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Coexpression of Human α- and Circularly Permuted β-Globins Yields a Hemoglobin with Normal R State but Modified T State Properties[†]

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ABSTRACT: For the first time, a circularly permuted human β-globin (cpβ) has been coexpressed with human α-globin in bacterial cells and shown to associate to form α-cpβ hemoglobin in solution. Flash photolysis studies of α-cpβ show markedly biphasic CO and O_2 kinetics with the amplitudes for the fast association phases being dominant due the presence of large amounts of high-affinity liganded hemoglobin dimers. Extensive dimerization of liganded but not deoxygenated α-cpβ was observed by gel chromatography. The rate constants for O_2 and CO binding to the R state forms of α-cpβ are almost identical to those of native HbA ($k'_{R(CO)} \approx 5.0\ \mu M^{-1}\ s^{-1}$; $k'_{R(O_2)} \approx 50\ \mu M^{-1}\ s^{-1}$), and the rate of O_2 dissociation from fully oxygenated α-cpβ is also very similar to that observed for HbA ($k_{R(O_2)} \approx 21-28\ s^{-1}$). When the equilibrium deoxyHb form of α-cpβ is reacted with CO in rapid mixing experiments, the observed time courses are monophasic and the observed bimolecular association rate constant is ∼1.0 $\mu M^{-1}\ s^{-1}$, which is intermediate between the R state rate measured in partial photolysis experiments (∼5 $\mu M^{-1}\ s^{-1}$) and that observed for T state deoxyHbA ($k'_{T(CO)} \approx 0.1$ to 0.2 $\mu M^{-1}\ s^{-1}$). Thus the deoxygenated permutated β subunits generate an intermediate, higher affinity, deoxyHb quaternary state. This conclusion is supported by equilibrium oxygen binding measurements in which α-cpβ exhibits a P_{50} of ∼1.5 mmHg and a low *n*-value (∼1.3) at pH 7, 20 °C, compared to 8.5 mmHg and $n \approx 2.8$ for native HbA under identical, dilute conditions.

In the 2005 Nationwide Blood Collection and Utilization Survey Report, the American Association of Blood Banks reported on the shrinking margin between the number of available units of blood approved for administration and the number of transfusions (*1*). Between 1989 and 2004 this margin decreased by 53%, and the 2004 margin was the smallest ever reported by this survey (*1*). In response to this trend, there is increased interest in developing a safe and effective red blood cell (RBC) substitute. Given the unique properties of RBCs, these endeavors face many design challenges. RBCs contain a high concentration of hemoglobin (Hb) inside a protective membrane, which provides Hb a circulatory half-life of several months and protects tissues from oxidative damage (*2*). Additionally, RBCs contain methemoglobin reductase, which prevents the accumulation of the oxidized form of Hb, which is incapable of transporting oxygen. Finally, allosteric effector molecules inside the RBC, primarily 2,3-bisphosphoglycerate (2,3-BPG), modulate the oxygen binding affinity of Hb to allow complete saturation with oxygen in the lungs and significant delivery of a large fraction of this bound oxygen to respiring tissues.

Three main classes of RBC substitutes have been investigated in response to these design challenges (*2*, *3*): perfluorocarbon emulsions, liposome encapsulated hemoglobin solutions, and extracellular hemoglobin-based oxygen carriers (HBOCs). The advantage of using cell-free Hb as an RBC substitute is that it is 2−3 times more efficient at delivering oxygen in blood vessels than Hb encapsulated in large red cells (*4*). HBOCs also have a much longer shelf life due to removal of bacterial and viral contaminants and are more stable at higher temperatures, allowing the use of HBOCs under more adverse conditions, such as battlefields and remote accident locations (*3*).

Several challenges must be overcome in the development of HBOCs, including the source of the starting material, the oxygen affinity of these preparations, and toxicity issues associated with the intravenous administration of cell-free Hb solutions (*5*). Protein engineering strategies to address these challenges have been extensively reviewed (refs (*2*−*9*) and references cited therein). The results of multiple studies suggest that the ideal HBOC should minimize renal toxicity (*10*−*13*), oxidative stress (*14*−*16*), and vasoactivity (*10*−*15*, *17*−*20*) and possess O_2 binding characteristics which allow it to effectively transport the ligand from

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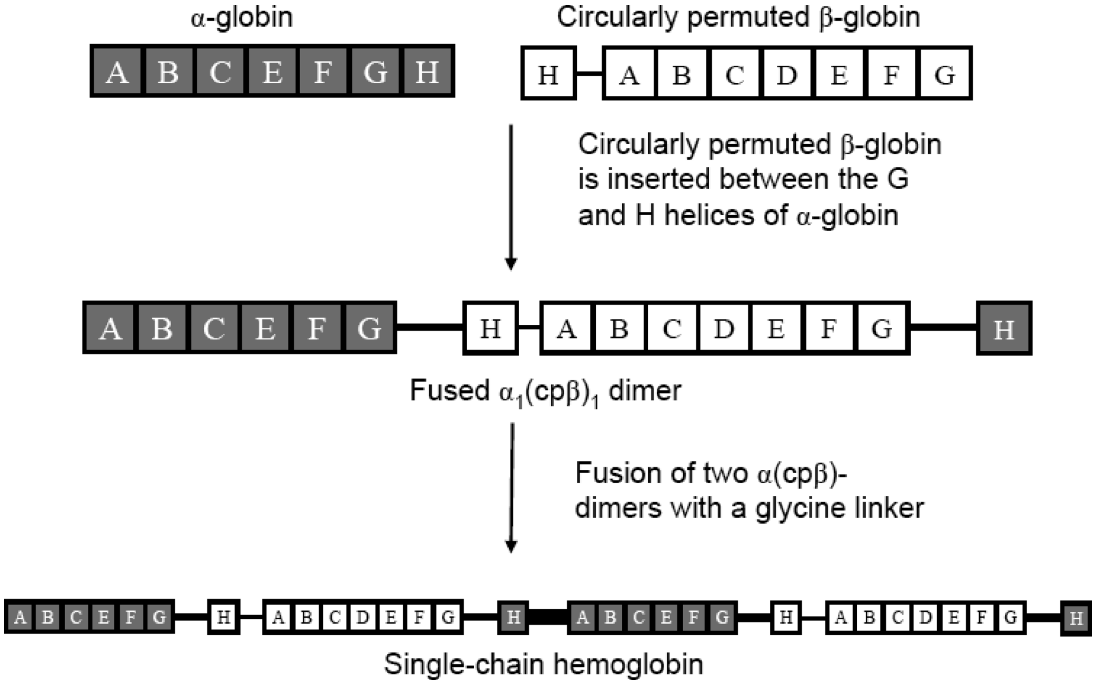
Scheme 1: Proposed Construction of the Single-Chain Hemoglobin[a]



[a] (1) Generation of a circularly permuted β-globin with new termini in the G-H loop, (2) insertion of cpβ-globin into the G-H loop of α-globin to create a fused $\alpha_1(cp\beta)_1$ dimer, and (3) fusion of two $\alpha_1(cp\beta)_1$ dimers by glycine insertion (thick line) to create a single-chain hemoglobin.

the lungs to the tissues in the absence of the allosteric effectors found in erythrocytes.

The long-term goal of our work is the development of a recombinant polymeric Hb derived from fusions of a single globin gene containing the α and β subunit sequences. This design will help to overcome some of the challenges described above. Several studies have demonstrated that solutions of polymeric Hbs with molecular masses ≥128000 Da attenuate the hypertension observed upon administration of cell-free Hb due to reduced extravasation of the larger molecular mass protein into the endothelium (*18*−*21*). In addition, polymeric Hbs are able to transport more oxygen at lower colloid osmotic pressure compared to simple Hb tetramers, and covalent genetic cross-linking between globin subunits completely prevents the dissociation of Hb into $\beta\alpha$ dimers, inhibiting loss from the bloodstream and renal damage.

Polymerization of Hbs for use as HBOCs has been achieved using well-established chemical cross-linking agents, primarily glutaraldehyde, and either donated human hemoglobin or bovine hemoglobin collected from animals (*3*, *20*). The resulting polymeric HBOC preparations contain uniform molecular mass distributions, all above 128000 Da, but do not represent a solution of a single type of protein molecule. We envision the production of recombinant polymeric Hbs of defined and uniform molecular mass by genetically cross-linking sets of human α and β chains into a single Hb "monomer". Because recombinant Hb can be produced by large-scale fermentation (*22*−*25*), the supply would not depend on donated blood. In addition, the recombinant Hb (rHb) molecule can also be engineered to address toxicity issues, modulate functional properties, increase affinity for the heme prosthetic group, and enhance the stability of the globin fold (*2*−*4*, *17*, *26*−*36*).

The simplest idea for the design of a single-chain Hb involves inserting a peptide linker between the globin subunits. The distance separating the termini of the α_1 and α_2 subunits can be spanned by one (*10*) or two (*35*) amino acids. Likewise, the termini between the β_1 and β_2 subunits can be spanned by four amino acids (*35*). A much longer peptide would be required to link the termini of the α- and β-globins, since these termini lie on opposite sides of the Hb tetramer. Our strategy for linking the α- and the β-globins without the need for a very long linker is to use circular permutation to relocate the termini of the β subunit to a site near the $\alpha_1-\beta_1$ interface and then insert the permuted β subunit into the G-H loop of the α subunit (Scheme 1).

This strategy is based on the following observations: (1) the G-H loops of both subunits in an α_1-β_1 dimer are on the surface of the Hb molecule and are separated by less than 15 Å, which could be spanned by five amino acids; (2) protein termini can be relocated by circular permutation, and circular permutations of both human α-globin (*36*) and sperm whale myoglobin (swMb)[1] (*37*, *38*) have been reported; (3) the $\alpha_1-\beta_1$ interface, unlike the $\alpha_1-\beta_2$ interface, is relatively immobile during the R to T transition in Hb and is

[1]Abbreviations: cpβ, circularly permuted human β-globin that has Glu121 as the N-terminus, Lys120 as the C-terminus, and eight amino acids connecting the original N- and C-termini; α-cpβ, the heterodimer including human α-globin and circularly permuted human β-globin; rHb0.0, recombinant human hemoglobin; HbA, wild-type human hemoglobin; swMb, sperm whale myoglobin; ESIMS, electrospray ionization mass spectrometry; $K_{R(O_2)}$, equilibrium association constant for O_2 binding to R state hemoglobin; $k'_{R(O_2)}$, association rate constant for O_2 binding to R state hemoglobin; $k_{R(O_2)}$, rate constant for O_2 dissociation from R state hemoglobin; $k'_{T(O_2)}$, association rate constant for O_2 binding to T state hemoglobin; IPTG, isopropyl thiogalactoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; IMAC, immobilized metal affinity chromatography; HPSEC, high-pressure size exclusion chromatography; IEC, ion-exchange chromatography; HPLC, high-pressure liquid chromatography.

therefore a more attractive site for creating new covalent links between the globins (*39*); (4) the globin fold appears to tolerate a wide variety of cross-links and backbone rearrangements which yield folded proteins with reversible ligand binding function (*34*−*38*, *40*−*43*); and (5) the wild-type termini in α-globin can be fused to yield a functional rHb (*10*, *35*, *36*).

Others have described the structural and functional consequences of creating recombinant α−α and/or β−β fusion proteins (*10*, *35*, *36*) and chimeric globins (*34*); however, we are not aware of any studies which describe the structural and functional consequences of fusing entire α- and β-chains via recombinant technology or the circular permutation of human β-globin. Here we report for the first time that circularly permuted β-globin (cpβ) can be expressed, associates with α-globin, and binds ligands reversibly. These results represent an important proof of concept to support the generation of a 64 kDa single-chain Hb as outlined at the bottom of Scheme 1. The longer term goal is then to link these single-chain Hb monomers to make higher order single-chain Hb oligomers with MWs ≥ 128000 Da from single large genes expressed in bacteria.

EXPERIMENTAL PROCEDURES

General. DH5α and BL21(DE3) strains of *Escherichia coli* were used for cloning and expression experiments. Enzymes were purchased from New England Biolabs. Synthetic oligonucleotides were ordered from Sigma-Genosys (The Woodlands, TX). The DNA sequences of all genes were confirmed by dideoxy sequencing carried out at Davis Sequencing LLC (Davis, CA). *E. coli* cells were transformed using a Bio-Rad Gene Pulser II set to a resistance of 200 ohms, a potential of 1.25 kV/mm, and a capacitance of 25 μF. Mass spectrometry was performed using an Applied Biosystems API 2000 triple quadrupole mass spectrometer, and observed masses were calculated from the data using the Bayesian Protein Reconstruct tool in the instrument manufacturer's BioAnalyst software. Protein concentrations of oxyhemoglobins in solution were calculated from visible absorbance data acquired at pH 7.0 using $\varepsilon_{415} = 125\ mM^{-1}\ cm^{-1}$ per heme (*44*).

Gene Construction. The genetic constructs used in this study were derived from the optimized coexpression vector, pDLIII-13e, developed by Hoffman et al. (*24*). The pDLIII-13e plasmid contains the sequences for human α- and β-globin genes using codons and promoter sequences optimized for protein expression in *E. coli*. The cpβ-globin gene was constructed via primer extension from a tandem gene template as described previously for the circular permutation of myoglobin (*37*). Plasmid pDML1 contains the tandem gene. Plasmid pALA1 contains the cpβ-globin gene, which encodes the eight amino acid linker GlySerGlyGlyGlnGlyGlyGly between the wild-type β-globin termini and has the new N- and C-termini located at Glu121 and Lys120, respectively. The cpβ-globin gene from pALA1 was then cloned into pDLIII-13e in place of the normal β-globin gene to make the α- and cpβ-globin coexpression vector pALA2. The details of the construction of pDML1, pALA1, and pALA2 are given in Supporting Information.

Coexpression of α- and cpβ-Globins. BL21(DE3) cells were transformed with the α-cpβ coexpression vector pALA2. Bacterial fermentations were carried out in shake flasks. Each liter of Luria−Bertani medium (LB) containing 12.5 μg/mL tetracycline was inoculated with 10 mL of overnight cell culture. Cells were incubated at 37 °C until midlog phase (OD_{600} = 0.5−0.7) and then cooled to 25 °C prior to induction with 0.1 mM IPTG. Hemin solution was prepared by dissolving 0.05 g of hemin (Alfa Aesar) per liter of culture in 3−4 mL of 20 mM NaOH, followed by filtration through a sterile 0.2 μm cellulose acetate membrane. Half of the hemin solution was added at induction, and the other half was added at 2 h postinduction. The cultures were incubated for 4 h postinduction and then harvested by centrifugation at 4000*g* for10 min. The cell pellet was then resuspended in 30 mL of lysis buffer (17 mM NaCl, 50 mM Tris-HCl, pH 8.5), flash frozen in liquid nitrogen, and stored at −80 °C.

Expression of Recombinant α- and β-Globins. Recombinant human hemoglobin, "rHb0.0" (*10*), derived from plasmid pDLIII-13e, was expressed in BL21(DE3) cells as described above, except that the induction temperature was 37 °C rather than 25 °C.

Protein Purification. Resuspended cell pellets were thawed and then lysed by sonication for 90 s with a Sonifier model 450 (Branson Instruments, Inc.) equipped with a 1.8 cm diameter horn using a 50% duty cycle and a power output level of 6. The crude lysate was then centrifuged at 21000*g* for 20 min. The pH of the supernatant was then adjusted to between 8.0 and 8.5, and zinc acetate from a 0.5 M stock was added to a final concentration of 2−4 mM. The lysate was then centrifuged again at 40000*g* for 20 min and filtered through a 0.45 μM filter (Millipore Durapore).

The clarified cell lysates were immediately purified as described previously for myoglobin (*37*) using a column charged with 3 mL of Chelating Sepharose Fast Flow resin (GE Healthcare Lifesciences). Purified protein was collected and then concentrated to a final volume of 0.3−1.0 mL in Centricon YM-10 concentrators (Amicon). The protein samples were then either flash frozen in liquid nitrogen and stored at −80 °C or immediately purified by HPSEC or IEC.

α-cpβ hemoglobin for ligand binding studies was purified by ion-exchange chromatography (IEC) using a Mono Q 5/50 GL column (GE Healthcare Lifesciences) and a Waters 650E HPLC system operating at a flow rate of 1 mL/min. The column was equilibrated with 20 mM Tris-HCl, pH 8.5 (IEC buffer "A"). The IMAC-purified protein sample was buffer exchanged into IEC buffer "A", concentrated to a volume of 100−200 μL, and then loaded onto the equilibrated column. The desired protein was eluted using a linear gradient of 0−30% IEC buffer "B" (20 mM Tris-HCl, 300 mM NaCl, pH 8.5) over 3 min, followed by a linear gradient of 30−80% IEC buffer "B" over 25 min. Fractions with significant absorbance at 280 nm were collected, pooled, concentrated, and frozen as described above.

Protein samples were purified by HPSEC using a Superdex 75 HR 10/30 column (GE Healthcare Lifesciences) and a Waters 650E HPLC system operating at a flow rate of 1 mL/min. The mobile phase was 20 mM Tris-HCl and 120 mM NaCl, pH 8.5, unless otherwise specified. A sample volume of 250−300 μL was injected and the absorbance monitored at 280 nm. Fractions with significant absorbance at 280 nm were collected, concentrated, and flash frozen as described above. Purified protein samples for ESIMS analysis were subjected to HPSEC using a 20 mM ammonium acetate buffer, pH 7.0.

Analytical HP-SEC. Analytical HPSEC was performed using the Superdex 75 HR 10/30 column connected to a Varian ProStar HPLC system. Data analysis was performed using the manufacturer's software package. Proteins were eluted from the column at a flow rate of 0.5 mL/min in either 50 mM sodium phosphate and 150 mM NaCl, pH 6.7, or 25 mM Tris-HCl and 0.5 M $MgCl_2$, pH 7.4. For each buffer system three independent injections of a gel filtration standard spanning a molecular mass

range of 1350−670000 Da (Bio-Rad) were used for construction of calibration curves (log of the molecular mass of each protein vs its average retention time). The molecular masses of HPSEC-purified α-cpβ and rHb0.0 were calculated from their retention times using the appropriate calibration curve. For analyses performed with deoxygenated mobile phase, oxy protein was applied to the HPSEC column which was equilibrated with 50 mM sodium phosphate and 150 mM NaCl, pH 7.0, that had been sparged with nitrogen and then supplemented with 5 mM sodium dithionite.

Ligand Binding Studies of α-cpβ and HbA. The native HbA used in the ligand binding studies was purified from red blood cells as described (*45*). Flash photolysis and stopped-flow experiments to determine R and T state CO and O_2 binding rate constants were conducted at 20 °C using the methods and analyses described by Olson et al. (*46*, *47*). Equilibrium oxygen binding curves were measured using a Shimadzu spectrophotometer equipped with an Imai cell and an O_2 electrode (*48*). The system was calibrated with 100% nitrogen, 20% oxygen, and 100% oxygen before each measurement. Hb samples were injected into the cuvette, and the formation of deoxygenated protein was monitored by absorbance changes at 560 or 430 nm at 20 °C. Absorbance data were collected and stored at preset changes in P_{O_2} as measured by a picoammeter system. Spectra from 400 to 700 nm were collected at 100% and 0% oxygen concentrations to monitor the extent of oxygenation.

RESULTS

Protein Expression, Purification, And Characterization. Expression yields for α-cpβ were calculated following the IMAC purification step and found to be roughly 1.6 mg of α-cpβ/L of cell culture. Under similar fermentation conditions rHb0.0 yielded 15.0 mg of rHb0.0/L of cell culture. Following SEC, both α-cpβ and rHb0.0 were purified to ≥95% homogeneity as judged by SDS−PAGE (see Supporting Information). Reversed-phase HPLC analysis of purified α-cpβ and rHb0.0 shows the same ratio of heme to globin protein, and the UV−vis spectra of both proteins in the oxygen-bound state are essentially identical (Figure 1). These results suggest that heme binding to α-cpβ is not significantly perturbed.

The apparent molecular masses of the purified proteins were estimated by analytical HPSEC. When compared to rHb0.0, the oxygenated form of α-cpβ shows a predominant species with a retention time that corresponds to a dimer (23.2 min) and another species that elutes as a tetramer (20.5 min; gray trace in Figure 2). When the experiment was repeated in the presence

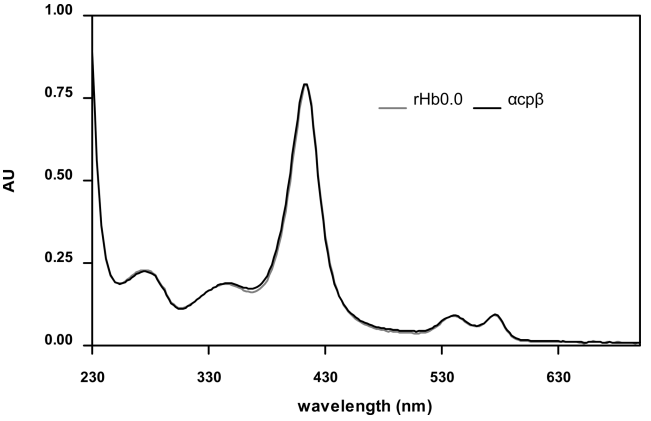
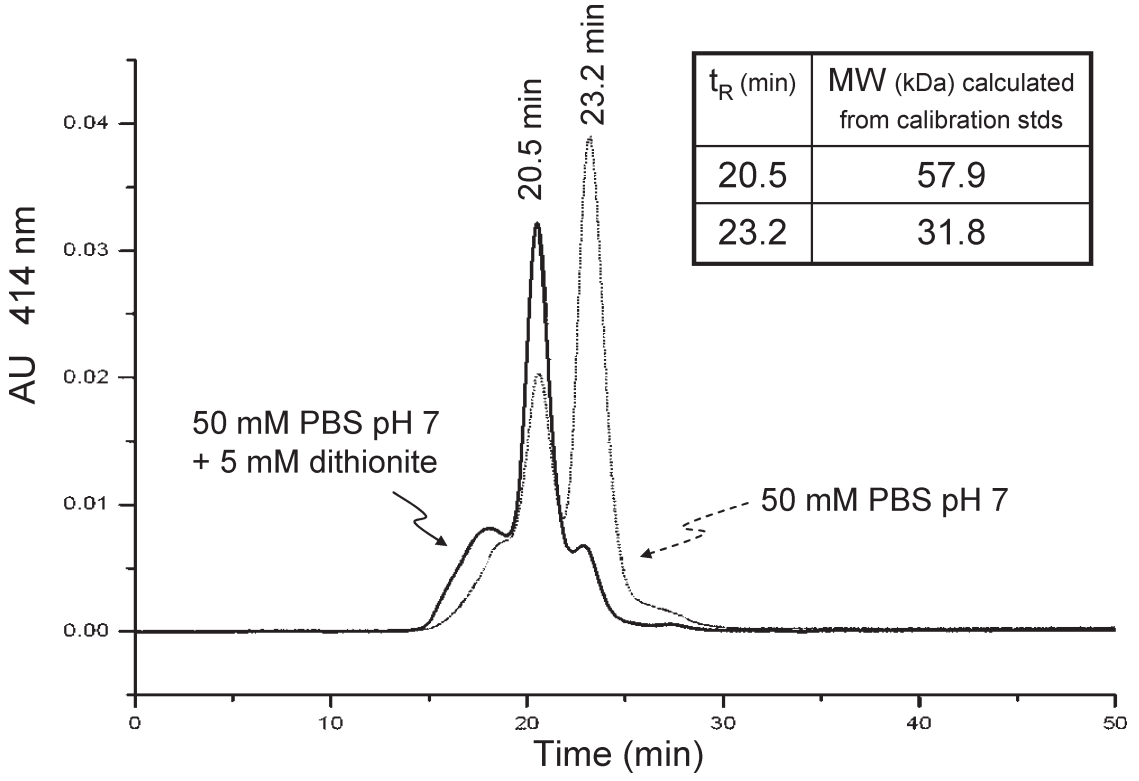


FIGURE 1: UV−visible spectra of HPSEC-purified α-cpβ (black line) and rHb0.0 (gray line) recorded in 10 mM sodium phosphate and 150 mM NaCl, pH 7.0.



t_R (min)	MW (kDa) calculated from calibration stds
20.5	57.9
23.2	31.8

FIGURE 2: HPSEC of oxy and deoxy α-cpβ. Samples of oxy α-cpβ (160 μM in heme) were subjected to analytical HPSEC using mobile phases that were either equilibrated with air (gray trace) or deoxygenated by sparging with N_2 and addition of 5 mM sodium dithionite (black trace). Inset: Apparent molecular masses (kDa) calculated from calibration standards.

of 0.5 M $MgCl_2$, which causes wild-type hemoglobin to dissociate into $\beta\alpha$ dimers (*49*), the later eluting peak was confirmed to correspond to a dimer (data not shown). Under these conditions, both α-cpβ and rHb0.0 elute as a single peak when coinjected. The ratio of the putative $\alpha_1(cp\beta)_1$ dimer to $\alpha_2(cp\beta)_2$ tetramer was shown to be dependent on the concentration of the α-cpβ injected onto the HPSEC column, with tetramer accounting for roughly 15% to 25% of the total when heme concentration varied from 20 to 160 M, respectively (data not shown).

The black trace in Figure 2 shows that α-cpβ elutes predominantly as a tetramer when applied to an HPSEC column equilibrated in deoxygenated buffer. Both chromatograms in Figure 2 show evidence for higher molecular mass species that appear to diminish in concentration as the sample becomes more oxygenated. These data suggest that α-cpβ can form a stable tetramer under deoxy conditions; but, the dimer is favored when oxygen is present.

The protein-containing peaks from analytical HPSEC were also characterized by ESIMS. When the mass spectra of the rHb0.0 and α-cpβ samples are compared (Figure 3), the same peaks corresponding to wild-type α-globin can be seen in each spectrum. In contrast, the peaks corresponding to wild-type β-globin differ from those corresponding to cpβ-globin, reflecting the 657 amu mass difference between the two subunits. The observed molecular masses agree closely with the expected molecular masses in all cases (Table 1). Reversed-phase HPLC analysis shows similar peak areas for both globin chains in α-cpβ (data not shown), suggesting the expected 1:1 ratio of subunits in the heterodimer.

Ligand Binding Studies. Full photolysis of CO and O_2 from both HbA and α-cpβ was used to estimate k'_T, the pseudo-first-order rate constant for ligand recombination to the low-affinity T conformation, and partial photolysis was used to measure k'_R, the pseudo-first-order rate constant for ligand recombination to the high-affinity R state, which is defined experimentally as the last step in ligand binding (i.e., Hb_4X_3 + X) (*47*). Data for full and partial photolysis of CO or O_2 from HbA and α-cpβ are shown in Figure 4. In both full-photolysis experiments ligand binding to α-cpβ is dominated by a fast phase, whereas for native HbA the slower phase has the greatest amplitude. The dominance of the fast phase for α-cpβ is almost certainly due to the presence, prior to photolysis, of a large fraction of liganded dimers, which do not associate rapidly enough after photolysis to form T state tetramers before ligand rebinding occurs. In contrast, native HbCO is mostly a tetramer at the concentrations used in the laser photolysis experiments (~100 μM), and the switch from R to T occurs very rapidly; thus, the majority of the CO molecules rebind to slowly reacting T state tetramers (*47*). The association rate constants for O_2 binding are roughly 10 times larger than those for CO binding regardless of quaternary state and mutation. As a result, the amplitude of the slow phase for O_2 rebinding to native HbA after 100% photolysis is smaller than that observed for CO rebinding because the tetramers have less time for the R to T switch before the ligand starts to rebind (*47*). In all cases, the rates of the fast and slow phases depend on ligand concentration, and this dependence can be used to calculate bimolecular association rate constants for the R (fast phases) and T (slow phases) states (Tables 2 and 3).

The values of k'_R calculated for the fast phases for O_2 (~50 μM^{-1} s^{-1}) and CO (~5.0 μM^{-1} s^{-1}) rebinding to HbA and α-cpβ are not significantly different (see Tables 2 and 3), suggesting that both proteins have similar R state active sites and iron reactivities. The values obtained from partial photolysis experiments (Figure 4B,D) support this conclusion and verify the

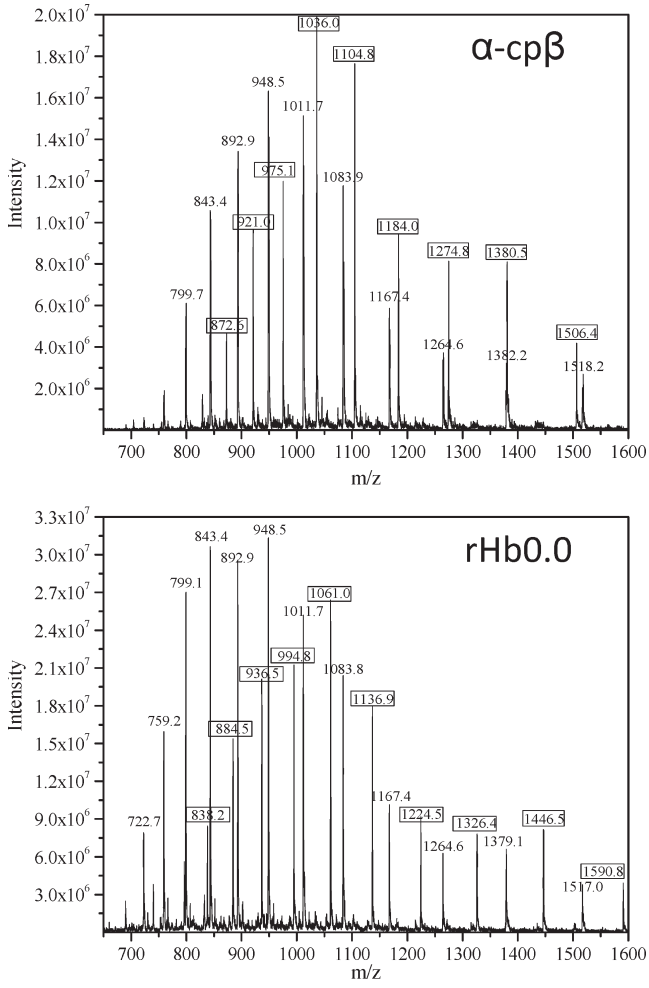


FIGURE 3: ESIMS mass spectra of HPSEC-purified α-cpβ (7.5 nmol; top) and rHb0.0 (11.6 nmol; bottom). In both spectra the peaks for the β-globin are labeled with boxes. The spectra were taken in 50% (v/v) acetonitrile in water containing 0.1% (v/v) formic acid.

Table 1: Observed Molecular Masses for Wild-Type α- and β-Globin and cpβ-Globin Calculated from the ESIMS Spectra of rHb0.0 and α-cpβ

	molecular mass (Da)	
	expected	observed
wild-type α-globin	15159.42	15161.19 (α-cpβ), 15166.83 (rHb0.0)
wild-type β-globin	15900.28	15902.55
cpβ-globin	16556.94	16559.10

apparent rate constant obtained from the full photolysis experiment. In these analyses we have not attempted to resolve the differences between the α and β subunits, which have been shown to react somewhat differently in both the R and T states (*50*). These differences in wild-type Hb and native HbA are small (less than 2-fold) and difficult to measure without creating mutant or metal hybrid Hb tetramers. The key results shown in Figure 4 are that the partial photolysis time courses are almost identical for the native and mutant proteins, indicating that the permutation does not markedly affect the R state ligand binding properties of the cpβ subunit. Otherwise, biphasic time courses would have been observed for the mutant.

The lack of effect of circular permutation on the R state active site of the β subunits was confirmed by measuring the rate of O_2 dissociation from α-cpβ. Time courses for replacement of O_2 by

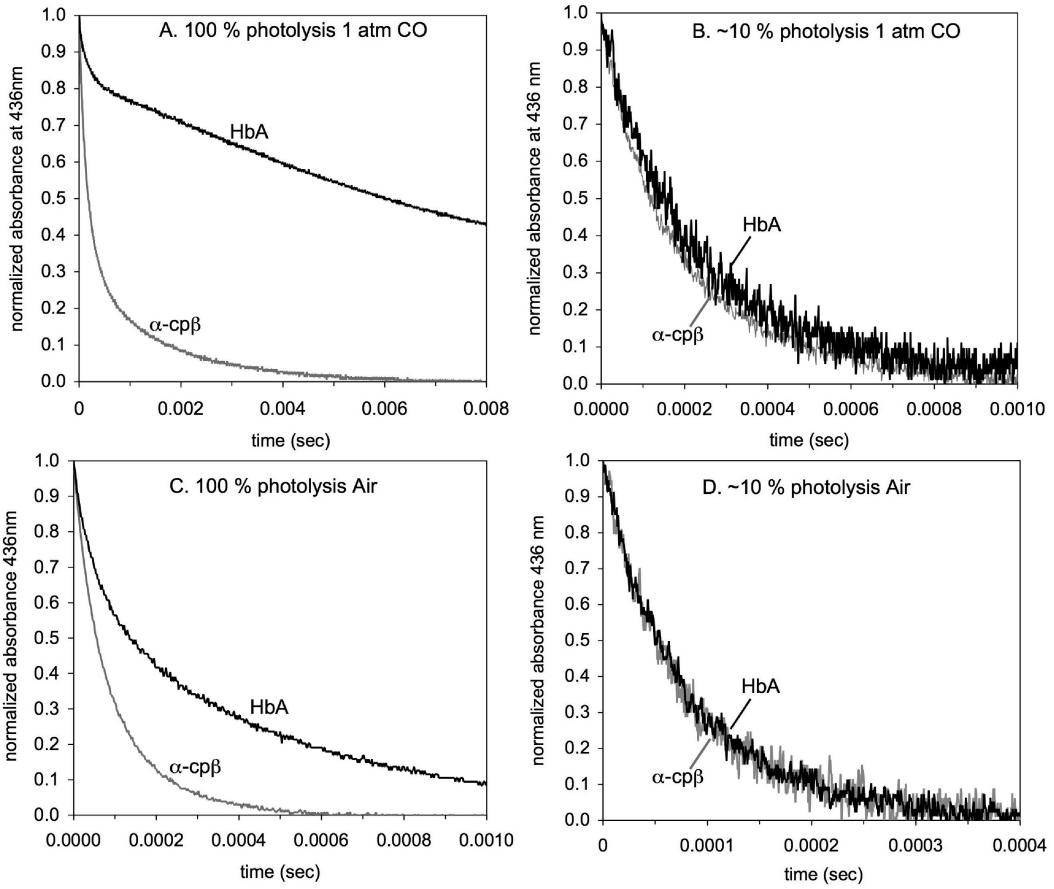


FIGURE 4: Time courses for full photolysis of CO from HbA and α-cpβ (panel A) and partial photolysis of CO (panel B) from HbA and α-cpβ (4% photolysis for HbA and 12% photolysis for α-cpβ). Experiments were conducted in CO-saturated 0.1 M potassium phosphate and 1 mM EDTA, pH 7.0 at 20 °C, in the presence of sodium dithionite. The concentrations of heme in the HbA and α-cpβ samples were 58 and 48 μM, respectively. Time courses for full photolysis (panel C) and partial photolysis (11% photolysis for both; panel D) of O_2 from HbA and α-cpβ. Experiments were conducted in air-equilibrated 0.1 M potassium phosphate and 1 mM EDTA, pH 7.0 at 20 °C. The concentrations of heme in the HbA and α-cpβ samples were 73 and 56 μM, respectively.

Table 2: Second-Order Rate Constants for the Faster and Slower Phases of CO Binding to HbA and α-cpβ^a

CO binding[b]	$k'_{T(CO)}$ (μM^{-1} s^{-1})	$k'_{R(CO)}$ (μM^{-1} s^{-1})
HbA[c]	0.12	6.0
HbA 100% photolysis	0.090 (85)	8.3 (15)
HbA 4% photolysis		4.7
deoxy HbA + 23 μM CO	0.22	
α-cpβ 100% photolysis	0.72 (34)	6.6 (66)
α-cpβ 12% photolysis		5.2
deoxy α-cpβ + 23 μM CO	0.85	

[a] The percentages of fast R state versus slow T state rebinding for 100% photolysis are given in parentheses. [b] The values were calculated from full and partial photolysis data as well as stopped-flow data. [c] From Olson et al. (*47*).

CO are shown in Figure 5A and were obtained by mixing HbO_2 samples with solutions containing high concentrations of CO (*47*). The observed replacement rate is given by

$$r_{obs} = \frac{k_{R(O_2)} k'_{R(CO)}[CO]}{k'_{R(CO)}[CO] + k'_{R(O_2)}[O_2]} \quad (1)$$

The R state O_2 dissociation rate constants for native HbA and α-cpβ were calculated from eq 1, using the measured values of r_{obs} obtained from fitting the replacement time courses to a single exponential expression and independently determined values of

Table 3: Rate Constants for O_2 Binding to HbA and α-cpβ and for O_2 Dissociation from HbA and α-cpβ^a

O_2 binding[b]	$k'_{T(O_2)}$ (μM^{-1} s^{-1})	$k'_{R(O_2)}$ (μM^{-1} s^{-1})	$k_{R(O_2)}$ (s^{-1})	$K_{R(O_2)}$ (μM^{-1})
HbA[c]	5−10	66	20	3.2
HbA 100% photolysis	7.1 (60)	60 (40)	28	2.1
HbA 11% photolysis		43	28	1.5
α-cpβ 100% photolysis	19 (31)	61 (69)	21	2.9
α-cpβ 11% photolysis		46	21	2.2

[a] The percentages of fast R state versus slow T state rebinding for 100% photolysis are given in parentheses. [b] Values for $k'_{T(O_2)}$, $k'_{R*(O_2)}$, and $k'_{R(O_2)}$ were calculated from the observed pseudo-first-order rate constants generated from laser flash photolysis. Values for $k_{R(O_2)}$ were calculated using the observed first-order rate constants for replacement of O_2 with CO as well as the values for $k'_{R(CO)}$ and $k'_{R(O_2)}$ calculated from full and partial photolysis. Values for the equilibrium constant for O_2 binding, $K_{R(O_2)}$, were calculated from $k_{R(O_2)}$ and $k'_{R(O_2)}$. [c] From Olson et al. (*47*).

$k'_{R(CO)}$ and $k'_{R(O_2)}$ obtained from the laser photolysis experiments shown in Figure 4. The computed values of $k_{R(O_2)}$ were 24−29 s^{-1} for HbA and 20−22 s^{-1} for α-cpβ and are almost identical (Table 3).

Previous work with metal-substituted, chemically modified, or mutant hybrid hemoglobins has shown that the rate constants for O_2 dissociation from R state α and β subunits in native HbA are ~15 and ~30 s^{-1}, respectively (*50*). However, these differences are difficult to define in the native and wild-type proteins,

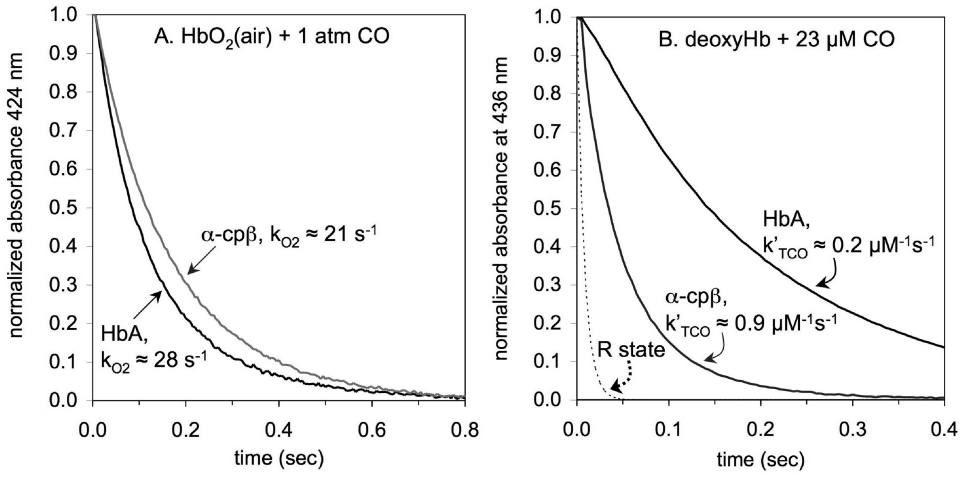


FIGURE 5: Normalized time courses for stopped-flow measurement of O_2 displacement by CO (panel A) and CO binding to deoxyHbA and deoxy α-cpβ (panel B). Samples were prepared in 0.1 M potassium phosphate buffer and 1 mM EDTA, pH 7.0, and data were collected at 20 °C. Air-equilibrated samples were rapidly mixed with buffer equilibrated with 100% CO and containing sodium dithionite to a final concentration of 464 μM CO, 131 μM O_2, and 3.5 μM heme for the HbA sample and 2.5 μM heme for the α-cpβ sample. Deoxy samples containing sodium dithionite were mixed with 5% (v/v) CO in N_2 to a final concentration of 23.2 μM CO and 3.5 μM heme for HbA and 2.5 μM heme for α-cpβ.

and the observed time courses appear monophasic and are often analyzed as simple exponential processes (*47*). As shown in Figure 5A, the time course for O_2 dissociation from α-cpβ is also monophasic and only a little slower than that for dissociation from native HbA. Thus, if anything, the circular permutation causes the rate of O_2 dissociation to be somewhat slower from the mutant β subunit, but the effect is minimal (Table 3).

In contrast to the R state ligand binding parameters, there are significant differences between the values of k'_T for the slow phases of ligand rebinding to HbA and α-cpβ after complete laser photolysis. For both O_2 and CO binding, the values of k'_T are 3−8-fold higher for α-cpβ than for HbA, indicating that the iron atom in deoxygenated α-cpβ is much more reactive than it is in deoxygenated HbA. This observation was confirmed in the stopped-flow experiments in which an equilibrium deoxyHb sample was mixed with low concentrations of CO and the reaction followed at 430 nm. As shown in Figure 5B, the time course of CO binding to native deoxyHbA shows acceleration at the beginning of the reaction and an apparent overall bimolecular rate constant equal to ~0.2 μM^{-1} s^{-1} (Table 2). In contrast, CO binding to deoxy α-cpβ is a simple exponential process with a single rapid rate corresponding to $k'_{T(CO)} \approx 0.9$ $\mu M^{-1}s^{-1}$. We looked for but could not find a fast R state phase in these experiments by mixing the deoxy sample with very low concentrations of CO, and no absorbance change appeared to be lost in the "dead time" of the apparatus. The observed bimolecular rate constant for CO binding is significantly lower than that for binding to the R state where $k'_{R(CO)} \approx 5\,\mu M^{-1}$ s^{-1} for both native HbA and α-cpβ (dashed line in Figure 5B).

The 5-fold smaller association rate constant for CO observed in the rapid mixing experiments compared to that measured after partial photolysis suggests strongly that deoxygenated α-cpβ forms a T state-like tetramer, but one with higher iron reactivity. Thus, circular permutation either decreases the allosteric isomerization constant for the R to T transition in the deoxy state (i.e., the *L*-value) or markedly alters the T state to a conformation with more reactive iron atoms. The lack of biphasic character to CO binding in the rapid mixing experiments suggests that there are no large active site changes in the T state β subunits, but instead the genetic modifications are causing a decrease in stability of

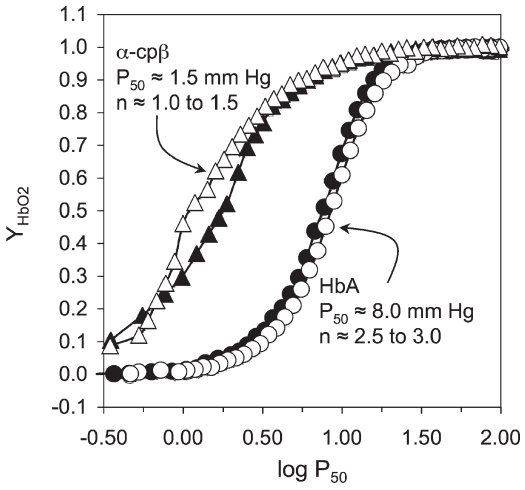


FIGURE 6: Fractional saturation with oxygen vs log of oxygen partial pressure (mmHg) for HbA (circles) and α-cpβ (triangles). Samples were prepared in 0.1 M potassium phosphate buffer and 1 mM EDTA, pH 7.0, and data were collected at 20 °C. Samples were either 10 μM (solid symbols) or 30 μM (open symbols) in heme.

the T quaternary state, which enhances the amount of faster reacting R state conformations.

The conclusion that α-cpβ adopts a higher affinity conformation compared to HbA is supported strongly by the equilibrium oxygen binding curves shown in Figure 6. At both 10 and 30 μM α-cpβ shows a 5−8-fold lower P_{50} and much less cooperativity than native HbA. This increase in O_2 affinity and loss of cooperativity correlates with the increase in the rate of CO and O_2 binding to the fully deoxygenated mutant and with the large amount of oxygenated α-cpβ dimers in concentrated solutions.

DISCUSSION

The strategy outlined in Scheme 1 for generation of a single-chain Hb requires, as a first proof of concept, that the proposed circular permutation of human β-globin yields a functional globin subunit. Precedent for this idea comes from the work of Sligar and co-workers, who reported the circular permutation of a di-α-globin sequence to yield a functional Hb (*36*).

Our laboratory has shown previously that relocating the termini in swMb to the G-H loop by circular permutation does not appear to significantly alter the structure of the heme binding pocket or its ligand binding properties (*37*). The results reported here show that oxygenated cpβ-globin forms a stable heterodimer and a tetramer when deoxygenated. The spectral properties of the deoxy, oxy, and carbon monoxide forms of the mutant are identical to those of native and wild-type HbA. This mutant Hb also reversibly and stably binds CO and O_2 with rates in the liganded state that are similar to those observed for R state HbA. These results demonstrate unambiguously that cpβ-globin can fold into a fully functional conformation.

Attempts to express cpβ-globin by itself did not yield any soluble protein. This result is probably a consequence of reduced thermodynamic stability of the cpβ apoglobin compared to wild-type β apoglobin. It has been shown for swMb and HbA that expression yields are correlated to thermodynamic stability (*51*, *52*). Also, the circular permutant of swMb mentioned above yields a protein that is 5 kcal/mol less stable and expresses poorly compared to the wild-type swMb (*37*). Although β subunits can be directly expressed and will associate to form stable β_4 homotetramers (*53*), the yields are much lower than those observed for Hb production when α and β genes are expressed simultaneously. It is likely that circular permutation of the β-globin destabilizes the subunit even further, resulting in enhanced degradation of cpβ chains that are not associated with an α subunit in the bacterial cell.

As hoped, coexpression of cpβ-globin with α-globin did yield the desired holohemoglobin with the α subunit serving as an effective stabilizing partner for the cpβ-globin. This strategy, along with a decreased induction temperature, allowed production of α-cpβ. However, the yield was lower than that for recombinant wild-type α- and β-globins (rHb0.0) expressed under similar conditions, a result which is consistent with the hypothesis that the cpβ-globin is less stable than its wild-type counterpart.

In contrast to oxygenated rHb0.0, which forms $\alpha_2\beta_2$ heterotetramers in solution at concentrations $\geq 10\ \mu$M, liganded holo-α-cpβ isolated from bacterial cells appears to be primarily an $\alpha_1(\text{cp}\beta)_1$ heterodimer at relatively high heme concentrations. Although tetramer formation is impaired, the interactions between the α- and cpβ-globins in the dimer are robust. The oxy α-cpβ protein elutes from HPSEC with retention times consistent with those predicted for an $\alpha_1(\text{cp}\beta)_1$ dimer and an $\alpha_2(\text{cp}\beta)_2$ tetramer, and reversed-phase HPLC and ESIMS analyses of these column fractions confirm the presence of both globin chains (Figure 3, Table 1).

Under deoxygenated conditions where no ligand is bound, α-cpβ forms a tetramer (Figure 2). Higher molecular mass species also form; however, they appear to dissociate to lower molecular mass species in the presence of oxygen. Such higher molecular mass oligomers may form as the result of "domain swapping" (*54*), in which the H helix of one cpβ subunit substitutes for the H helix of another cpβ subunit in a second tetramer. It is noteworthy that the unbound state is more susceptible to the formation of larger oligomers. Perhaps the close juxtaposition of the linker sequences of the two cpβ subunits in the putative α-cpβ tetramer leads to steric destabilization of the packing of the cpβ H helices and perturbation of intramolecular packing interactions. As a result, "domain-swapping " interactions with homologous subunits in other tetramers might become possible; however, this idea is speculative.

Although we currently have no direct structural evidence that association of the α- and cpβ-globins is along the same interface found in the $\alpha_1\beta_1$ dimers of HbA, this assumption seems reasonable because the ligand binding function of α-cpβ appears to be intact, with rate constants similar to those for native $\alpha_1\beta_1$ dimers and tetramers in the R state (Tables 2 and 3). The MWC model for cooperative ligand binding (*55*) and the Perutz mechanism (*56*) suggest that an $\alpha_1\beta_1$ dimer should not exhibit cooperative ligand binding. The tetramer is proposed to be the minimum unit of cooperativity because the shifts in the $\alpha_1-\beta_2$ and $\alpha_2-\beta_1$ interfaces are primarily responsible for the concerted transition from the T to the R state upon ligand binding.

Addition of 0.9 M $MgCl_2$ (*57*), raising the pH to above 10.6 (*58*), or mutagenesis of β37 Trp to Glu (*59*) all disrupt the $\alpha_1-\beta_2$ interface, and ligand binding to the resultant $\alpha_1\beta_1$ dimers is very rapid, noncooperative, and high affinity (i.e., equivalent to the R quaternary state). Our results show that ligand binding to the α-cpβ dimer exhibits almost identical properties (Figure 6). Sanders et al. (*36*) reported that a recombinant hemoglobin derived from a circularly permuted di-α-chain associated with two wild-type β-globin subunits has an increased O_2 affinity (P_{50} = 0.9 mmHg) and decreased cooperativity (n = 2.0) compared to HbA. The higher cooperativity of this permuted di-α-β_2 hemoglobin, compared to α-cpβ is likely achieved as a result of stable quaternary contacts between the four globin subunits, which are not present in a dimer. However, it should be noted that CO binding to the equilibrium deoxygenated form of α-cpβ does show a single moderately slow phase, indicating formation of a T-like, low-affinity quaternary structure. The HPSEC data shown in Figure 2 provide good evidence that this lower affinity state is an unliganded tetramer.

It is not surprising that tetramer stability is reduced for the α-cpβ construct. In the deoxy conformation of HbA the α-carboxylate of βHis146 forms a stabilizing salt bridge with the ε-amino group of αLys40 (*56*). The βHis146 carboxylate is converted to an amide bond as part of the linker that connects the H and A helices in the cpβ subunit. The loss of this salt bridge would be expected to reduce the stability of the quaternary structure of the $\alpha_2(\text{cp}\beta)_2$ tetramer. The properties of α-cpβ are quite similar to Hb lacking βHis146 ("desβHis146 Hb"), which displays on and off rates for O_2 binding that are similar to those for R state HbA (*60*). DesβHis146 deoxyHb and the naturally occurring mutant deoxyHb Hiroshima (βH146D) also bind CO significantly faster than deoxy HbA (*60*) at rates very similar to that of α-cpβ (see panel B of Figure 5). Treatment of HbA with carboxypeptidase A removes the βTyr145 and βHis146 ("HbCPA"). HbCPA displays completely noncooperative O_2 binding with ultrahigh affinity (P_{50} = 0.42 Torr) (*61*). Given the similarities in the functional properties between α-cpβ and these mutant or biochemically modified Hb variants, particularly desHis Hb, it is reasonable to assume that the loss of the βHis146 α-carboxylate in α-cpβ is responsible for most of the observations presented in the current work: (1) the predominance of dimers over tetramers in solution, (2) loss of cooperativity, and (3) ligand binding properties consistent with higher affinity conformations.

The key observation in support of using circularly permuted β subunits to construct a single-chain Hb gene is that there is no significant perturbation of ligand binding to the dimeric and R state forms of α-cpβ. The values of $k'_{\text{R(CO)}}$, $k'_{\text{R}(\text{O}_2)}$, $k_{\text{R}(\text{O}_2)}$, and $K_{\text{R}(\text{O}_2)}$ for α-cpβ are remarkably similar to those for native HbA (Tables 2 and 3). Thus, permutation of the β-globin does not

alter the distal pocket and reactivity of the iron atom in the R state. This conclusion is consistent with results reported for the circular permutation of swMb, in which relocating the termini to the G-H loop did not significantly alter ligand binding or heme-pocket structure (*37*).

Hemoglobins with covalently linked di-α-chains (*10*), circularly permuted di-α-chains (*36*), and covalently linked di-α- and di-*β*-chains (*35*) have been described. However, a single Hb gene containing two α and two *β* domains has not been investigated as a possible HBOC. Our strategy for generating a single-chain Hb requires the incorporation of a circularly permuted *β* subunit. This subunit has new termini positioned such that they can be inserted into the G-H loop of α-globin. The results in Figures 1−6 demonstrate that a circularly permuted *β*-globin can associate with α-globin and form an oligomer that can bind O_2 rapidly, reversibly, and stably. The expression and characterization of this circularly permuted *β*-globin is a critical first step in the generation of a single-chain Hb; however, these results also show that there are design issues that must be addressed. Foremost among these is enhancing the extent of tetramer formation in the liganded state. The linker sequence in the cp*β*-globin is likely responsible for hindering tetramer formation; thus, it will need to be redesigned. The current linker was designed to be (1) sufficiently long to span the distance between the wild-type termini and (2) sufficiently flexible to adopt whatever conformation may be required for interaction with other subunits. Computational methods (*62*) could prove useful for the design of an optimized linker sequence, and coexpression of the cp*β*-globin with the di-α-chain described by Looker et al. (*10*) may also promote the desired heterotetramer formation. It is also possible that mutagenesis could be used to increase P_{50} and strengthen the tetramer as has been shown for the Hb Providence mutation, *β*K82D (*52*, *63*).

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SUPPORTING INFORMATION AVAILABLE

Experimental methods for cloning of the genes for tandem repeat of *β*-globin, cp*β*-globin, and the coexpression vector pALA2, amino acid sequence of cp*β*-globin, and SDS−PAGE of purified proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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