peptide from the tryptic map was isolated and infused into the LCQ mass spectrometer. The double-charged ion (528.8) was fragmented to elucidate the position and structure of the modification. MS/MS fragmenation spectra (see Figure 5) showed an unusual pattern of a neutral loss of 44 Da followed by an additional loss of 18 Da. These mass losses corresponded to mass gains of 28 and 10 Da on the unmodified peptide. The neutral loss of 44 and 18 Da is usually associated with decarboxylation and loss of water, respectively. The observed B ions, starting from B_2, showed gains of 10, 28, or 72 Da, confirming the presence of the modification within the first two residues. The series of Y ions was consistent with unmodified peptide, except the Y_7 and Y_8 ions. The Y_7+10 mapped a mass gain of 10 Da to His2 and the Y_8 ion mapped a mass gain of both 10 and 28 Da to the Met1 position. A mass gain corresponding to Y_7+28 was not found. The N-terminus was shown to be blocked to Edman sequencing.[26] Reduction with cyanoborohydride did not change either the elution position or the mass of the M+72 peptide. The data are consistent with an imidazolidinone structure for the modification.

Effects of pH on the Stability—The propensity of the deoxy-rHb in the presence of 2 mM ascorbate to aggregate and precipitate during 4 °C storage was studied at pH 7 and pH 8 by DLS. Comparison of the day 0 samples at pH 7 and pH 8 indicated that their size profiles are similar with no apparent aggregation (see Figure 6). Aggregates of ~100 and 500 nm were observed in the pH 8 sample after 2 weeks of incubation at 4 °C. Following an additional 2 weeks of storage, the size of the aggregates appeared to increase, and by 6 weeks a precipitate was visible in the bottoms of the vials. The pH 7 samples did not display any indications of aggregation or precipitation for up to 8 weeks of storage.

Effects of Tween 80 on Aggregation—The ability of Tween 80 to prevent protein aggregation of deoxy-rHb1.1 at a liquid surface interface was examined by shaking-induced aggregation. Samples not containing Tween 80 demonstrated an increase in the number of aggregates per milliliter at ≥2 μm with increasing shaking speed, resulting in an ~500-fold increase between 0 and 225 rpm (Figure 7A). Even greater increases were observed for aggregates ≥10 μm (Figure 7B) and ≥25 μm (Figure 7C), resulting in ~2000- and ~4000-fold increases, respectively. Addition of Tween 80 significantly decreased aggregation of the deoxyHb when shaken at speeds up to 170 rpm. At this speed, a slight increase in aggregates was observed compared with the 0 rpm sample, but no difference was seen between any of the Tween 80-containing solutions. Increasing the speed to 225 rpm produced a level of aggregation in the hemoglobin sample containing 0.019% Tween 80 similar to that observed without surfactant. In contrast, at 225 rpm, the samples containing 0.045−0.095% Tween 80 did not demonstrate an increase in aggregate levels above that observed for 170 rpm. Furthermore, these samples produced only an ~10-fold increase in counts for aggregates ≥2 μm (Figure 7A) and ≥10 μm (Figure 7B) and an ~5-fold increase for aggregates ≥25 μm (Figure 7C) compared with those observed prior to shaking.

Long-Term Storage in Vials—*Overall Stability of the Hemoglobin during Long-Term Incubation at 4 °C in Vials*—Based on the observations made during the acute stability studies, four sets of samples of rHb1.1 were prepared under deoxy conditions, defined as 150 ppm oxygen, followed by addition of 2.0 mM ascorbate. Polysorbate 80, 0.03%, was added to preclude aggregation of the protein during storage. The levels of ascorbate dropped steadily during the 12-month storage period from approximately 1.75 to 0.25 mM (see Figure 8, panel A). In accordance with the presence of ascorbate, the concentration of metHb remained at <2% (limit of quantitation) of the total protein during the course of the study (data not shown). Starting samples had a P_{50} value of approximately 32 mmHg and did not change for the first 6 months of storage (see Figure 8, panels B and C). Between 6 and 12 months, the P_{50} dropped slightly to 30 mmHg. The Hill Coefficient measured at n_{max} demonstrated a slight decrease between 0 and 6 months from an initial value of 2.2 to 2.0. A further change was observed at 12 months, with the n_{max} further decreasing to 1.8.

The propensity of the protein to aggregate during the incubation was measured by both SEC and DLS. We did not observe a change in the multimeric hemoglobin level (see Figure 8, panel D) or changes in the overall chromatographic HPSEC profile of the hemoglobin during the first 6 months of storage (data not shown). Between 6 and 9 months an increase in the level of multimer was observed which increased further between 9 and 12 months. Higher order aggregates (>2 μm) were not seen in the samples until 12 months incubation (see Figure 8, panel E). An initial apparent diameter of 6.2 nm was measured by DLS at the beginning of the study, with an apparent increase to 6.8 nm and the appearance of species with apparent diameters of 212, 240, and 306 nm at the end of 12 months. Precipitation was not observed on the vial walls during the study period (data not shown).

Characterization of the Multimeric Species—The multimer observed by SEC analysis was further characterized. Hemoglobin samples following 15 days and 1 year incubation at 4 °C were treated with 0.1 M DTT for 15 min and analyzed by SEC (Table 1). The DTT treatment of the 15-day sample resulted in reduction of 50% of the multimer, whereas only 25% of the multimer was reduced by DTT following a 1-year incubation. Prior to deoxygenation and addition of ascorbate, multimer present in the hemoglobin was completely reducible by DTT treatment (data not

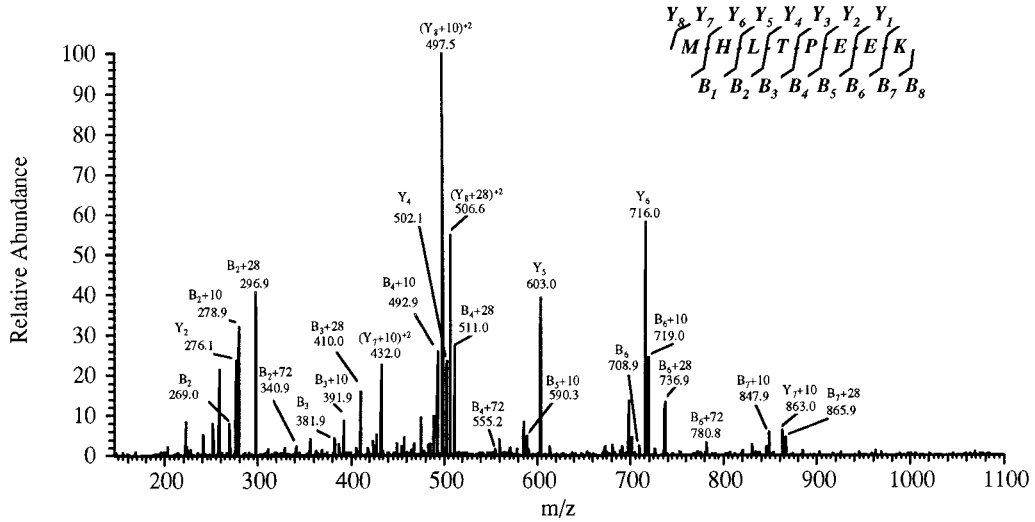


Figure 5—MS/MS fragmentation spectra of the M+72 difference peptide. The double-charged ion *m/z* 528.8 was chosen and fragmented. Due to the limits of the LCQ, the B_1 ion or its adducts were to small to detect. The B and Y ions were annotated according to the legend in the upper right of the figure.

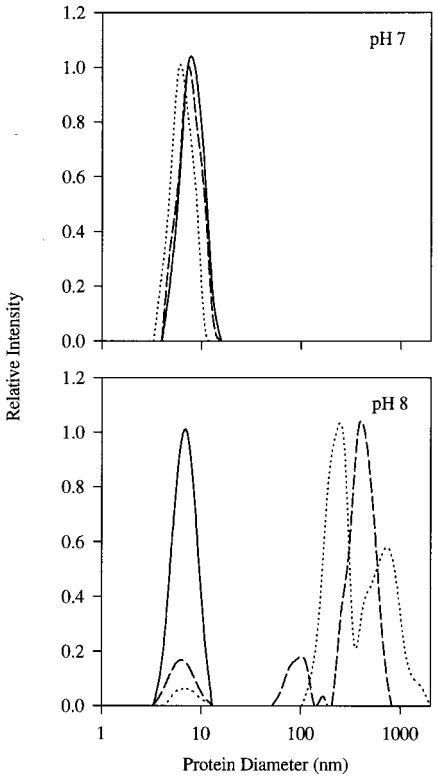


Figure 6—Dynamic light scattering analysis on the effect of pH during 4 °C storage: (solid line) starting material after equilibration at the indicated pH; (dashed line) after 4 °C incubation for 2 weeks; (dotted line) after 4 °C incubation for 4 weeks.

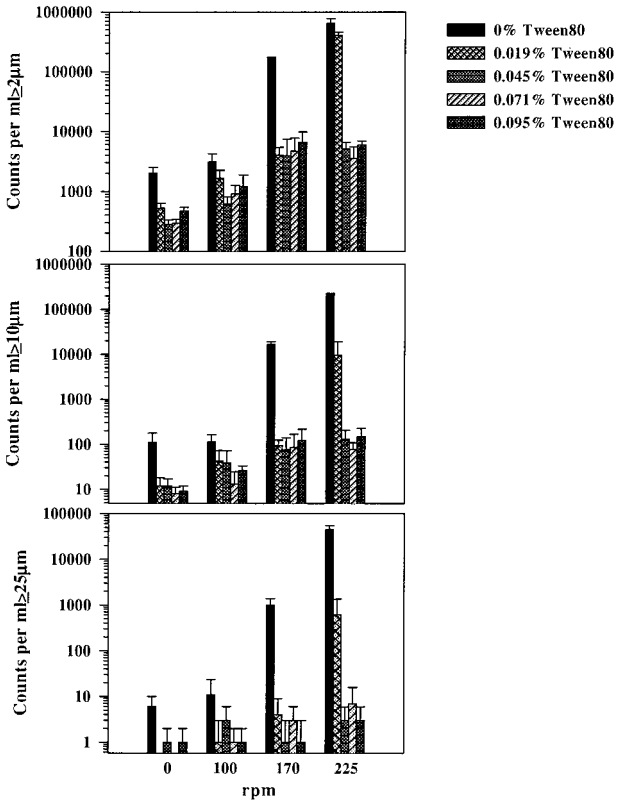


Figure 7—Effect of Tween 80 on protein aggregation of deoxy Optro during shaking in vials. Samples were prepared as described in *Materials and Methods* and shaken at the indicated speeds for 1 h at room temperature. Protein aggregation was measured using the Hiac/Royco particle counter. Triplicate vials were prepared for each Tween 80 concentration and analyzed separately. Values are reported as the average of values from the three vials ± standard deviation.

shown). Following treatment with DTT, the remaining multimer was isolated from the SEC column and analyzed by SDS-PAGE. As shown in Figure 9 (panel A), the isolated monomer peak only contained the β- and di-α-globin bands. A number of bands were present in the isolated non-DTT-reducible multimer peak with molecular weights ranging between 45 and 68 kDa. The origin of the cross-linked hemoglobins observed by SDS-PAGE was further investigated. Addition of ascorbate directly to oxyHb followed by overnight incubation at room temperature produced a cross-linking pattern similar to that observed for the 1-year samples (see Figure 9, panel B, lane 3). Saturation of the hemoglobin with carbon monoxide (lane 5) or deoxygenation (lane 8) prior to ascorbate addition prevented additional formation of the higher molecular weight bands. Addition of hydrogen peroxide to the CO-Hb sample (lane 6) produced a banding pattern similar to that observed with ascorbate and oxyHb. Deoxy-Hb incubated with ascorbate demonstrated a small increase in the intensity of the banding pattern over that seen in deoxyHb alone.

The DTT-reducible multimer was characterized by pepsin mapping. The SEC purified multimer from freshly prepared rHb1.1 was separated by C3 RP-HPLC and the

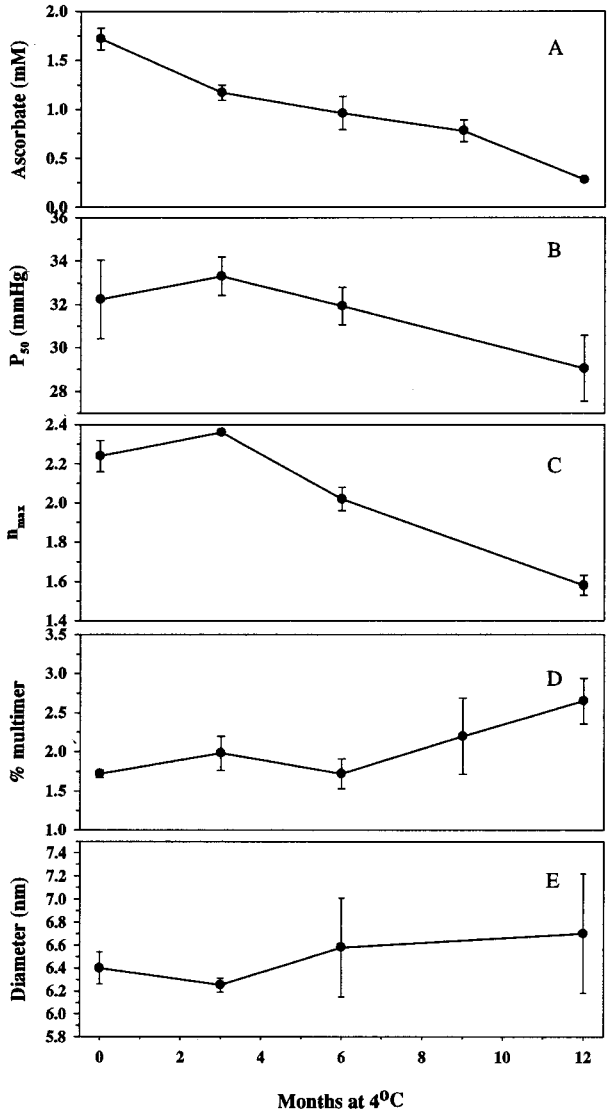


Figure 8—Change in ascorbate, P_{50}, n_{max}, percent multimer, and apparent diameter of samples of rHb during long-term storage in vials. All values are the average from four samples analyzed at the indicated time points. The value for ascorbate at 12 months is from a single sample. Data were analyzed for statistically relevant differences during the storage period using a one-way analysis of variance (ANOVA) and a Newman-Keuls post-hoc analysis. Panel A is the ascorbate concentration ($p < 0.05$ for 0 versus 3, 6, and 9 months; $p < 0.05$ for 3 versus 6 and 9 months; 6 and 9 months were not differerent). Panel B is the parameter P_{50} ($p < 0.05$ for 0, 3, and 6 versus 12 months; all others were not different). Panel C is the Hill coefficient, n_{max} ($p < 0.05$ for all samples versus each other). Panel D is the percent multimeric hemoglobin ($p < 0.05$ for 0, 3, 6, and 9 months versus 12 months). Panel E is the apparent diameter of the hemoglobin during incubation at 4 °C (samples were not statistically different from each other).

Table 1—Size Exclusion Chromatographic Analysis of Multimeric rHb Before and After Treatment with Dithiothreitol[a]

treatment	% multimer (–DTT)	% multimer (+DTT)	% change
15 days incubation at 4 °C	1.47	0.75	–50%
1 year incubation at 4 °C	2.84	2.05	–25%

[a] Analysis were performed on single samples.

β globin and di-β-globin were isolated and cleaved with pepsin. The pepsin-generated peptides were separated by C18 RP-HPLC, and a single difference peak in the di-β-globin map was found (see Figure 10, panel B). The same pepsin digest was incubated at pH 8.0 with 0.1 M DTT and

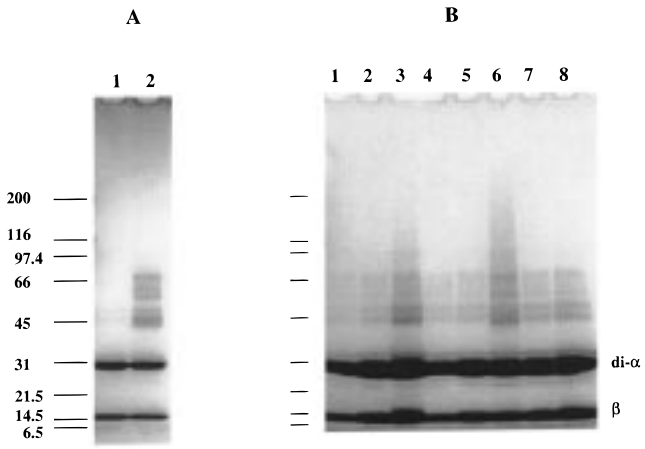


Figure 9—SDS–PAGE analysis of isolated monomer and multimer and reactions of rHb1.1 with ascorbate. Panel A: Hemoglobin (rHb1.1), incubated with 2 mM ascorbate for 1 year at 4 °C under reduced oxygen tension was reduced with DTT, and the nonreducible multimer was separated from the monomer by SEC and isolated: lane 1, isolated monomer; lane 2, isolated multimer. Panel B: Hemoglobin (rHb1.1) at 75 mg/mL, prepared as oxy, carbonmonoxy, or deoxy, was incubated overnight at room temperature with 2 mM ascorbate and then precipitated with acid acetone and resuspended in reducing buffer and analyzed: lane 1, rHb1.1 control; lane 2, oxy-rHb1.1 at room temperature overnight; lane 3, oxy-rHb1.1 + ascorbate; lane 4, CO-rHb1.1 at room temperature overnight; lane 5, CO-rHb1.1 + ascorbate; lane 6, CO-rHb1.1 + 4 mM hydrogen peroxide; lane 7, deoxy-rHb1.1 at room temperature; lane 8, deoxy-rHb1.1 + ascorbate.

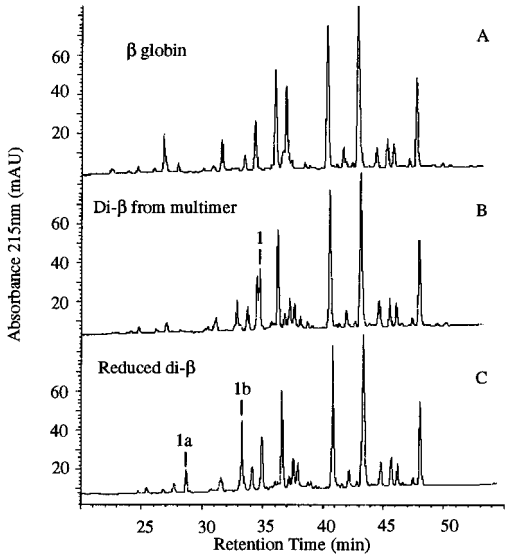


Figure 10—Pepsin maps of DTT-reducible cross-linked β-globin. Multimeric rHb1.1 was isolated by SEC and further separated by C3 RP-HPLC: panel A, pepsin-treated β globin isolated from RP-HPLC; panel B, pepsin-treated cross-linked β-globin from RP-HPLC (arrow denotes the difference peptide); panel C, isolated difference peptide from B after reduction with DTT.

rechromatographed. Two new difference peptides were generated, the first eluting at 28.7 min (1a, Panel C) and the second eluting at 32.6 min (1b, Panel C). Sequencing of the isolated peptides by Edman degradation identified the first peptide as β89−102 and the second as β86−102. Both of these peptides contain cysteine at position 93, suggesting the hemoglobin was cross-linked through Cys93 of the β-globins from separate monomeric hemoglobins.

Characterization of Globin Modification During One Year Incubation at 4 °C in vials—Modification of the hemoglobin primary structure was further examined by RP-HPLC analysis and in-line mass spectrometry (RP/LC-MS) to detect potential storage induced modification of the protein primary structure. Comparison of the control hemoglobin

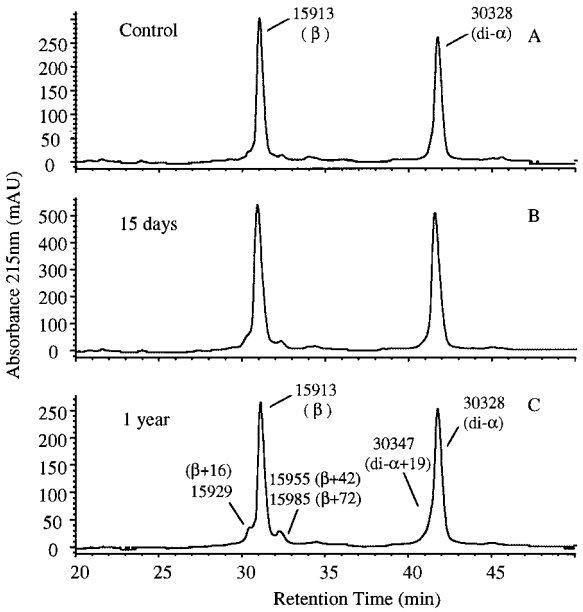


Figure 11—Reversed-phase HPLC analysis of rHb1.1 during 1 year of storage at 4 °C in vials. Samples were prepared and stored as already described. At the indicated period of time, samples were removed and aliquots were stored at –80 °C until analysis. Samples were prepared for analysis by acid/acetone precipitation immediately upon thawing and analyzed by RP-HPLC and RP/LC-MS as described in *Materials and Methods*.

with the samples stored in ascorbate for 1 year shows modification to both shoulders of the β-globin peak and the leading shoulder of the di-α-globin peak (see Figure 11). The main β globin peak showed a characteristic mass of 15 913 Da. The adduct eluting at 28.8 min in front of the main β peak had a mass increase of 16 Da, likely due to oxidation. The quantity of this adduct varied during the study period. Two β-globin adducts were observed in the peak eluting at 30.6 min and included mass additions of 42 and 72 Da. The peak eluting at that position increased from 2.2 to 4% during the study period. Cross-linked β globins were observed with masses of 31 825 and 31 908 Da. Only one modification was observed on the di-α-globin peak occurring on the leading shoulder with a mass increase of 19 Da compared to 30 328 Da observed for the main di-α peak. The main di-α peak is composed of the methylated (30 338 Da) and the unmethylated (30 324 Da) forms of the di-α-globin.[27]

Long-Term Storage in Bags—Studies of deoxy-rHb1.1 were also carried out with deoxyHb stored in oxygen-impermeable containers to prevent ascorbate degradation. Aggregation was not definitively observed with the long-term studies in vials, so polysorbate 80, 0.03%, was again included in the formulations to preclude aggregation of the protein during storage. The initial ascorbate levels present in each of the three lots were not significantly different from those present following 12 months incubation at 4 °C (Table 2). In accordance with the presence of ascorbate, the concentration of met-rHb remained at <2% (limit of quantitation) of the total protein during the course of the study. Changes were also not observed in the P_{50} and n_{max} measurements, with average values of 32 mmHg for the P_{50} and 2.17 for the n_{max} during the incubation period (Table 2). Additionally, we did not observe an increase in the multimer content (Table 2) or changes in the overall chromatographic HPSEC profile of the hemoglobin (data not shown).

Dynamic light scattering was used to ascertain the presence of higher order aggregates not detected by HPSEC. As seen in Figure 12, an initial diameter of 5.9–6.2 nm was measured by DLS at the beginning of the study, with

Table 2—Ascorbate, P_{50}, n_{max}, and Multimer Data for Deoxy-rHb1.1 Stored in Oxygen-Impermeable Bags[a]

lot	months at 4 °C	ascorbate (mM)	P_{50} (mmHg)	n_{max}	multimer (% of total Hb)
1	0	1.65 ± 0.03	32.5 ± 0.5	2.21 ± 0.03	2.7 ± 0.5
	12	1.67 ± 0.01	32.9 ± 0.2	2.21 ± 0.03	1.7 ± 0.1
2	0	1.42 ± 0.02	33.5	2.44	2.1 ± 0.3
	12	1.39 ± 0.01	33.4 ± 0.4	2.15 ± 0.04	2.1 ± 0.5
3	0	1.70 ± 0.02	32.0	2.01	1.3 ± 0.2
	12	1.64 ± 0.01	32.8 ± 0.1	2.22 ± 0.01	1.8 ± 0.4

[a] Three bags were analyzed at each time point, and the average ± standard deviation are reported. Samples without standard deviation are from analysis of a single analysis only.

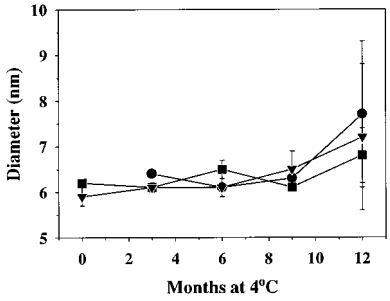


Figure 12—Dynamic light scattering analysis of rHb1.1 during 1 year of storage at 4 °C in bags. At the indicated periods of time, samples were removed and immediately analyzed for aggregates <0.2 μm by DLS as described in *Materials and Methods*. Three bags of each lot were analyzed at each time point, and the average ± standard deviation are reported.

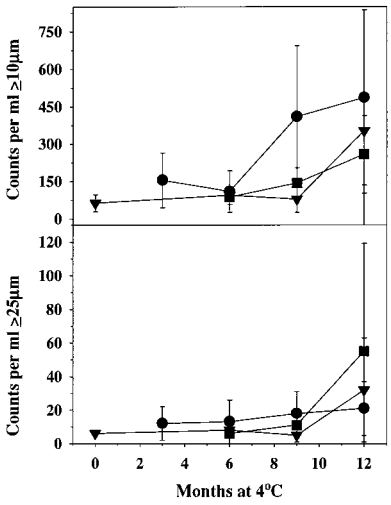


Figure 13—Light obscuration analysis of rHb1.1 during 1 year of storage at 4 °C in bags. At the indicated period of time, samples were removed and immediately analyzed for aggregates ≥10 and ≥25 μm by light obscuration as described in *Materials and Methods*. Three bags of each lot were analyzed at each time point and the average ± standard deviation are reported.

no discernible change through 9 months of storage. At 12 months, the mean diameter of the rHb in each of the lots increased to an average diameter of 7.2 nm, indicating the presence of aggregates. Distinct peaks were not differentiated by the instrument, rather the aggregation was detected as an overall increase in the size of the protein. Aggregates of 10 μm (Figure 13A) and 25 μm (Figure 13B) demonstrated a similar pattern with minimal changes through 9 months and a sharp increase between 9 and 12 months. Increases in the levels of aggregates were not associated with measurable precipitation of the protein because the soluble protein concentration did not decrease significantly during the course of the study (data not shown).

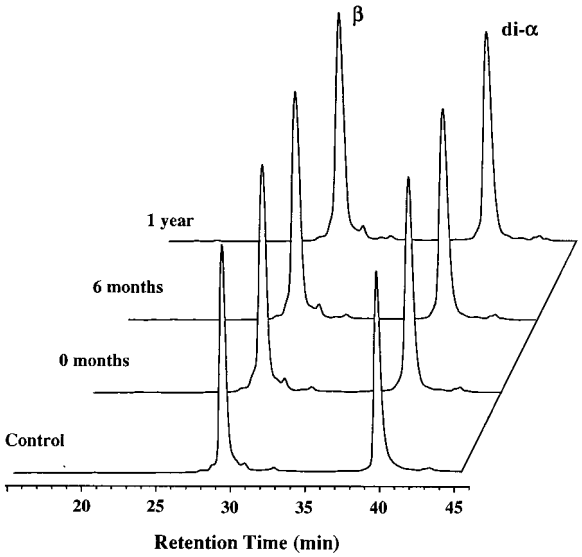


Figure 14—Reversed phase HPLC analysis of rHb1.1 during 1 year of storage at 4 °C in bags. At the indicated period of time, samples were removed and aliquots were stored at –80 °C until analysis. Samples were prepared for analysis by acid/acetone precipitation immediately upon thawing and analyzed by RP-HPLC and RP/LC-MS as described in *Materials and Methods*.

Modification of the hemoglobin primary structure was again examined by RP/LC-MS to detect potential storage-induced degradation of the protein primary structure. Comparison of the control hemoglobin with the hemoglobin formulated in ascorbate (Figure 14, compare panels A and B) shows modification to both the leading and lagging shoulders of the β-globin peak, with a corresponding decrease in the ratio of β- to di-α-globin peak heights from 1.14 to 1.07, respectively. Once the hemoglobin was formulated and put in storage conditions, RP-HPLC profiles did not reveal the presence of new peaks nor decreases in the height ratio of the β-globin-to-di-α-globin peaks. Ascorbate/oxygen-mediated modifications that were previously described were observed in the samples but did not appear to change qualitatively during the study period. We were unable to quantitate these adducts (in the hemoglobin) by RP/LC-MS due to a lack of mass resolution. The RP/LC-MS analysis of the β- and di-α-globin peaks demonstrated a consistent molecular weight for each of the globin chains during storage of 15 913 Da for the β globin and 30 330 for the di-α-globin.

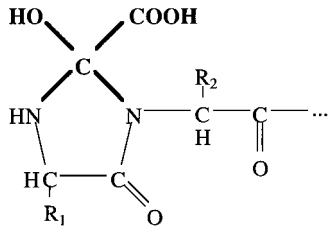
Discussion

The purpose of this study was to investigate the effects of ascorbate, oxygen, and pH on the long-term stability of deoxyHb. Storage of deoxyHb has been investigated by other researchers.[28,29] Kramlova et al.[29] attempted to store stroma-free hemoglobin following bubbling of nitrogen through the solution. After 1 year at 4 °C, the metHb content increased from 5 to 41%. De Venuto[28] stored hemoglobin at 25 °C in sealed glass ampules following three cycles of gas evacuation and flushing with nitrogen. In those studies, the metHb content increased from 6.1 to 52.8% during the 8-week storage period. It is likely that the hemoglobin in those investigations oxidized due to incomplete deoxygenation of the solutions. Keilin[30] demonstrated that the rate of autoxidation increases dramatically if the hemoglobin is only partially deoxygenated. Investigations by Brantley et al.[2] using myoglobin confirmed these observations and demonstrated that under saturating oxygen conditions autoxidation occurs through a unimolecular dissociation of the neutral superoxide radical ($HOO^{\bullet}$) from the heme. At low oxygen concentrations, autoxidation may occur through the unimolecular reaction as well as a bimolecular reaction. The bimolecular reaction is facilitated by the structure of partially deoxygenated Mb, which allows a water molecule to coordinate with His64 and the ferrous iron of the deoxy heme. The water molecule is capable of stabilizing the ferric state of the oxidized heme, thereby facilitating the extraction of an electron from the ferrous iron by an approaching oxygen. When rHb1.1 was equilibrated with 15 000 ppm of oxygen in the headspace and stored at 4 °C, the autoxidation rate increased by approximately 50% over that normally observed for oxy-rHb1.1. Equilibrating the hemoglobin with 150 ppm of oxygen in the headspace of our containers did not completely prevent autoxidation, although it did decrease the rate by approximately 5-fold from that observed with 15 000 ppm of oxygen in the headspace (see Figures 1 and 2). Further reduction of autoxidation was accomplished by inclusion of the reducing agent, ascorbate. The ascorbate likely reacted with excess oxygen present in the solution and reduced any ferric hemoglobin back to the ferrous state.

Reaction of reducing agents such as ascorbate with oxygen can produce superoxide.[31] During our studies, inclusion of 0.5 and 1 mM ascorbate in formulations containing 15 000 ppm of oxygen in the headspace increased the autoxidation rate compared with the rate in solution without ascorbate (see Figure 1). The increased oxidation rate observed for the first 8 days was likely due to production of superoxide by ascorbate and oxygen until all the ascorbate in the solution was consumed. Superoxide quickly disproportionates to hydrogen peroxide, which can react with and oxidize ferrous Hb. In the sample of hemoglobin incubated with 2 mM ascorbate, it is likely that ascorbate consumed the oxygen and reduced the metHb. Both reactions conceivably occurred in competition, resulting in the slower rate of metHb reduction than that seen with 5 mM ascorbate.

The protein modifications observed during the long-term studies in vials (Figures 8–11) were similar to those observed during the acute studies and are likely related to the reaction of ascorbate with oxygen which produces superoxide.[31] Oxygen may have entered the reaction vials during the study period through the permeable butyl rubber stoppers. The protein cross-linking observed by SDS-PAGE (Figure 9) was likely related to formation of hydrogen peroxide from the superoxide anion because the cross-linking pattern in the presence of H_2O_2 was similar to that when the oxyHb was incubated with ascorbate. Additionally, a similar type of cross-linking pattern was observed when HbAo was incubated with hydrogen peroxide (data not shown), suggesting that the cross-linking was not due to a difference in susceptibility of the two hemoglobins to H_2O_2. The cross-linking of the protein would explain the decrease in the P_{50} and n_{max} observed during our long-term studies. A decrease in the oxygen binding parameters of recombinant hemoglobin has been observed after heterogeneous intramolecular cross-linking of the globins.[32]

Chemical modifications of the hemoglobin observed during the acute studies (see Figures 3 and 4) were also present in the material used for long-term stability following addition of ascorbate (see Figures 10 and 14), and increased during storage of the rHb1.1 in vials (see Figure 10). The modifications did not appear to increase when the hemoglobin was stored in an oxygen-impermeable storage system (Figure 14, compare panels B–D). Reduction of metHb by ascorbate produces dehydroascorbate, which could subsequently react with low levels of residual oxygen and produce other potentially highly reactive



R_1 = Met
R_2 = His

Figure 15—Proposed structure of the M+72 addition to the N-terminal tryptic peptide of the β-globin. The section in boldtype represents the proposed +72 addition.

byproducts that could modify the protein.[33−36] A number of new modifications of hemoglobin were detected by C3 RP/LC-MS analysis. The most significant mass gains were 16 and 72 Da. Mass gains of 58 Da (carboxymethylation) and 130 Da (dehydroascorbate addition) could not be definitively assigned by LC-MS (data not shown). Only one difference peak was observed during peptide mapping and was assigned as a 72 Da addition to the N-terminus of the β chain. This result may indicate that other modifications are labile or heterogeneously distributed across β and di-α-globins. Heterogeneous modifications not detectable by tryptic mapping have also been observed.[32] The mass increase of 16 Da is likely due to oxidation of side chains of labile amino acids.[37,38] Amino acid analysis indicated the presence of a low level of labile oxo-histidine in the sample equilibrated with 15 000 ppm of oxygen and incubated with 5 mM ascorbate (data not shown).

Efforts were made to identify the M+72 modification at the N-terminus of the β-globin. The results from the MS/MS fragmentation together with the protein chemistry (blocked to Edman degradation and not reducible by borohydride) suggest that the modification is distributed between the Met1 and His2 residues, which is consistent with an imidazolidinone structure. Imidazolidinone structures have been postulated to form at the N-terminus of the β globin in reactions of human hemoglobin A_0 with aldehydes.[39−42] Our results also indicate that the modification is likely to contain labile carboxy and hydroxy groups, which is consistent with the observed neutral loss of 44Da- and 18 Da-derived fragments. The proposed structure for the modification is presented in Figure 15. The structure indicates a similarity to an oxalate-derived modification. However, incubation of hemoglobin with oxalate did not produce any trace of the M+72 modification (data not shown). Examination of the known ascorbate degradation pathway[34−36] did not indicate the presence of degradation byproducts that could directly derivatize the hemoglobin and result in a mass increase of 72 Da. A number of ascorbate degradation products containing carbonyl groups could initially form a Schiff's base with the N-terminus followed by rearrangement to the imidazolidinone structure and further degradation (i.e., oxidation) to produce the observed mass gain.

Surfactants may stabilize protein structure by binding to hydrophobic surfaces on proteins [44−46] or by competing with proteins for adsorption at liquid−surface interfaces.[47−49] Studies by Kerwin et al.[43] demonstrated that Tween 80 does not bind to the rHb1.1, suggesting that the decreased aggregation observed during the shaking studies (Figure 7) was due to competition of the surfactant with the liquid−surface interfaces. Therefore, because the hemoglobin would be stored for long periods of time while in contact with the ethyl vinyl acetate surface of the bag, Tween 80 was added to the hemoglobin solutions to circumvent possible aggregation during the study period. In contrast to the long-term study in vials in which little aggregation was observed by DLS, a variable degree of aggregation was observed by both DLS and light obscuration when the hemoglobin was stored in the bags. This difference may be due to differential affinities of the surfactant and the hemoglobin for either the glass or ethyl vinyl acetate surfaces. Work by Hlady and co-workers[49,50] has demonstrated that the interaction of proteins with surfaces is dependent on the surface chemistry. It is not likely that the aggregation was due to the initial modification of the hemoglobin following ascorbate addition (Figure 14) because similar observations were made during the long-term studies of deoxy-rHb in vials (Figure 11), which contained a much higher degree of protein modification at the end of the study period. The concentration of Tween 80 used in our formulation may have been too low to successfully prevent interaction of the hemoglobin with the ethylvinyl acetate bag surface, eventually leading to aggregation. It is also possible that the N-terminal methionine and the "glycine linker" between the α subunits, which are not present in HbAo, add to the instability of the protein, making it more prone to aggregation. However, we do not feel that this is the case because comparison of the α-carbons of the X-ray crystallographic structures of deoxy-rHb1.1 and HbAo have a root-mean-square (RMS) deviation of 0.187 with the met1-Cα and an RMS deviation of 0.170 without the met1-Cα (E. Brucker, personal communication).

The data presented here demonstrate a significant advance in our ability to successfully store hemoglobin-based oxygen-carrying therapeutics. To our knowledge this is the first evidence that a deoxygenated hemoglobin can be stored for an extended period of time in the presence of reducing agents without metHb formation or precipitation. Stability against met-rHb formation was increased >50-fold by deoxygenation and reduction of the protein with ascorbate. Storage of the deoxy-rHb in vials resulted in modification to the protein primary structure (Figure 11), decreased P_{50} and n_{max} values (Figure 8), and formation of cross-linked protein (Figure 9). These modifications were all likely related to the influx of oxygen through the vial stopper and were not observed following storage of the hemoglobin in the foil overwrapped containers.

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