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A recombinant polymeric hemoglobin with conformational, functional, and physiological characteristics of an in vivo O_2 transporter

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Bobofchak, Kevin M., Toshiaki Mito, Sarah J. Texel, Andrea Bellelli, Masaaki Nemoto, Richard J. Traystman, Raymond C. Koehler, William S. Brinigar, and Clara Fronticelli. A recombinant polymeric hemoglobin with conformational, functional, and physiological characteristics of an in vivo O_2 transporter. *Am J Physiol Heart Circ Physiol* 285: H549–H561, 2003. First published April 10, 2003; 10.1152/ajpheart.00037.2003.—With the objective of developing a recombinant oxygen carrier suitable for therapeutic applications, we have employed an *Escherichia coli* expression system to synthesize in high-yield hemoglobin (Hb) Minotaur, containing α-human and β-bovine chains. Polymerization of Hb Minotaur through S-S intermolecular cross-linking was obtained by introducing a Cys at position β9 and substituting the naturally occurring Cys. This homogeneous polymer, Hb Polytaur, has a molecular mass of ~500 kDa and was resistant toward reducing agents present in blood. In mice, the circulating half-time (3 h) was fivefold greater than adult human Hb (HbA). The half-time of autooxidation measured in blood (46 h) exceeded the circulating retention time. Hypervolemic exchange transfusion resulted in increased arterial blood pressure similar to that with albumin. The increase in pressure was less than that obtained by transfusion of cross-linked tetrameric Hb known to undergo renovascular extravasation. The nitric oxide reactivity of Hb Polytaur was similar to HbA, suggesting that the diminished pressor response to Hb Polytaur was probably related to diminished extravasation. Transfusion of 3% Hb Polytaur during focal cerebral ischemia reduced infarct volume by 22%. Therefore, site-specific Cys insertion on the Hb surface results in uniform size polymers that do not produce the large pressor response seen with tetrameric Hb. Polymerization maintains physiologically relevant oxygen and heme affinity, stability toward denaturation and oxidation, and effective oxygen delivery as indicated by reduced cerebral ischemic damage.

hemoglobin recombinant; hemoglobin synthetic; hemoglobin polymeric; blood substitutes; transfusional fluids; artificial oxygen carriers; hemoglobin retention time; exchange transfusion; extravasation; focal cerebral ischemia

IN MEDICINE there is an increasing need for transfusional fluids. The use of stroma-free solutions of adult human (HbA) or bovine (HbBv) hemoglobins as a red blood cell replacement is being explored (10, 21, 46). Solutions of stroma-free Hb contain tetrameric (molecular mass 64 kDa) and dimeric (molecular mass 32 kDa) molecules at equilibrium. Because of the rapid filtration of the dimers through the kidneys, the retention time of infused solutions of Hb is very short. In addition, Hb molecules extravasate through the endothelium, scavenging nitric oxide (NO) from the interstitial fluid. The latter is believed to be the main reason for the increase in mean arterial pressure observed during the administration of stroma-free Hb solutions (13). Chemical modifications have been employed in attempts to convert HbA and HbBv into therapeutically useful oxygen carriers (1, 11, 14, 20). These solutions generally are a heterogeneous mixture of different size polymers, and small size components may still extravasate in peripheral tissues, thus scavenging NO to produce vasoconstriction (3, 31). Also, the introduction of chemical cross-linkers may result in toxicity or immunogenic response (12).

Alternatively, Hb can be expressed in microorganisms (19, 29, 34, 44). The continuous development of recombinant techniques will allow the production of Hb in microorganisms in a potentially limitless supply, free from mammalian infectious agents. This approach also offers the possibility to design and construct mutant Hbs with specific conformational and functional characteristics that render them suitable for applications as therapeutic agents in different clinical situations. In the search for recombinant Hb molecules possessing properties suitable for therapeutic applications, we previously described a polymer of HbA, Hb Prisca ($\alpha_H\beta_H^{S9C+C93A+C112G}$), containing six to eight tetrameric molecules intermolecularly cross-linked through S-S bonds introduced at position β9 on the Hb

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surface (16). Hb Prisca was modeled after the naturally occurring mutant Hb Porto Alegre ($\alpha_H\beta_H^{S9C}$) (7). Hb Prisca has an oxygen affinity similar to that of plasma-based HbA. It was obtained by refolding recombinant β-globin, initially expressed in *Escherichia coli* as a fusion protein. Refolding occurred in the presence of heme and native α-subunits (19). The recombinant hemoglobins obtained with this system had biophysical characteristics nearly identical to that of natural Hb; however, the yield of the protein was too low for the implementation of in vivo studies.

Alternatively, Hb can be expressed in *E. coli* in a soluble form, thus allowing the possibility to produce recombinant Hbs in an amount sufficient for animal experimentation (13). For the study presented in this article, we constructed Hb Minotaur, containing α-human and β-bovine chains. This hybrid Hb was selected because of the high yield of expression and protein recovery. Here we describe the construction and characterization of a homogeneous and stable polymer of Hb Minotaur, with functional characteristics suitable for an artificial oxygen carrier. We determined the plasma retention time and investigated whether polymerization would decrease the hypertensive response associated with peripheral vasoconstriction typically observed after infusion of nondissociable tetrameric cross-linked Hb solutions. The efficacy as an oxygen carrier was evaluated by measuring infarct volume after transient focal cerebral ischemia.

MATERIALS AND METHODS

Synthesis of bovine β-globin gene. The βA allele of HbBV has been constructed from 21 synthetic deoxy oligonucleotides that were synthesized and gel purified by DNAgency (Berwyn, PA). The protein sequence was that previously determined by Schroeder et al. (43) and by Schimenti and Duncan (42). The DNA codon usage was optimized for *E. coli* expression, and restriction enzyme recognition sites were incorporated into the sequence at potentially useful locations. The gene was constructed in four parts, I, II, III, and IV, of blunt-ended, double-stranded DNA. The fragments were then joined in pairs, i.e., I-II and III-IV, by first digesting parts I and II with *Bst*EII and III and IV with *Pst*I, followed by ligation. I-II was digested with *Nco*I and *Kpn*I and inserted into a vector obtained by treating pNFMb with *Nco*I and *Kpn*I. The resulting plasmid was designated pNFBov/2. pNFMb is a plasmid constructed by replacing the human β-globin gene of pJKO5 with the sperm whale myoglobin gene from pMb413 (38). After treatment of gene parts III and IV with *Pst*I, they were ligated to give part III–IV, which in turn was digested with *Kpn*I and *Eco*RI, and inserted into pNFBov/2, which had been digested with the same two enzymes. The resulting plasmid pNFBovine was sequenced by the DNA Sequencing Facility in the Department of Genetics, University of Pennsylvania, verifying the sequence shown in the accompanying figure (Fig. 1). For expression of the fusion protein NS1-FX-β bovine, *E. coli* strain AR120 was transformed with the plasmid and induced with nalidixic acid as previously described for pJKO5 (19).

For expression of the hybrid hemoglobin $\alpha_H\beta_{Bv}$ designated Hb Minotaur, the Arg codon of the Factor X recognition sequence in pNFBovine was changed from CGC to CAT, creating a *Nde*I site at the *Met*1 codon. The pAlter Mutagenesis system of Promega was utilized for this and other mutagenesis reactions. The β^{bovine} gene was then extracted by digesting with *Nde*I and *Hin*dIII and inserted into pDLIII-13e (24), which had been completely hydrolyzed with *Hin*dIII and partially hydrolyzed with *Nde*I. The resulting plasmid has the human β-globin gene of pDLIII-13e replaced by the bovine β-globin gene and is designated $pDL\alpha_H\beta_{Bv}$. During the purification procedure, an excess of β_{Bv}-chains was observed relative to α_H-chains. In a effort to more nearly equalize expression of the two globins, a second α^{human} gene was inserted into the plasmid. This was accomplished by taking advantage of two *Xba*I sites, one on each side of the α^{human} gene. Partial digestion of the plasmid with *Xba*I provided the vector (5,639 bp) and complete digestion provided the insert (508 bp). The sequence of the plasmid containing two α^{human} genes is identical regardless of which *Xba*I site receives the insert. The amount of hemoglobin isolated from expression of this plasmid is ~40% greater than from the plasmid containing only one α^{human} gene.

Expression and purification of tetrameric Hb molecules in E. coli. Soluble hemoglobin was expressed in $pDL\alpha_H\beta_{Bv}$ or in $pDL\alpha_{2H}\beta_{Bv}$. Cells were grown in a 5-liter fermentor in DM-4 medium (30) supplemented by continuous glucose feeding. With these conditions, we obtain ~30.0 g of cells per liter of medium. After equilibration with CO, harvesting, and two cycles of freezing and thawing, the cells were suspended in 3 vol/g cells paste of buffer containing (in mM) 40 Tris, 1 benzamidine, 0.3 dithiothreitol, and 0.1 triethylenetetramine (TETA) at pH 7.4. The cells were lysed by treatment with lysozyme (1 mg/ml) and benzonase (50 μl/l). Nucleotides were precipitated in 0.5% polyethylenimine. Hb is purified by a two-step ammonium sulfate precipitation (45% and 75%), followed by chromatography on a CM SepharoseFF column connected to a ÄKTA Prime chromatography system (Amersham Pharmacia Biotech). The protein and the column were equilibrated with (in mM) 10 phosphate buffer, pH 6.5, 1 benzamidine, 0.3 dithiothreitol, 1 EDTA, and 0.1 TETA. A linear gradient uses 10 mM dibasic phosphate as a second buffer. The Hb obtained is pure as determined by reversed-phase HPLC (Fig. 2*A*) indicating the presence of the heme peak (E_T = 11.3 min) followed by only two peaks. Edman degradation (10 cycles) confirmed the purity and identified the peak with E_T = 46.4 min as α_H-globin and the peak with E_T = 72.6 min as β_{Bv}-globin. In the absorption spectrum of the purified protein shown in Fig. 2*B*, the presence of contaminating proteins or residual nucleotides was not detected in the far UV region. The absorption spectrum of the oxy derivative, shown in the *inset* of Fig. 2*B*, indicates the absence of the adduct of heme and hydrogen sulfide (sulfHb).

Protein polymerization. Polymerization spontaneously occurred through the oxidation of thiol groups and formation of S-S bonds between Cys residues introduced at position β9 of tetrameric Hb. Polymerization was carried out under one atmosphere of O_2 in 20 mM Tris, 1.0 mM benzamidine, and 0.1 mM TETA, pH 8.4. The Hb in the oxygenated form was concentrated to ~40–60 mg/ml and filtered through a 0.45-μm filter into a sterilized amber bottle. The extent of polymerization was measured using a prepacked (1.6 × 60 cm) Fractogel EMD BioSec column (EM Industries). The elution buffer was 20 mM phosphate and 300 mM NaCl at pH 7.2. The column detects molecular mass differences between 5 and 900 kDa and was calibrated using a set of known standard proteins. Peak areas were calculated using PrimeView data collection software (Amersham Pharmacia Biotech). Polymerization time varied with temperature from 2 days at 30°C to 4 wk at 4°C. Methemoglobin formed during

Fig. 1. Sequence of the synthetic bovine globin gene

the polymerization process was reduced using the enzymatic system (23).

Endotoxin removal. For samples to be used in animal experimentation, the protein was dialyzed against lactate-Ringer solution. After filtration through a 0.45-μm filter, endotoxins were removed by gentle mixing for 18 h with Detoxy-gel (Pierce) following the procedure described by the manufacturer that reduces the endotoxin content to less than

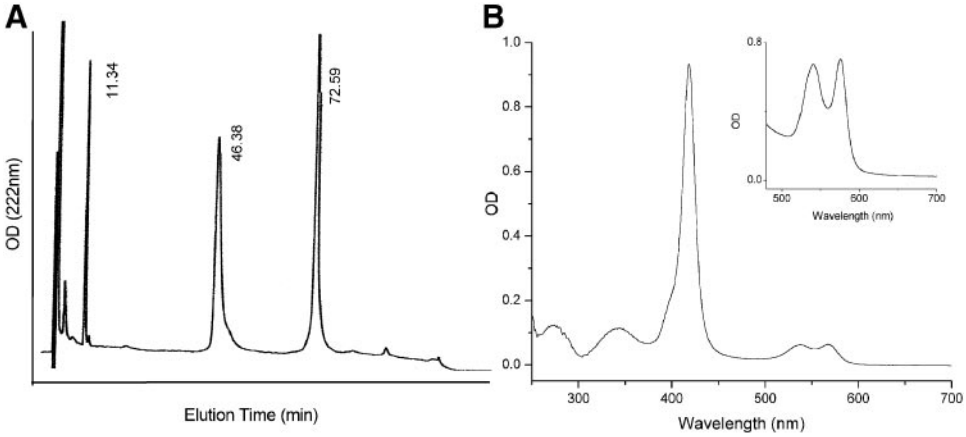

Fig. 2. Purity of hemoglobin (Hb) Polytaur. *A*: reversed-phase HPLC of purified Hb Polytaur on a Vydac C4 column. Buffer consisted of 20% CH_3CN, 0.1% trifluoroacetic acid (*buffer A*), 60% CH_3CN, and 0.1% trifluoroacetic acid (*buffer B*) with a gradient of 44%B to 75%B over 120 min. *B*: UV/Vis Spectrum of purified carbonmonoxyHb Polytaur. Ratio between the Soret (419) and tyrosine-tryptophan (280) peaks is the same as in adult human Hb (HbA), indicating the absence of contaminating proteins or nucleotides. *Inset*: absorption spectrum of oxyHb Polytaur, indicating the absence of the sulfoHb peak at 622 nm.

2 EU/ml. The endotoxin content of our samples was, however, not determined. The protein, was then stored in sterile glass vials at −80°C. Methemoglobin formation upon storage was minimal.

CD spectroscopy. The CD spectra were recorded at 15°C in 20 mM phosphate buffer at pH 7.0 on an AVIV model 202 spectropolarimeter (Aviv Associates, Lakewood, NJ). Each spectrum represents an average of eight scans. The Hb samples in the carbonmonoxy form were prepared by filtration through a Sephadex G25 column equilibrated with CO-saturated buffer. Immediately before use, 10 mg of dithionite dissolved in 100 μl of buffer were adsorbed on the top of column to completely reduce any methemoglobin present. This treatment did not affect the degree of polymerization. Soret spectra were measured between 470 and 380 nm; Hb concentration was 0.10 mg/ml.

Thermal denaturation. An AVIV model 202 spectropolarimeter with Peltier temperature controller was used to determine the median temperature (T_m) of denaturation. The Hb samples, in the carbonmonoxy form, were at a concentration of 0.1 mg/ml in 10 mM phosphate buffer at pH 7.0. The change in ellipticity was recorded at 222 nm under continuous stirring in a capped 1-cm cuvette. The temperature was increased at a rate of 10°C/min from 25°C to 100°C with a step size of 2.0°C. Equilibration at each temperature step was continued for 30 s before data collection, which was averaged over an additional 30 s.

Autooxidations. These measurements were performed either in buffer or in whole human blood. Measurements in buffer were carried out in a HP 8452A diode array spectrophotometer at 37°C with a protein concentration of 1.0 mg/ml. Buffer was 0.1 M phosphate at pH 7.0 with 1 mM EDTA and beef liver catalase at a molar ratio of 0.003 M heme^{-1} (28). Spectra were recorded every 30 min. Turbidity was corrected using as baseline the spectrum of a cuvette wrapped with parafilm (16). The absorption spectra were decomposed linearly into a series of prerecorded standard spectra. The fraction of oxyhemoglobin was plotted as function of time and then fit to a first- or second-order kinetic equation using Origin 6.1 (OriginLab, Northampton, MA).

For the measurements in whole blood, human blood (K. M. Bobofchak) collected in citrate was divided in 2-ml aliquots in capped 4-ml test tubes and equilibrated at 37°C. To each tube, 200 μl of a 3% solution of Hb in the polymerization buffer were added. The tubes were rotated slowly using a Labquake shaker (Labindustries) and 100-μl blood aliquots were collected at intervals and immediately centrifuged at 3,000 *g*, and the supernatant was collected and diluted with buffer. The absorption spectra, recorded between 480 and 380 nm in an AVIV spectrophotometer, were decomposed linearly into prerecorded spectra. The control for these measurements contained plasma from the same blood sample to which 100 μl of polymerization buffer were added, but no Hb.

Heme affinity. The affinity of Hb for heme was measured following the method of Hargrove et al. (22) using apomyoglobin $His^{64} \rightarrow Tyr$. Spectra were recorded using an HP 8452A spectrophotometer with a water-controlled temperature cell holder. Hb concentration was 3 μM in heme and apomyoglobin was 15 μM, temperature 25°C. The protein was buffered in 150 mM phosphate and 0.45 M sucrose, pH 7.0. Spectra were recorded every 5 min for 600 min. As heme is released from Hb and bound to apomyoglobin, a decrease and red-shift of the Soret peak at 406 nm is observed. The absorbance at 406 nm was plotted and fitted with a second-order kinetic equation using the program Origin version 6.1 (OriginLab) to obtain the rate constants.

Oxygen affinity. A Hemox model B (TCS Medical Products) with computer interface was used for the measurements. Oxygen dissociation curves were obtained using the enzymatic deoxygenation method of Vandegriff et al. (51). Measurements were carried out in 0.1 M phosphate and 0.1 M NaCl at pH 7.4 at 37°C at a protein concentration of 1 mg/ml. The protein was equilibrated with 1 mM protochatechuic acid in a cuvette, which was then sealed with a custom-made adaptor containing a silicon membrane to eliminate air from the cell. Deoxygenation was initiated by injection of protochatecuate-3,4-dioxygenase through the membrane in a ratio of 0.06 U/mol heme. Raw data were fitted to the Adair function to determine the P_{50} and Hill coefficient, using the program Origin version 6.1 (OriginLabs). The percentage of ferric Hb was measured spectroscopically before and after each experiment; any runs with >10% methemoglobin were discarded.

NO reactivity. The rate constant for the combination of Hb with NO was measured by stopped flow at 436 nm. The instrument SX-18-MV from Applied Photophysics, (Leatherhead, UK) has a dead time of ~2 ms and can measure rate constants up to ~250 s^{-1}. Buffer was 0.1 M bis-Tris, pH 7.2, containing 1 mM EDTA.

A 2 mM stock solution of NO was prepared by allowing the gas (at 1 atm and 25°C) to dissolve in degassed water; this solution was then diluted with degassed buffer to obtain NO concentrations ranging from 5 to 20 μM. The effective concentration of the stock solution was checked by titration with HbO_2 in an anaerobic, gas-free cell, because the gas converts stoichiometrically oxyhemoglobin into methemoglobin.

Hb solutions were prepared by diluting the protein to a final concentration of 1.5 μM (heme) in deoxygenated buffer. Immediately before the measurements, ~0.1 mg/ml of sodium dithionite was added to the samples to ensure the complete oxygen removal from the buffer. The extent of polymerization was not modified by dithionite in the time frame of the measurements (<10 min). Dithionite destroys NO at a much slower rate than that for the combination of the gas with unliganded Hb (rate constant, k = 1,400 $M^{-1}s^{-1}$) (30). This entails an error <10% on the measured rate constants.

Transfusion experiments. Transfusions were performed on C57Bl/6 mice (~25 g body wt). All procedures were approved by the institutional animal care and use committee. Mice were anesthetized with 1.0–1.5% halothane administered via face mask, and a femoral artery was catheterized. After the surgery was completed, the inspired halothane concentration was reduced to 0.7%. Rectal temperature was maintained with a heating lamp. To determine the plasma retention time of the hemoglobin, 200 μl of a 3% Hb solution were infused over a 5-min period. Arterial blood samples (50 μl) were drawn at 10, 20, 40, 60, 80, 120, and 180 min after the transfusion for analysis of plasma Hb concentration. Plasma was diluted 60-fold with a CO-saturated buffer containing 1 mg/ml dithionite to reduce any methemoglobin present. Hb concentration was determined at 419 nm using the molar extinction coefficient of carboxymonoxy-heme (ϵ_{heme}) = 190,000 M^{-1} cm^{-1}.

In a second experiment, an exchange transfusion was performed with either a 5% albumin solution or a 3% polymerized Hb solution to determine whether there are differences in the arterial blood pressure response. As a positive control, an additional group of mice was transfused with a 6% sebacoyl cross-linked tetrameric Hb solution (8), which has previously been shown to increase arterial pressure in cats (48). The exchange transfusion through the femoral artery catheter was performed by infusing and withdrawing fluid in

three-step increments over a 20-min period. The total amount of fluid infused was 650 μl, and the amount withdrawn was 350 μl. The exchange transfusion was hypervolumetric to allow subsequent blood sampling (50 μl) at 15, 40, 80, and 120 min after the transfusion without inducing hypovolemia. Differences in mean arterial pressure between groups transfused with albumin and the two hemoglobin solutions were compared by two-way analysis of variance over time. Comparisons among the three groups of the change in arterial pressure from baseline were made at each time point by one-way analysis of variance and the Newman-Keuls multiple range test at the 0.05 significance level. Data are presented as means ± SE.

In a third in vivo experiment, an exchange transfusion was performed with either a 5% albumin solution or a 3% polymerized hemoglobin solution during transient focal cerebral ischemia. A control group with no transfusion was also studied. Focal ischemia was produced by inserting a 6-O monofilament with a blunted tip 6 mm into the internal carotid artery so that in flow into the middle cerebral artery was occluded. At 10 min of ischemia a 700-μl exchange transfusion was performed over a 20-min period. After we obtained a blood sample at 45 min of ischemia, an additional 250 μl were infused. Catheters were removed, incisions were closed, and anesthesia was discontinued. At 2 h of ischemia, the mouse was briefly reanesthetized with halothane, and the monofilament was withdrawn from the internal carotid artery to allow reperfusion. At 24 h the mice were killed by an overdose of halothane anesthesia, and the brains were harvested and cut into five coronal slabs (2-mm thick). These slabs were incubated at 37°C in a 1% solution of 2,3,5-triphenyltetrazoleum chloride for 20 min followed by fixation in 10% buffered formalin. Infarct volume corrected for tissue swelling was measured by integrating regions that stained red with this vital dye in the ipsilateral hemisphere and subtracting this value from the volume of the contralateral hemisphere. Comparisons of infarct volume among the control, albumin-, and Hb-transfused groups were carried out by analysis of variance and the Newman-Keuls multiple range test.

RESULTS

Polymerization. β^{bovine} gene was used for expressing Hb Minotaur containing α-human and β-bovine subunits ($\alpha_H\beta_{Bv}$). This expression system was selected because of its high yield of protein expression and recovery introduced by the β_{Bv} gene.

For the polymerization of Hb Minotaur, the mutant ($\alpha_H^{V1M+C104S}\beta_{Bv}^{A9C+C93A}$) was constructed and designated as Hb Polytaur. In the α_H-chains α1Val was replaced by Met to closely approximate the conformation of the natural α-chains (27), and α104Cys was replaced by Ser, the residue present at this site in HbBv. In the β_{Bv}-chain, β9Ala was replaced by Cys (this residue is external and exposed to the solvent), and β93Cys was replaced by Ala (52). The substitutions of the naturally occurring Cys were introduced to facilitate the in vivo refolding and to confine the intermolecular cross-linking to the Cys residues introduced at the β9 position. In Fig. 3*A* the elution profile of size exclusion chromatography of Hb Minotaur and of Hb Polytaur is shown. The dotted line corresponds to the molecular mass of tetrameric Hb Minotaur in equilibrium with its dimeric form. The solid line shows the elution profile of Hb Polytaur after 30 days of polymerization at 4°C. The symmetric shape of the peak at 47 ml indicates the presence of a major homogeneous fraction with a molecular mass of ~500 kDa, corresponding to a polymer of seven to eight tetrameric Hb molecules. A small peak at 60 ml (<10% total protein) indicates the presence of some nonpolymerized tetrameric Hb. After removal of this fraction by gel filtration, Hb Polytaur maintains an invariant elution profile.

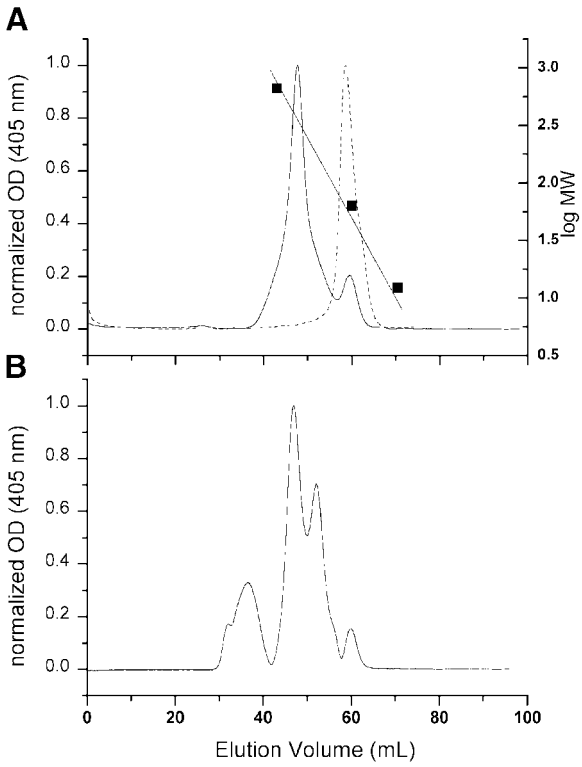

Fig. 3. Polymerization of Hb Polytaur and S-S bond stability. *A*: size exclusion chromatograph of Hb Minotaur (dashed line) and Hb Polytaur (solid line) on a prepacked (1.6 × 60 cm) Fractogel EMD BioSec column; 0.5 ml of a 2 mg/ml Hb solution was injected. Buffer consisted of 20 mM phosphate and 300 mM NaCl, at pH 7.2, 4°C, and flow rate of 0.5 ml/min. Standards (■): cytochrome *c* (molecular weight 12,400), cross-linked HbA (molecular weight 64,500), and thyroglobulin (molecular weight 669,000). Linear fit of the standards was used to calculate the molecular weight of the hemoglobins. *B*: size-exclusion chromatograph of Hb Polytaur after being mixed with blood for 24 h at 37°C and corrected for plasma absorbance. Conditions same as in *A*. Two new peaks are present, one with elution volume (V_E) = 52.2 ml for molecular mass of 250 kDa, the other with V_E = 36.6 ml for molecular mass of 860 kDa. The fraction with V_E = 60.1 ml represents <10% of the total protein as in *A*.

Because polymerization was obtained through the formation of intermolecular S-S bonds, we determined the stability of the polymer toward the reducing agents present in the blood. The S-S bonds are ~2.5 Å long, and our working hypothesis was that their accessibility would be hindered by the numerous interactions resulting from the intermolecular cross-linking of the

[1] In β_{Bv} the histidine present at position β2 in HbA is missing. We assign a number to this position in Hb Polytaur for consistency.

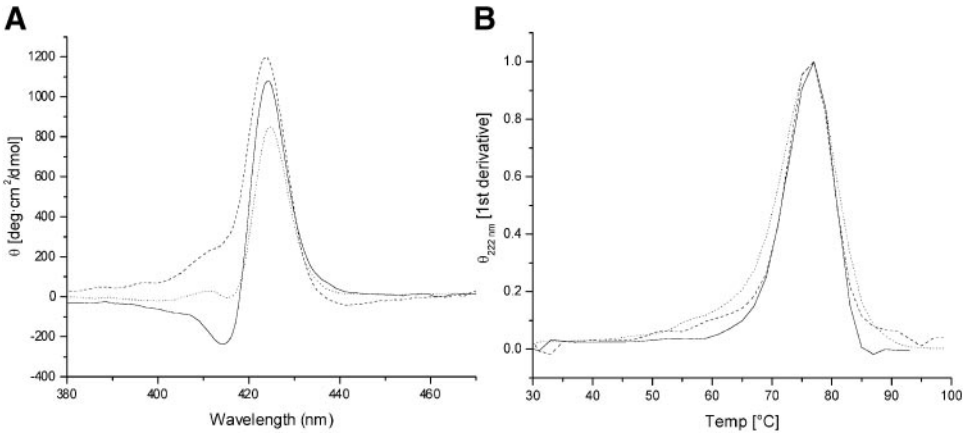

Fig. 4. CD Spectra. *A*: CD spectra of the carbonmonoxy derivatives of Hb Minotaur (solid lines), Hb Polytaur (dotted lines), and HbA (dashed lines). Conditions were the following: protein concentration, 0.1 mg/ml in 20 mM phosphate buffer, pH 7.0, 15°C. *B*: thermal stability of Hb Minotaur (solid lines), Hb Polytaur (dotted lines), and HbA (dashed lines). Conditions were same as in *A*. Change in ellipticity (θ) was recorded at 222 nm from 25 to 100°C with a step size of 2.0°C. Figure represents the first derivative of the temperature scanning, with the peaks corresponding to the median temperature of denaturation (T_m).

subunits. To test the polymerization stability, the protein in 40 mM phosphate buffer at pH 7.4 was mixed with human blood at a ratio of 1 ml 3% Hb solution to 9 ml blood for 24 h at 37°C. In a control sample only buffer was added to the blood. The samples were centrifuged at 800 *g*, and a plasma aliquot was analyzed on a gel filtration column. Optical density changes were recorded at 405 nm in the heme-sensitive Soret region of the spectrum. Figure 3*A* shows the elution profile of Hb Polytaur used for this experiment, and Fig. 3*B* shows the elution profile of the plasma sample containing Hb Polytaur corrected for the absorption profile of the control plasma sample. Two new peaks are observed: one with an elution volume (V_E) equaling 38 ml, corresponding to a molecular mass of 860 kDa, and a second with a V_E equaling 52 ml, corresponding to a molecular mass of 125 kDa. The amount of tetrameric Hb (V_E equaling 60 ml) present in the plasma was not increased.

Conformational characteristics and stability to denaturation. The conformation of the heme pocket of Hb Minotaur and Polytaur was investigated by observing the CD spectra in the Soret. In this region the spectra are sensitive to the position of the heme in relation to the aromatic residues lining the heme pocket. HbA and HbBv have similar CD spectra (18), and HbA is taken as the reference spectrum. A decreased ellipticity is observed in Hb Minotaur and Hb Polytaur with respect to HbA (Fig. 4*A*). Spectral differences are also present between the two recombinant hemoglobins. The stability toward denaturation of the carbonmonoxy derivatives of these hemoglobins was investigated as a function of temperature. Figure 4*B* shows the traces of the temperature-scanning measurements. The three hemoglobins have a very similar resistance to heat denaturation with a T_m ~76°C.

Autooxidation. Autooxidation rates were measured at 37°C either in phosphate buffer or in whole blood. In phosphate buffer, HbA autooxidation was described by a single exponential with a rate of 0.02 h^{-1}. In Hb Minotaur and Hb Polytaur, the autooxidation was described by two exponentials with ~20% of the protein having a fast autooxidation rate 50- to 150-fold increased with respect to HbA. The rate of the remaining fraction (~80%) was also increased, about eightfold with respect to HbA. The half-time of autooxidation, $T_{½\,ox}$, is 33 h for HbA and 3.2 h for Hb Minotaur and Hb Polytaur. Representative first-order plots are shown in Fig. 5*A*, and the results of the data analysis are listed in Table 1.

For the measurements in whole blood, fewer points could be collected, and the autooxidation rate was described by a linear decay as shown in Fig. 5*B*. The $T_{½\,ox}$ was 160 h for HbA and 46 h for Hb Polytaur

Table 1. *Autooxidation and heme transfer rates of HbA, Hb Minotaur, and Hb Polytaur*

Protein	Autooxidation (Buffer)			Autooxidation (Blood)		Heme Transfer	
	k, h^{-1}	%	$T_{1/2ox}$, h	k, h^{-1}	$T_{1/2ox}$, h	k, h^{-1}	%
HbA	0.02 ± 0.0006	100	33.0	$0.0043 \pm 4.5 \times 10^{-5}$	160.00	2.1 ± 0.12	50
						0.2 ± 0.02	50
Hb Minotaur ($\alpha_H\beta_{Bv}$)	2.90 ± 0.6	20	3.5			3.8 ± 0.22	50
	0.16 ± 0.003	80				0.47 ± 0.031	50
Hb Polytaur ($\alpha_H^{V1M+C104S}$ $\beta^{A9C+C93A}$)	1.06 ± 0.1	25	3.2	$0.015 \pm 5.5 \times 10^{-4}$	46.0	0.96 ± 0.066	30
	0.17 ± 0.003	75				0.11 ± 0.014	30
						$0.00 \pm 1 \times 10^{-9}$	40

Blood autooxidation measurements were carried out either in buffer (0.1 M phosphate at pH 7.0 plus 1 mM EDTA) at a protein concentration 60 μM in heme or in whole blood at a protein concentration of 150 μM in heme. Temperature was 37°C. $T_{1/2ox}$, autooxidation half-time. Percent values represent percentage of species. The affinity of hemoglobin (Hb) for heme transfer was measured following the method of Hargrove et al. (22) using apomyoglobin $His^{64} \rightarrow Tyr$. Hb concentration was 3 μM in heme and apomyoglobin 15 μM. Protein was buffered in 0.15 M phosphate, 0.45 M sucrose, pH 7.0, temperature = 25°C. k, Rate constant; HbA, human hemoglobin.

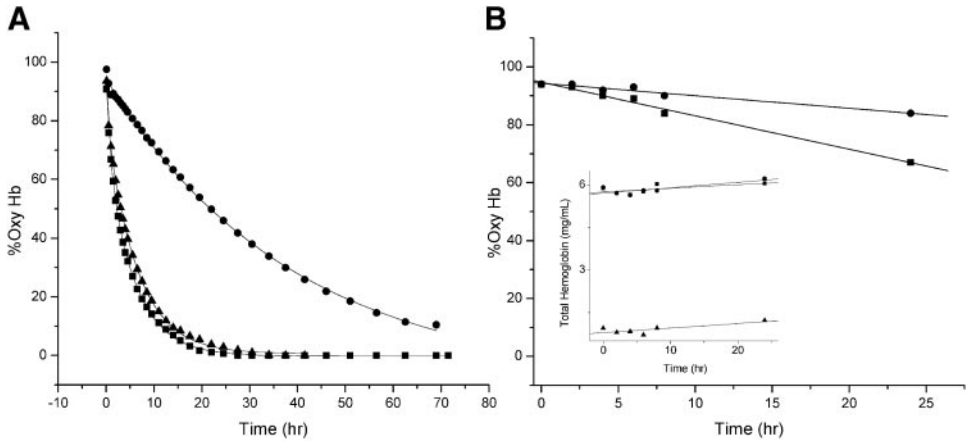

Fig. 5. *A*: buffer. Autooxidation rate of HbA (●), Hb Minotaur (▲), and Hb Polytaur (■) are shown. Data were fit with the program Origin version 6.1, using a first- or second-order kinetics equation and a Levenberg-Marquardt algorithm for minimization (R^2 for HbA: 0.999, Hb Minotaur: 0.999, Hb Polytaur: 0.999). Protein concentration was the following: 1 mg/ml in 0.1 M phosphate, pH 7.0, 37°C. *B*: blood. Autooxidation rate of HbA (●) and Hb Polytaur (■) are shown. Data were fit linearly to obtain the rate (SD of fit for HbA: 0.8823; Hb Polytaur: 1.0594) To 2 ml of blood, 200 μl of a 3.0% solution of Hb in the polymerization buffer were added. At intervals, aliquots were collected and the absorption spectra recorded between 470 and 380 nm and corrected for plasma absorption. Analysis was carried out on the absorption spectra decomposed linearly into a series of prerecorded standard spectra. *Inset*, plasma Hb concentration with added: HbA (●), Hb Polytaur (■), and buffer (▲). Linear fit shows similar slopes, indicating nearly zero hemolysis of the erythrocytes in all samples (SD of fit for HbA: 0.141, Hb Polytaur: 0.134, buffer: 0.122).

(Table 1). These data were not distorted by the release of Hb from the erythrocytes during the incubation time of the measurements. As shown in the *inset* of Fig. 5*B*, Hb is released in minimal and similar amounts from all samples, indicating that, under the conditions of this experiment, the addition of free Hb to the plasma did not increase hemolysis of the erythrocytes.

Heme affinity. The rate of heme transfer was determined according to the method of Hargrove et al. (22), in which the transfer occurs to an apomyoglobin in the solution. Representative time courses are shown in Fig. 6. As listed in Table 1, in HbA and Hb Minotaur the data were described by a fast and a slow rate of the same fixed amplitude (0.50) and one order of magnitude difference. The fast and a slow rate constants are assigned to heme release from the α- and β-chains, respectively (22). In Hb Minotaur the rate of heme transfer was twofold increased with respect to HbA. In contrast, in Hb Polytaur 40% of the total heme was not released by the protein. In the remaining 60% the heme transfer rates were twofold decreased with respect to HbA.

Oxygen affinity. The oxygen affinities of solutions of HbA, Hb Minotaur, and Hb Polytaur are similar under physiological conditions (37°C at pH 7.4 in the presence of 0.1 M NaCl) with a P_{50} of ~16–18 mmHg (Fig. 7). The *inset* in Fig. 7 shows a decreased cooperativity of the recombinant hemoglobins with respect to HbA. This is usually observed with recombinant hemoglobins and is particularly evident in this hybrid system.

NO reactivity. Figure 8 shows the time course of NO binding to HbA and Hb Polytaur at 7.5 μM NO (concentration before mixing). The traces, analyzed by a nonlinear least squares minimization routine, were found to be compatible with a single second-order process; most often even a pseudo first-order approximation was found to provide a satisfactory fit to the experimental data. The rate constant for the combination of NO with the deoxygenated forms of HbA and Hb Polytaur is similar, 19–25 $\mu M^{-1}s^{-1}$, respectively. This value is very close to that reported in the literature for HbA (k = 25 $\mu M^{-1}s^{-1}$) (32, 33).

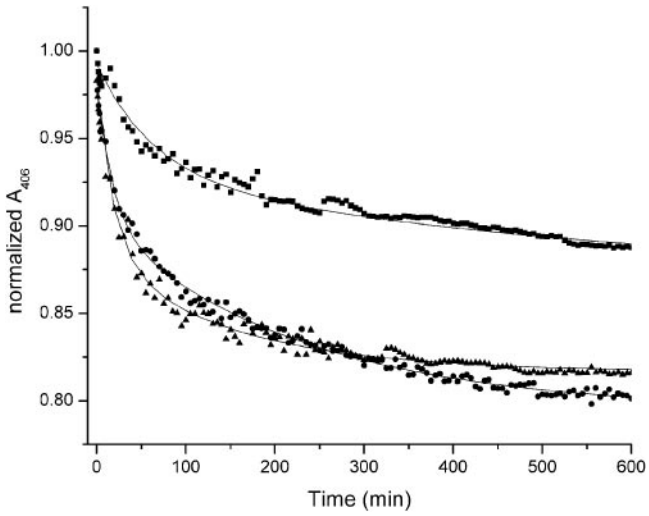

Fig. 6. Heme transfer. Affinities of HbA (●), Hb Minotaur (▲), and Hb Polytaur (■) for heme are shown. Hb concentration was 3 μM in heme and apomyoglobin 15 μM at temperature 25°C. Buffer consisted of 150 mM phosphate, 0.45 M sucrose, pH 7.0. Data were fitted with the program Origin version 6.1, using a second-order kinetics equation and a Levenberg-Marquardt algorithm for minimization (R^2 for HbA: 0.992 Hb Minotaur: 0.988; Hb Polytaur: 0.990). Rate of heme loss was the same for HbA and Hb Minotaur. Heme was not released by 40% of Hb Polytaur; in the remaining 60% of the protein the rate of heme loss was twofold decreased with respect to HbA.

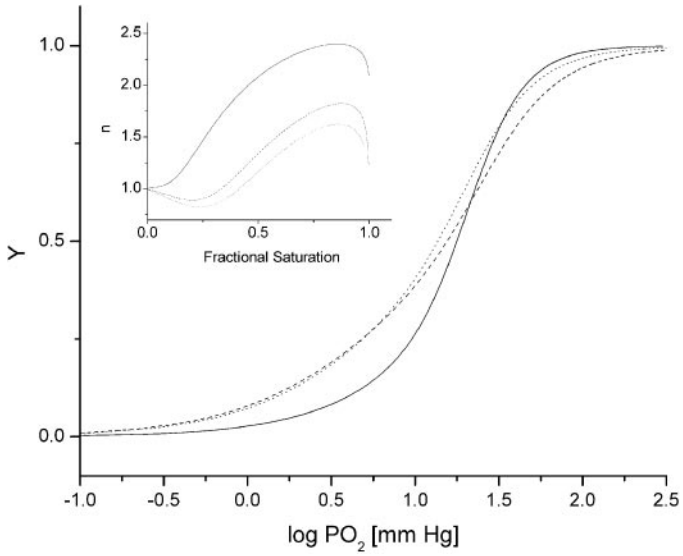

Fig. 7. Oxygen affinity. Oxygen equilibrium curves of HbA (solid line), Hb Minotaur (dotted line), and Hb Polytaur (dashed line) are shown. *Y*, fractional saturation of Hb with oxygen. Oxygen dissociation curves were obtained at a protein concentration of 1 mg/ml in 100 mM phosphate + 100 mM NaCl, pH 7.4, 37°C, using the enzymatic deoxygenation method of Vandegriff et al. (26). *Inset*: Hill coefficient plotted as a function of *Y* for each of the Hbs.

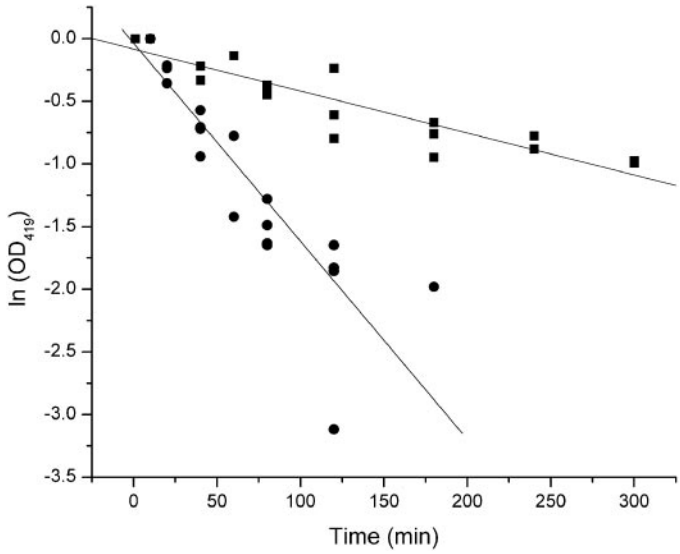

Fig. 9. Retention time in circulation in mice. Time points collected for mice infused with HbA (●) and Hb Polytaur (■). Infusion volume was 10% of the total blood (~200 μl). HbA was used as a control. Data were fit linearly using Origin version 6.1 to obtain the rate constants (slope) and, subsequently, the half-time. Retention half-time was 3.0 h for Hb Polytaur and 0.5 h for HbA.

Plasma retention time. Figure 9 shows the pooled data of plasma Hb concentration normalized by the initial concentration after transfusion. Mice transfused with Hb Polytaur had a fivefold greater retention time than mice transfused with HbA. When half-time was analyzed individually on each mouse ($n = 4$ per group), the half-time was significantly greater after Hb Polytaur transfusion (176 ± 23 min) than after HbA transfusion (34 ± 4 min). Hemoglobinuria was evident after HbA but not after Hb Polytaur transfusion.

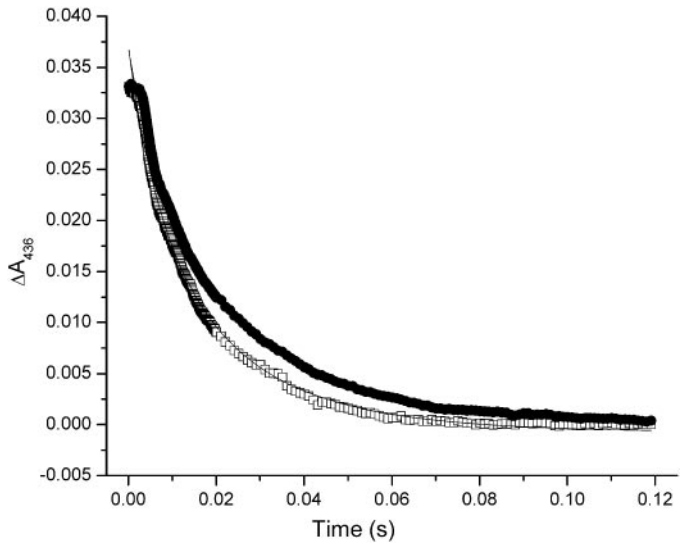

Fig. 8. NO binding. Time course of nitric oxide (NO) combination with deoxygenated HbA (●) and Hb Polytaur (□) is shown. ΔA_{436}, change in absorption at 436 nm. Data analysis was performed using a first-order rate equation minimized using the FMINS routine (Matlab). Overall fit is good, with minimal systematic deviations and high significance of the estimated parameters; accordingly, semilogarithmic linearization yields the following values of R^2: HbA 0.996, Hb Polytaur 0.994. Experimental conditions were 0.1 M bis-Tris buffer containing 1 mM EDTA. Hb concentration was 0.7 μM (per heme after mixing); NO concentration was 5 μM (after mixing), 23°C. Solution of Hb contains 0.01 mg/ml dithionite, whereas the NO was dithionite free; we carefully degassed both solutions to remove oxygen.

Exchange transfusion. Exchange transfusion with a 3% solution of Hb Polytaur in mice ($n = 5$) resulted in a decrease in arterial hematocrit from 42 ± 1% to 35 ± 1% at 15 min after completion of the transfusion. Hematocrit decreased further (31 ± 1%) with subsequent blood sampling over a 2-h period. Similar decreases occurred after exchange transfusion of a 5% albumin solution ($n = 5$) and a 6% solution of sebacoyl-cross-linked tetrameric Hb ($n = 6$) (8). The plasma concentration of Hb Polytaur was 1% and 0.5% at 15 min and 24 h after transfusion, respectively. The plasma samples were analyzed spectrophotometrically; methemoglobin formation was not observed. The exchange transfusion was hypervolumetric (650 μl infused; 350 μl withdrawn) to allow for subsequent blood sampling in the mouse. Mean arterial blood pressure increased in all three groups (Fig. 10*A*). Two-way analysis of variance with repeated measures indicated a significant effect of time ($P < 0.001$) and a significant interaction among the three treatment groups over time ($P < 0.001$). Data were further analyzed by performing one-way analysis of variance with the Newman-Keuls multiple range test at each time point. At 15 min after the hypervolumetric transfusion, the change in arterial pressure with the sebacoyl tetrameric Hb (25 ± 3 mmHg) was greater than the increase with albumin (11 ± 2 mmHg), whereas the increase seen with Hb Polytaur (16 ± 5 mmHg) was not different from that seen with albumin. The in-

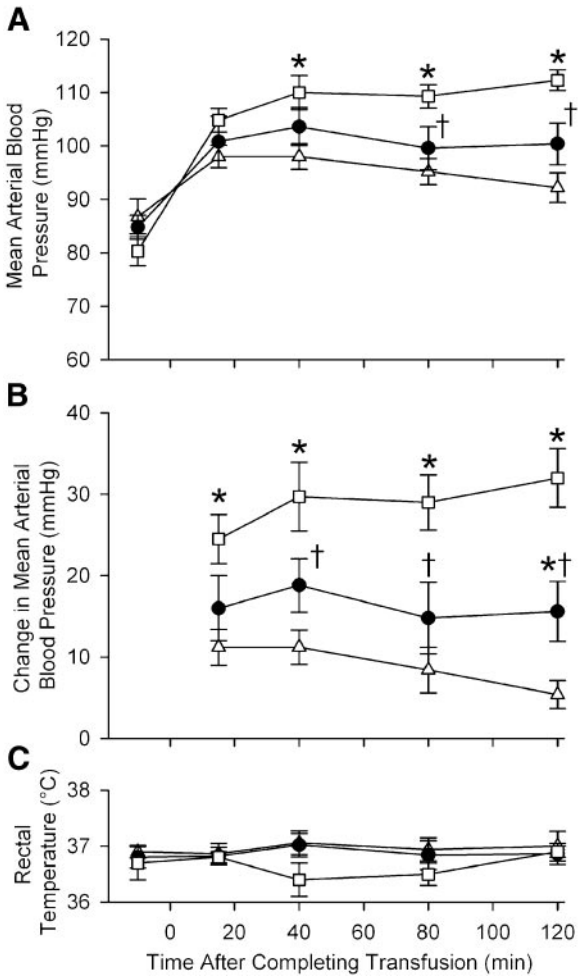

Fig. 10. Exchange transfusion in mouse. Time course of mean arterial blood pressure (*A*), change in mean arterial pressure from the pretransfusion baseline (*B*), and rectal temperature (*C*) after hypervolumetric exchange transfusion with solutions of 5% albumin (△, $n = 5$), 3% Hb Polytaur (●, $n = 5$), or 6% sebacoyl-cross-linked tetrameric Hb (□, $n = 6$) in anesthetized mice. Values are means ± SE. *$P < 0.05$ from albumin group; +$P < 0.05$ from sebacoyl-cross-linked Hb group by ANOVA and Newman-Keuls test.

crease in pressure was sustained during subsequent blood sampling over a 2-h period in both Hb groups but gradually subsided in the albumin group (Fig. 10*B*). Consequently, the change in arterial pressure became significantly different between the albumin and Hb Polytaur groups 2 h after transfusion. The change in arterial pressure was significantly greater in the sebacoyl Hb group than in the Hb Polytaur group from 40 min through 120 min after the transfusion. There was no change in rectal temperature after transfusion in any group and no difference among groups (Fig. 10*C*).

Focal cerebral ischemia. Exchange transfusion with 5% albumin after the onset of focal cerebral ischemia decreased hematocrit from 41 ± 1% to 31 ± 1% and decreased arterial Hb concentration from 11.1 ± 0.5 to 7.0 ± 0.5 g/dl. Exchange transfusion with 3% Hb Polytaur decreased hematocrit from 45 ± 1% to 35 ± 1% and decreased arterial Hb concentration from 11.7 ± 0.5 to 9.4 ± 0.3 g/dl. As expected, the change in total blood hemoglobin concentration (equivalent gram of tetramer) was less in the Hb-transfused group. Infarct volume after 2 h of transient ischemia was similar in the control and albumin-transfused groups but was reduced by ~22% in the Hb Polytaur transfused group (Fig. 11).

DISCUSSION

Recombinant technology techniques may eventually become a major supply source for Hb to be used as blood replacement in therapeutics. Our initial approach has been to design Hb molecules that would form homogeneous polymers with good oxygen delivery capability. The advantage of a homogeneous product is that it can be subjected to extensive biophysical characterization, which is essential to the understanding of its physiological response. The combination of the biophysical and physiological investigation is necessary to the tailoring of Hb molecules to specific therapeutic applications.

Polymer engineering. For the design of Hb Polytaur, we have used the same approach described for Hb Prisca (16), consisting of the substitution of the endogenous Cys residues and introduction of a novel Cys on the Hb surface to elicit specific intermolecular cross-linking. The substitution of the endogeneous Cys residues was necessary for obtaining the homogeneous polymerization shown in Fig. 3*A*. This peak has an estimated molecular mass of 500 kDa, corresponding to the polymerization of approximately seven tetrameric Hb molecules. A similar value was obtained by dynamic light scattering for polymerized Hb Prisca in which the tetramers assemble in a globular shape. Polymerization of Hb Prisca and Hb Polytaur follows the same approach; these systems most likely have the

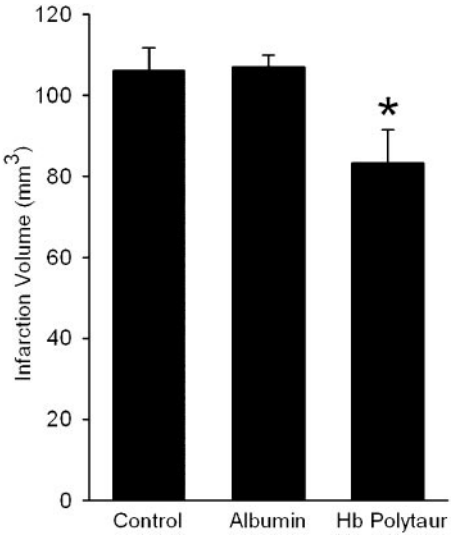

Fig. 11. Infarct volume (± SE) after 2 h of focal cerebral ischemia in control mice with no transfusion and in mice transfused with either 5% albumin ($n = 7$) or 3% Hb Polytaur ($n = 7$) during ischemia. Infarct volume was significantly smaller in the Hb Polytaur group. *$P < 0.05$ from control and albumin groups by ANOVA and Newman-Keuls test.

same polymerization assembly. Some residual tetrameric Hb (<10%) is observed, which can be eliminated by gel filtration. After removal of the tetrameric fraction by gel filtration, the elution profile of Hb Polytaur remains invariant, suggesting that the tetrameric residual is not the result of an equilibrium between the polymeric and the tetrameric forms but is due to a limiting rate of tetrameric Hb incorporation into the polymer. On mixing of Hb Polytaur with whole blood, the amount of tetrameric Hb present in the plasma remains very small (Fig. 3*B*). However, some opening of the S-S bonds by the reducing agents present in blood occurs as indicated by the appearance of a peak with a molecular mass of 250 kDa. A similar polymer with a molecular mass of 250 kDa was observed in hemolysates of erythrocytes containing HbA and Hb Porto Alegre in which the glutathione reductase activity was threefold increased (47). We also obtained a second new peak corresponding to a protein with an average molecular mass of 850 kDa. This peak is not observed in experiments in which blood was mixed with HbA. It may be due to fragments of Hb Polytaur captured by the plasma proteins or to the assembly of the species with molecular masses of 500 and 250 kDa. Whatever its origin, the important result is that the amount of tetrameric Hb capable of extravasation is not increased in the plasma. It is worth stressing that, for these experiments, we used a large excess of blood over free Hb, which should maximize S-S bond reduction.

Conformational characteristics and stability. The CD spectra in the Soret region spectrum of Hb Minotaur and Polytaur (Fig. 4*A*) indicate differences in the tertiary structure around the heme pocket in both proteins with respect to HbA. Differences in the Soret CD spectra are also present between Hb Minotaur and Polytaur. They may be due to conformational effects resulting from the amino acid substitutions introduced in Hb Polytaur. Alternatively, they may indicate that the intermolecular cross-linking elicits conformational changes that propagate to the heme pocket.

The high helical content and the quaternary assembly contribute in conferring on the HbA molecule a high stability to temperature denaturation (Fig. 4*B*). The stability is well maintained in Hb Minoatur and Polytaur and is consistent with the high expression level of these hybrid hemoglobins. This is an important aspect for the long-term storage of these proteins. Notably, in Hb Prisca containing α- and β-human chains the stability to temperature denaturation was decreased 8°C with respect to HbA (16).

Autooxidation. The autooxidation rate of Hb is concentration dependent, with Hb dimers having about a 20-fold higher autooxidation rate than the tetramers (53). At the concentration used in our experiments (1.0 mg/ml), the amount of dimers present in HbA is <10% and does not contribute significantly to the autooxidation rate measured, which appears to be monoexponential. Heterogeneity in the rate of autooxidation has been observed in recombinant Hbs (17). In Hb Minotaur and Hb Polytaur, the autooxidation rates are described by two exponentials of different amplitude and a 10-fold difference in the autooxidation rate (Table 1, Fig. 5*A*). The increased rate of autooxidation present in Hb Minotaur could be explained by the presence of a very large dimeric population; however, Hb Polytaur, which is not in equilibrium with its dimeric form, has an autooxidation rate similar to that of Hb Minotaur and is ~8- to 10-fold faster than HbA. The similarity of these data suggest that the large increase in the autooxidation rate observed in Hb Minotaur and Hb Polytaur is an intrinsic property of the hybrid molecule.

Measurements made in vitro provide information about the intrinsic properties of proteins, which may be affected by components present in an in vivo environment. Therapeutic applications of Hb solutions involve injection into the circulation. Reducing systems are present in the blood, which may affect the autooxidation rate of proteins dissolved in the plasma. Our data indicate that, in the presence of blood, the autooxidation rates of stroma-free HbA and Hb Polytaur are 5- and 10-times decreased, respectively, compared with the rates measured in buffer (Table 1, Fig. 5*B*) confirming a previous report on the protective role of blood components toward heme oxidation (45). In blood the half-time of autooxidation of Hb Polytaur is increased from ~4 h in buffer to ~46 h. This value is within the retention time in circulation of other polymerized hemoglobins, ~20 h in humans (9, 25, 26).

Heme affinity. Hb Polytaur has a remarkable increase in heme affinity with ~40% of the total heme not released from the protein. This effect can be attributed to heme stabilization by intermolecular cross-linking because it was not observed in Hb Minotaur (Fig. 6). This observation is consistent with a globular assembly of Hb Polytaur similar to that proposed for Hb Prisca (16), which would make the hemes less accessible to solvent. Heme release triggers oxidative stress and cytotoxicity in the endothelial cells (2, 4), and these negative effects should be reduced in Hb Polytaur.

Oxygen affinity. The oxygen-binding curves of Hb Minotaur and Polytaur show an oxygen affinity similar to that of HbA but a decreased cooperativity (Fig. 7). In the construction of hybrid Hbs containing HbBv β-chains, we hypothesized that they would have an oxygen affinity lower than that of HbA, regulated by the Cl^- in the solvent. However, this was not observed. In the presence of 100 mM Cl^-, HbA, Hb Minotaur, and Hb Polytaur have the same oxygen affinities. The absence of a decreased oxygen affinity in the hybrid hemoglobins is tentatively attributed to the nine amino acid differences present at the $\alpha_1\beta_1$ interface between HbA and HbBv (17). We are currently investigating this hypothesis with hybrid hemoglobins having either human or bovine residues at the $\alpha_1\beta_1$ interface.

The optimal oxygen affinity and the relevance of cooperativity in oxygen transport and delivery are currently under scrutiny (50). The assumption that a plasma-based O_2 carrier should have an O_2 affinity similar to the red blood cell-based hemoglobin may not

necessarily hold under all circumstances. Plasma may account for a considerable resistance to O_2 diffusion in small vessels, and hemoglobin in the plasma may facilitate O_2 unloading by increasing the effective O_2 solubility, even at low Hb concentrations in the plasma (36, 37). A low O_2 affinity would promote precapillary O_2 loss and raise perivascular P_{O_2}, which could produce counterregulatory arteriolar constriction and uneven capillary perfusion (41, 50). Our observation that infusion of a 3% solution of Hb Polytaur decreases infarct volume implies effective O_2 delivery under ischemic conditions at a low plasma concentration (~1 g/dl) and high O_2 affinity (P_{50} ~17 mmHg).

Reactivity with NO. NO combines with reduced unliganded Hb without discriminating between the T and the R allosteric states, and its reaction is virtually diffusion limited, the energy barrier for bond formation being almost nil (35). Thus the experiments of rapid mixing described herein probe the rate of diffusion of this gas into the heme pocket. The observation that Hb Polytaur combines with NO at the same rate as that of HbA (Fig. 8) demonstrates that polymerization does not hinder the diffusion of diatomic ligands into the heme pocket (33). It should be pointed out that the overall affinity of NO for heme is largely determined by the slow dissociation rate constant, which was not determined in these experiments. The latter measure is more likely to correlate with O_2 affinity and cooperativity.

Plasma retention time. Hb may be cleared from the plasma in the urine, lymph, or reticuloendothelial system (6). The fivefold increase in the plasma retention time of Hb Polytaur compared with HbA is largely due to the lack of clearance in the urine and possibly in lymph (Fig. 9). It has been shown that removing chemically cross-linked tetramers and polymers of <300 kDa nominal molecular mass prevented extravasation and appearance in renal lymph draining renal peritubular capillaries (31). Because the Hb Polytaur solution has a homogeneous molecular mass of ~500 kDa with <10% Hb remaining unpolymerized, the amount cleared by the lymphatics is probably relatively small. The 3-h half-life of Hb Polytaur may appear to be too brief for some clinical applications. However, it should be recognized that the plasma retention of chemically modified Hb polymers varies logarithmically with body mass and could be substantially greater in humans.

Exchange transfusion. The hypervolemic exchange transfusion resulted in similar increases in arterial blood pressure in the albumin and Hb Polytaur groups (Fig. 10). The lack of a substantially greater increase in arterial pressure with Hb Polytaur than with albumin transfusion implies that the polymer does not produce the large pressor response often seen with other Hb solutions (3, 9, 49). The pressor response to exchange transfusions of polymeric Hb solutions can be prevented by removing molecular mass species <300 kDa, which also reduces extravasation in renal lymph (31). Thus the lack of a large pressor response with the Hb Polytaur solution is probably related to the large homogeneous size of the polymer, which presumably does not extravasate in most vascular beds. As discussed above, polymerization does not hinder NO diffusion into the heme pocket and is unlikely to interfere with binding of NO. Extravasated Hb will scavenge NO in the interstitial spaces surrounding vascular smooth muscle more effectively than Hb in the plasma and thereby increase vascular tone. Therefore, eliminating low-molecular-mass Hb components is important for preventing unwanted vasoconstriction in tissues such as the kidney and intestines. In support of this hypothesis, tetrameric cross-linked Hb extravasates in renal lymph (31), increases arterial pressure in the cat (48), and decreases renal and intestinal blood flow (49) but does not inhibit NO-dependent dilation evoked by acetylcholine or ADP in brain arterioles with tight endothelial junctions (3). Moreover, sebacoyl-cross-linked tetrameric Hb transfusion in the mouse was presently found to increase arterial pressure to a greater extent than Hb Polytaur. These results are consistent with Hb Polytaur producing less peripheral vasoconstriction than the sebacoyl tetramer.

A limitation of this study is that cardiac filling pressure and cardiac output were not measured in these mice to determine whether systemic vascular resistance changed after transfusion. The oncotic pressure of the 6% sebacoyl Hb solution is presumably greater than that of the 3% Hb Polytaur solution. Therefore, it is possible that rapid fluid shifts resulted in a somewhat greater cardiac output contribution to the pressor response in the sebacoyl Hb group compared with the Hb Polytaur group. Moreover, a greater oncotic pressure of 5% albumin compared with 3% Hb Polytaur may have helped sustain arterial pressure at a level similar to that obtained during the first 80 min after Hb Polytaur transfusion. The difference in arterial pressure at 120 min between the albumin and Hb Polytaur groups might be attributed to a subsequent decline in plasma volume in the albumin group or possibly to time-dependent extravasation and NO scavenging of the small amount of unpolymerized tetrameric Hb (<10%) remaining in the infused solution. Therefore, differences in arterial pressure responses could be influenced by shifts in blood volume as well as by changes in peripheral vascular resistance.

Transfusion of Hb Polytaur during 2 h of transient focal cerebral ischemia resulted in a decrease in infarct size relative to albumin-transfused mice with a similar 20–25% lowering of hematocrit (Fig. 11). This decrease in infarct volume presumably is attributable to increased oxygen delivery into the ischemic border regions. Plasma-based Hb is thought to increase the effective O_2 diffusivity in plasma and thereby facilitates O_2 unloading (36, 37). Moreover, with low blood flow associated with ischemia, red blood cell flux in individual capillaries may become heterogeneous, and a plasma-based O_2 carrier may improve the homogeneity of O_2 distribution. However, we cannot exclude that differences in arterial blood pressure among groups may have occurred after discontinuing the anesthesia and that an improved O_2 delivery was second-

ary to a higher blood pressure in the Hb Polytaur group during the remaining ischemia period.

We conclude that site-specific introduction of Cys on the Hb surface permits polymerization to a uniform size while maintaining physiologically relevant oxygen affinity, cooperativity, stability toward denaturation, heme affinity, and stability toward oxidation. The solution of Hb Polytaur can be used in vivo without producing hemoglobinuria. Replacing ~20% of the red blood cells with this plasma-based polymer resulted in stable arterial blood pressure. NO diffusion into the Hb Polytaur heme pocket is not hindered by polymerization, and the absence of a large pressor response may be attributed to the absence of extravasation. Reduced ischemic damage in the brain suggests that the polymer can act as an effective oxygen transporter despite a P_{50} of ~17 mmHg. Therefore, this unique polymer of recombinant Hb represents a viable alternative oxygen carrier that potentially could be developed into a transfusion fluid.

DISCLOSURES

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