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# Interfacial and Distal Pocket Mutations Exhibit Additive Effects on the Structure and Function of Hemoglobin<sup>†</sup>

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## **Abstract**

Protein engineering strategies seek to develop a hemoglobin-based oxygen carrier with optimized functional properties, including (i) an appropriate  $O_2$  affinity, (ii) high cooperativity, (iii) limited NO reactivity, and (iv) a diminished rate of autooxidation. The mutations  $\alpha L29F$ ,  $\alpha L29W$ ,  $\alpha V96W$  and  $\beta N108K$  individually impart some of these traits and in combinations produce hemoglobin molecules with interesting ligand-binding and allosteric properties. Studies of the ligand-binding properties and solution structures of single and multiple mutants have been performed. The aromatic side-chains placed in the distal-heme pocket environment affect the intrinsic ligand-binding properties of the mutated subunit itself, beyond what can be explained by allostery, and these changes are accompanied by local structural perturbations. In contrast, hemoglobins with mutations in the  $\alpha_1\beta_1$  and  $\alpha_1\beta_2$  interfaces display functional properties of both "R"- and "T"-state tetramers because the equilibrium between them is altered. These mutations are accompanied by global structural perturbations, suggesting an indirect, allostery-driven cause for their effects. Combinations of the distalheme pocket and interfacial mutations exhibit additive effects in both structural and functional properties, contribute to our understanding of allostery, and advance protein-engineering methods for manipulating the  $O_2$  binding properties of the hemoglobin molecule.

Human hemoglobin (Hb) serves as a classical model of allostery in proteins, and its study has contributed greatly to understanding the relationship between structure and function in biological molecules. The two-state model of allostery in proteins described by Monod, Wyman and Changeux was based, in part, on structural and functional studies in Hb (1). According to this two-state model, cooperative  $O_2$  binding results from a conversion of Hb between high affinity, "R"- and low affinity "T"-states, and allosteric control operates by changing the equilibrium between these two states, measured as the equilibrium constant, L. Comparison of x-ray crystal structures of Hb allowed Perutz to assign R- and T-states to quaternary structures of the Hb tetramer and establish a stereochemical description of allostery that has been widely used in explaining and understanding cooperative  $O_2$  binding by Hb (2).

A large amount of work suggests that a simple two-state mechanism does not fully account for cooperativity and allostery in  $O_2$  binding. Structural studies have detected conformations of Hb distinct from those originally noted, including R2 (3), RR2 and R3 (4) conformations. NMR studies suggest that the solution structure of HbCO A is a dynamic intermediate between R

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and R2 conformations (5), with the R structure representing an intermediate form between T and R2 structures (6), consistent with the pathway proposed by Srinivasan and Rose (7). Alternate structures may fit with a two-state model, but only if the structural ensemble sorts into only two functional states. The low-affinity T-state form of Hb appears to comprise at least two different forms with differing affinities (8,9). Trapping these states in a sol-gel has allowed detection of functional behaviors intermediate between classic R- and T-state traits (10). Numerous high-quality x-ray crystal structures have been reported for deoxy-Hb, but none appear to match the solution structure, which could possibly represent rapidly interconverting species (11). To account for inadequacies of the original two-state model, several new models of allostery in hemoglobin have been proposed including the global allostery (12,13), molecular code (14,15) and tertiary-two state (16) models.

Understanding of allostery in Hb can be advanced by the study of mutant recombinant Hbs (rHbs) possessing interesting new structural or functional properties. Such rHbs are being produced in a rational protein engineering approach to develop a hemoglobin-based oxygen carrier (HBOC). Promising rHbs should possess: (i) appropriate  $O_2$  affinity, in order to facilitate  $O_2$  delivery in the absence of the intracellular allosteric effector, 2,3-bisphosphoglycerate (2,3-BPG), (ii) resistance to auto-oxidation, in order to increase the functional half-life of the product and inhibit the production of radical oxygen species, and (iii) limited reactivity with nitric oxide (NO) to diminish the 'hypertensive side effect' (17). Hbs containing  $\alpha$ L29F,  $\alpha$ L29W,  $\alpha$ V96W, and  $\beta$ N108K substitutions possess many of these desirable qualities.

Hb Presbyterian, containing the  $\beta N108K$  substitution, was first found in three generations of the same family (18). Incorporation of this mutation into rHb conferred: (i) a pronounced chloride effect, (ii) an enhanced Bohr effect, (iii) lowered  $O_2$  affinity, and (iv) the capacity to switch to the low-affinity T-state conformation without changing ligation state (19). Acharya and coworkers found that the unusual T-state like character of the liganded form of transgenic (Tg) Hb ( $\beta 3N108K$ ) is: (i) detectable as an increase in geminate yield of carbon monoxide; (ii) enhanced by the allosteric effector phosphate; and (iii) manifested at both 'hinge' and 'switch' regions of the  $\alpha_1\beta_2$  interface, while the structure of the deoxygenated form is unperturbed compared to Hb A (20). Properties of Hb Presbyterian, including decreased  $O_2$  affinity and increased Bohr effect facilitate  $O_2$  delivery and led the research group at Somatogen to use the  $\beta N108K$  mutation in their first-generation rHb-based blood substitute product, rHb1.1 (21, 22). This group believed, as do we, that low affinity ( $P_{50}$  between 20 and 30 Torr) enhances  $O_2$  delivery, in both *in vitro* and *in vivo* (23,24). In contrast to this view is the belief that higher  $O_2$  affinity is required in a HBOC in order to prevent premature  $O_2$  delivery and overcompensation in auto-regulation of capillary flow (25).

The  $\alpha$ V96W substitution within the  $\alpha_1\beta_2$  interface that was created and characterized by our laboratory was found to increase  $P_{50}$  (26). Unlike many mutations within the  $\alpha_1\beta_2$  interface that disrupt H-bond or salt-bridge interactions and result in high  $O_2$  affinity and low cooperativity, the  $\alpha$ V96W substitution lowers  $O_2$  affinity while maintaining high cooperativity. We have found that the CO bound form could adopt the T-type quaternary structure without changing ligation state upon addition of the allosteric effector inositol hexaphosphate (IHP) and/or a decrease in temperature (26). X-ray crystal structures have shown that this Hb creates a water-mediated H-bond network between  $\alpha$ 96W and  $\beta$ 101E that appears to stabilize the T-state conformation, and that the R-state structure contains a  $\beta$ 101E- $\beta$ 104R interaction, which is normally seen only in T-state structures (27).

The lowered affinity and high cooperativity of  $\beta N108K$  and  $\alpha V96W$  substitutions have prompted our laboratory to explore the effect of the double mutation on the structure and function of Hb (19). We have found that rHb ( $\alpha V96W/\beta N108K$ ) has a greater tendency to

switch to the T-state conformation without changing ligation state than either single mutant, and that the double mutant has a lower  $O_2$  affinity than either single mutant. This combination of mutations appears to promote low  $O_2$  affinity by stabilizing the T-state of Hb while destabilizing or altering the high-affinity R-state, creating a promising prototype for a blood substitute molecule with a high  $P_{50}$  value. To advance this approach, we added the B10 mutation,  $\alpha L29F$ , to the double mutant (28). The L29F substitution in both myoglobin (Mb) and Hb was found previously to decrease the rates of both auto-oxidation and NO dioxygenation (29–32). However, this mutation markedly increases  $O_2$  affinity in both Mb and Hb to levels that would preclude  $O_2$  delivery in a normal capillary which has  $P_{O2}$  of ~30 to 50 Torr (30,33–35). Thus, we have combined the  $\alpha L29F$  mutation with the allosteric mutants to produce a rHb ( $\alpha L29F/\alpha V96W/\beta N108K$ ) triple mutant with lower affinity than Hb A and lower auto-oxidation rates than rHb ( $\alpha V96W/\beta N108K$ ) (28).

Here, we present results of flash photolysis, equilibrium binding,  $^1H\text{-NMR}$ , and rapid-mixing studies on Hbs containing  $\alpha L29F$ ,  $\alpha L29W$ ,  $\alpha V96W$ ,  $\beta N108K$ ,  $\alpha V96W/\beta N108K$ ,  $\alpha L29F/$   $\alpha V96W/\beta N108K$ , and  $\alpha L29W/\alpha V96W/\beta N108K$  mutations. The distal pocket mutations at the B10 helical position alter the intrinsic ligand binding properties of the  $\alpha$ -subunits, whereas the interface mutants affect the allosteric equilibrium, and both types of effects are additive in the multiple mutants.

### EXPERIMENTAL PROCEDURES

# **Hemoglobin Solutions**

Recombinant hemoglobins were designed, expressed and purified according to previously described procedures (36,37). The plasmids co-express methionine aminopeptidase (Met-AP), which removes the N-terminal methionine necessary for bacterial expression and results in production of authentic Hb. An oxidation-reduction step for the rHb molecule is included in the purification procedure in order to convert incorrectly oriented heme groups to the correct orientation (for details, see refs 36,37).

# **Experimental Conditions**

All experiments were performed in 0.1 M sodium phosphate buffer at pH 7.0 and 20 °C, with the exception of the geminate rebinding studies which were performed at 22 °C. Hb concentrations are reported on per heme or subunit basis.

### **Kinetic Measurements**

Association and dissociation rate constants were determined as described previously for Hbs (38–40). Flash-photolysis experiments monitored ligand rebinding as a change in the absorbance at 436 nm following photo-dissociation of ~10% of the bound ligands. Buffers equilibrated with 1 atm  $O_2$  (1.25 mM  $O_2$ ) or 1 atm CO (1.0 mM CO) ensured pseudo-first order conditions for the rebinding reaction to 0.1 mM Hb (heme basis). Partial photolysis was employed to produce a comparatively large population of tri-liganded molecules so that absorbance changes would reflect the last step of the ligand binding (i.e.,  $Hb_4X_3 + X \rightarrow Hb_4X_4$ ). Time courses were fit to a two-exponential expression, and the resulting observed rates divided by the ligand concentration to yield association rate constants. Dependence of the observed rates on ligand concentration was confirmed by experiments performed in buffers containing 0.25 mM  $O_2$  or 0.10mM CO.

Rate constants for CO dissociation from the rHbs were measured by rapid mixing techniques. rHbs in solutions containing 0.1 mM CO were mixed with buffer containing 2.0 mM (equilibrated with 1 atm) nitric oxide (NO). Free NO was present in excess and has an association rate constant approximately 10-fold greater than  $k'_{co}$  for Hb, allowing the CO-

rebinding reaction to be ignored. The CO dissociation rate constants were determined for  $\alpha$ -and  $\beta$ -subunits directly from the time courses by fitting with a two-exponential expression.

Rate constants for  $O_2$  dissociation were also measured by rapid mixing techniques. In this case, 0.1 mM (heme basis) Hb in buffer equilibrated with air was mixed with buffer solutions equilibrated with 1-atm  $N_2$ , 1-atm CO, and air using a SFM-400 stopped flow (Biologic, France). Proportions of mixing were manipulated to record a number of time courses over a range of [CO]/[O<sub>2</sub>] values. The time courses were fit to two-exponential expressions to measure fast and slow phases. Because the  $O_2$  rebinding reaction cannot be ignored, the rate constant,  $k_{RO2}$ , was obtained by fitting the dependence of the observed pseudo first order replacement rate constants,  $r_{obs}$ , on [CO]/[O<sub>2</sub>] to the following expression:

$$r_{\text{obs}} = \frac{k_{RO_2} \frac{[\text{CO}]}{[O_2]}}{\frac{[\text{CO}]}{[O_2]} + \frac{k'_{RO_2}}{k'_{RCO}}} \tag{1}$$

The relatively low final Hb concentration of 5 to 10  $\mu$ M results in some dimer formation and an over-estimation of R-state character for the low affinity mutants since the ability to switch to the T-state relies on an intact  $\alpha_1\beta_2$  interface.

# **Equilibrium Oxygen Binding Curves**

Oxygen-dissociation curves were measured using a Hemox Analyzer (TCS Medical Products, Huntington Valley, PA), as described previously (36,37) with modifications. Hb concentration was 60  $\mu$ M and 0.3  $\mu$ M catalase and superoxide dismutase were included to limit oxidation. Oxygen affinity, measured as P<sub>50</sub>, and cooperativity, measured as the Hill coefficient (n<sub>50</sub>), were determined from the resulting oxygen equilibrium curves (OECs) with an accuracy estimated at  $\pm$  10% based on reproducibility of measurements with Hb A.

### **NMR Spectroscopy**

 $^{1}$ H-NMR spectra were measured for Hbs equilibrated under 1-atm CO gas using a 300-MHz Bruker Avance DRX-300 NMR spectrometer. Hb concentration was 4 mM (heme basis), dissolved in 0.1 M sodium phosphate pH 7.0 with 5%  $D_{2}O$  added. The water signal was suppressed by using a jump-and-return pulse sequence (41). Proton chemical shifts are referenced to the methyl proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) indirectly by using the water signal, which occurs 4.85 ppm downfield from DSS at 20  $^{\circ}$ C, as the internal reference.

## **RESULTS**

## **Measurement of Association Rates**

Representative time courses for CO and  $O_2$  rebinding following partial flash-photolysis are shown in Figure 1. In these experiments, ~10% of the bound ligand was photo-dissociated. As a result, the last step of ligand binding (i.e.,  $Hb_4X_3 + X \rightarrow Hb_4X_4$ , where X is the ligand  $O_2$  or CO) contributes virtually all of the recorded absorbance change. This measurement is model-independent. Considered within the context of the two-state MWC model, the last step of ligand binding normally represents association with the high-affinity R-quaternary state of the Hb tetramer (1). Fitting using two exponentials allows calculation of bimolecular ligand association rate constants  $k'_{R\alpha}$  and  $k'_{R\beta}$  for  $\alpha$ - and  $\beta$ -chains, respectively (24). However, the set of mutant rHbs being examined contain substitutions which may promote low affinity T-state character in Hb, even in the fully saturated state, and thus, mixtures of  $k'_R$  and  $k'_T$  values

can occur. This possibility is considered in assigning apparent rate constants for the faster and slower phases ( $k_f$  and  $k_s$ , respectively) of ligand rebinding.

Placement of Phe or Trp at position B10 of the α-subunit produced dramatic decreases in the rate of the slow phase of O<sub>2</sub> binding in all four mutants containing these substitutions. The scale of these reductions is greater than that seen in rMb (L29W), which produces a 70-fold decrease in k'<sub>O2</sub> compared to wild-type, and matches the large decreases in k'<sub>O2</sub> seen previously in rHb ( $\alpha$ V1 M/ $\beta$ V1M/ $\alpha$ L29F) and rHb ( $\alpha$ V1M/ $\beta$ V1M/ $\alpha$ L29W) (42,43). The amplitude of the markedly slower phase accounts for roughly 50% of the total observed reaction in each of the mutants containing the single distal mutation. The relatively unperturbed faster phase, stoichiometric amplitude distribution, and agreement with previous results for distal-heme pocket mutations of Hb and Mb lead to straightforward assignments of the slow and fast phases to  $\alpha$ - and  $\beta$ -subunits of the Hb, respectively. Rate constants for ligand association were calculated by fitting time courses to two-exponential functions and are reported in Table 1, together with values previously reported for Hb A (44-46). Values of k'<sub>RaO2</sub> measured in all four samples containing aromatic side-chains substituted into the distal binding pocket are ~10to 100-fold smaller than the reported values of  $k'_{T\alpha O2}$ , indicating that the effect cannot be explained by allostery alone. The  $\alpha$ L29F and  $\alpha$ L29W mutations directly alter the intrinsic ligand-binding properties of the  $\alpha$ -subunit, and that effect is clearly conserved in the multiple mutants.

Similar experiments were performed using CO as the ligand, as illustrated in Figure 1B. Again, the time courses are markedly biphasic and exhibit a slow phase rate constant, which is 10- to 300-fold lower than the reported T-state values, making the assignment of the mutant B10  $\alpha$ -subunit phase straightforward. This shows that the distal-heme pocket substitutions interfere with ligand association in a ligandindependent fashion. Calculated rate constants are presented in Table 1. Interestingly, the rate constants for the faster phase of CO rebinding in the rHb ( $\alpha$ L29F/ $\alpha$ V96W/ $\beta$ N108K) and rHb ( $\alpha$ L29W/ $\alpha$ V96W/ $\beta$ N108K) triple mutants match those reported for  $k'_{T\beta CO}$  rather than  $k'_{R\beta CO}$  suggesting that even with only 10% photolysis, the Hb tetramers are still in the T or low affinity quaternary state. This pattern differs from that seen with the same rHbs using  $O_2$  as ligand, or that seen in the single rHb ( $\alpha$ L29F) and rHb ( $\alpha$ L29W) mutants that do appear to switch to the R-state when three ligands have been bound. Thus, the T-quaternary conformation may occur for the Hb4(CO)3 species during the partial photolysis experiments.

To test this idea, the time courses following full photolysis have been analyzed. In the case of Hb A, full photolysis allows a significant fraction of the unliganded tetramers to switch quaternary conformations during the dye laser pulse, leading to biphasic rebinding time courses with slower apparent rate constants due to the presence of a large fraction of tetramers in the T-state quaternary form. In the case of the triple mutants, the fast and slow rate constants measured for CO binding after full photolysis are nearly identical to the rate constants determined after 10% photolysis. The full photolysis values are 0.003 and 0.11  $\mu M^{-1}s^{-1}$  for rHb (αL29F/αV96W/βN108K) and 0.0002 and 0.03  $\mu M^{-1} s^{-1}$  for rHb (αL29W/αV96W/ βN108K), and can be compared to the partial photolysis values reported in Table 1. The lack of dependence of the measured rate constants on the degree of photolysis and the excellent agreement between the faster phase and reported values of k'TBCO demonstrate that these triple mutant Hbs exhibit functional properties of the T quaternary state, even with three ligands bound [Hb<sub>4</sub>(CO)<sub>3</sub>]. Such T-state behavior was not seen in the single  $\alpha$ L29 mutants, showing that the combined αV96W and βN108K substitutions cause retention of the T-state in triliganded intermediates. The appearance of the T-state functional properties after partial photolysis of the two triple mutants arises from a quaternary switch to the low affinity form in the newly produced Hb<sub>4</sub>(CO)<sub>3</sub> intermediate, which has time to occur due to ultra slow CO rebinding to the B10 mutants.

The βN108K single mutant exhibits a fast O<sub>2</sub> rebinding phase after partial photolysis, with a rate constant of  $54 \,\mu\text{M}^{-1}\text{s}^{-1}$ , and a slower phase with a rate constant of  $4.3 \,\mu\text{M}^{-1}\text{s}^{-1}$ . Assignments based on these time courses alone are not straightforward. The apparent rate constant of the faster phase is similar to the average of the two R-state subunit association rate constants. The apparent rate constant for the slower phase, 4.3 µM<sup>-1</sup>s<sup>-1</sup>, is similar to that for O<sub>2</sub> binding to the T-state subunits and suggests that this mutation by itself can slow down the rate of the last step in ligand binding by creating a population of Hb<sub>4</sub>(O<sub>2</sub>)<sub>3</sub> tetramers in the Tquaternary conformation. The amplitudes of the fast and slow phases of rebinding support this interpretation. Following partial photolysis of native HbCO A, the fast and slow phases of bimolecular rebinding contribute roughly equally to the total absorbance change. These phases arise from reactions of the  $\alpha$ - and  $\beta$ -subunits, which are similar in both: (i) spectral changes between liganded and unliganded states and (ii) geminate recombination yield. The slower phase of the βN108K mutant rebinding time course comprises only ~25% of the total signal, indicating that the phases are not due to roughly equal contributions of  $\alpha$ - and  $\beta$ -subunits as was seen in Hb A, rHb (αL29F), and rHb (αL29W). Instead, this slow phase probably reflects the appearance of T-state character in the  $Hb_4(O_2)_3$  species.

The T-state of Hb A has very low ( $\leq$  1%) CO rebinding geminate yield and a high overall quantum yield ( $\sim$ 1.0) for complete photo-dissociation (47). Thus, the presence of any T-state conformations will be amplified in partial photolysis experiments since the quantum yield for the R-state hemoglobin is roughly 0.7. Thus, in our view, the partial photolysis results for rHb ( $\beta$ N108K) indicate the presence of a major population of tetramers rebinding at the average value of  $k'_{RO2}$  (i.e., at R-state tetramer rates), mixed with a smaller fraction of molecules reacting at slow T-state like rate constants. This interpretation is also supported by full photolysis experiments in which 100% of the  $O_2$  is photo-dissociated and the two phases of rebinding occur at average R- and T-state rates. For rHb ( $\beta$ N108K),  $k_{slow}$  and  $k_{fast}$  following full photolysis were measured as 6.0 and 45  $\mu$ M $^{-1}$ s $^{-1}$ , respectively. These values agree well with rate constants of 4.3 and 54  $\mu$ M $^{-1}$ s $^{-1}$  measured in the partial photolysis experiments. Both full and partial photolysis results agree well with previously reported average rate constants of approximately 6 and 60  $\mu$ M $^{-1}$ s $^{-1}$  for the T- and R-state  $O_2$  association to Hb A, respectively, as shown in Table 1.

Rebinding of CO to rHb ( $\beta$ N108K) produces fast and slow phases with apparent rate constants of 3.2  $\mu$ M<sup>-1</sup>s<sup>-1</sup> and 0.34  $\mu$ M<sup>-1</sup>s<sup>-1</sup>. As with O<sub>2</sub> association, the rate constants for these two phases are similar to reported values of the last (R-state like) and first (T-state like) steps of CO binding, respectively. The fraction of fast and slow phases of the total absorbance change were 0.23 and 0.77, respectively, in rHb ( $\beta$ N108K), and not equal as is observed in Hb A. Thus, the effects of mutation on rebinding are consistent for O<sub>2</sub> and CO. Apparent rate constants for fast and slow phases presented in Table 1 reflect the R- and T-state behavior and not different subunits.

Similar behavior was observed for the  $\alpha V96W$  mutant.  $O_2$  rebinding time courses exhibit a large amplitude phase, with an apparent rate constant that matches the average of R-state  $\alpha$ - and  $\beta$ -subunit rate constants, and a smaller amplitude slow phase reflecting T-state rebinding. Least-squares fitting of partial photolysis  $O_2$  rebinding traces produced an average slow phase rate constant of  $10~\mu M^{-1} s^{-1}$ . The full photolysis time courses provided apparent rate constants of  $10~and~60~\mu M^{-1} s^{-1}$ . As was also seen with rHb ( $\beta N108K$ ), the major difference between full and partial photolysis time-courses is an increase in the relative amplitude of the slow phase with increasing photolysis. CO rebinding to rHb ( $\alpha V96W$ ) shows time courses similar to those for the  $O_2$  rebinding, including a slow T-state like phase with a small amplitude. After full photolysis, the two rate constants for CO binding to rHb ( $\alpha V96W$ ) were  $0.15~and~5.9~\mu M^{-1} s^{-1}$  in excellent agreement with those for the T- and R-state of Hb A.

 $O_2$  rebinding to rHb ( $\alpha$ V96W/ $\beta$ N108K) also proceeds in two phases with rate constants of 43 and 4.1  $\mu$ M<sup>-1</sup>s<sup>-1</sup>. These values are nearly identical to those measured for rHb ( $\beta$ N108K), and the same R vs. T trend is observed for k' $_{CO}$ . The effects of the  $\alpha$ V96W and  $\beta$ N108K mutations are additive on the amplitude of the slower phase. The slow phases for bimolecular  $O_2$  rebinding to rHb ( $\alpha$ V96W), rHb ( $\beta$ N108K) and rHb ( $\alpha$ V96W/ $\beta$ N108K) accounted for 10, 20 and 36 % of the observed absorbance change, respectively, after partial photolysis, with the double mutant, as expected showing the greatest extent of T-state behavior.

#### **Measurement of Dissociation Rates**

Dissociation rate constants were determined by observing displacement of ligands in rapid-mixing mixing experiments. For the  $O_2$  dissociation reaction, replacement of  $O_2$  from HbO<sub>2</sub> by CO was monitored after mixing at a variety of ratios of free ligands. The time courses were fitted to a two-exponential function and the dependence of observed rates  $(r_{obs})$  on ligand ratio was used to determine  $k_{O2}$  (Figure 2). Assignment of the resultant rate constants was straightforward for each sample since at least one phase matched rate constants previously reported for the  $\alpha$ - and  $\beta$ -subunits, and in these experiments the starting liganded state is  $Hb_4(O_2)_4$ , which even in the case of the  $\alpha_1\beta_2$  interface mutants normally exhibits R-state like properties.

Placement of Phe at the B10 position within the distal-heme pocket produces dramatic 70- and 40-fold decreases in  $k_{O2}$  for  $\alpha$ -subunits within tetrameric rHb ( $\alpha L29F$ ) and rHb ( $\alpha L29F/\alpha V96W/\beta N108K)$ , respectively, compared to  $\alpha$ -subunits within Hb A. This finding parallels the effect seen in Mb, where the L29F mutation caused a dramatic ~10-fold reduction in  $k_{O2}$  compared to the wild-type value (30). The effect in Mb was explained as due to electrostatic stabilization of bound  $O_2$  by the positive edge of the phenyl-ring multipole. This phenomenon appears to be conserved in  $\alpha$ -subunits, where the L29F substitution reduces  $k_{O2}$  more dramatically than in Mb. Placement of Trp at position 29 of  $\alpha$ -subunits within tetrameric rHb ( $\alpha L29W$ ) and rHb ( $\alpha L29W/\alpha V96W/\beta N108K$ ) lowered  $k_{O2}$  in  $\alpha$ -subunits to a much lesser extent, 10- and 5-fold, respectively, than the  $\alpha L29F$  mutation. Again, similar effects of the Leu (B10)Trp mutation are seen for single mutants of Mb and Hb A (24,42,48). The faster phase for  $O_2$  dissociation from each of the four samples containing aromatic substitutions within the  $\alpha$ -subunit distal-heme pocket matched the rate constant measured for the native  $\beta$ -subunit, reflecting R-state type function.

The slower phase of the ligand dissociation reaction for rHb ( $\alpha$ V96W), rHb ( $\beta$ N108K), and rHb ( $\alpha$ V96W/ $\beta$ N108K) (14, 15, and 16 s<sup>-1</sup>, respectively), matches the values of 12 and 16 s<sup>-1</sup> previously reported for the native  $\alpha$ -subunit of Hb A, leading to straightforward assignments. The amplitudes of the slow and fast phases are roughly equal for Hb A and rHb ( $\alpha$ V96W). However, the replacement reactions in rHb ( $\beta$ N108K) and rHb ( $\alpha$ V96W/ $\beta$ N108K) have a larger, ~65%, fast phase and ~3-fold larger rate constants, indicating that the  $\beta$ N108K interface mutation acts to facilitate O<sub>2</sub> dissociation, either by increasing the intrinsic rate of O<sub>2</sub> dissociation from the  $\beta$ -subunits and/or by conferring the T-state behavior to the fully oxygenated tetramer. The distal-pocket mutations suppress this effect, suggesting that the elevation in k<sub>O2</sub> is not due to an intrinsic change to the  $\beta$ -subunit itself, but instead is due to increased T-state character imparted by  $\beta$ N108K mutation.

Similar rapid-mixing experiments were employed to measure the replacement of bound CO through competition with NO. In this case, the high relative concentration of NO, the high association rate constants of NO, and low dissociation rate constants for NO allow direct observation of  $k_{\rm CO}$  (49). Time courses were fitted to a two-exponential function using equal amplitudes, and the apparent rate constants uniformly match those of Hb A. This indicates that the effect of the  $\beta N108K$  substitution on ligand dissociation, unlike its effect on association, is ligand-dependent.

# Measurement of Geminate Rebinding

After rapid photolysis of bound ligand from the heme group, a portion of the dissociated ligands rebind internally to the iron atom to which it was originally bound (i.e., a geminate pair). The rate and amplitude of this ultrafast, nanosecond geminate rebinding process is dependent on the ability of the surrounding protein structure to capture the ligand in apolar cavities or to eject it into the solvent phase by steric hindrance (50). Normalized CO and  $O_2$  geminate rebinding traces are presented in Figure 3 and show clear differences in the fraction geminate rebinding ( $F_{gem}$ ). The overall trend for  $F_{gem}$  is rHb ( $\alpha$ L29F/ $\alpha$ V96W/ $\beta$ N108K)  $\approx$  rHb ( $\alpha$ L29W/ $\alpha$ V96W/ $\beta$ N108K) < rHb ( $\alpha$ V96W/ $\beta$ N108K)

The alteration in  $F_{gem}$  for rHb ( $\alpha$ V96W), rHb ( $\beta$ N108K) and rHb ( $\alpha$ V96W/ $\beta$ N108K) reflects a decrease in iron reactivity and iron-ligand-bond formation by proximal restraints of in plane movement of the iron atom as result of these substitutions at the subunit interfaces. The effects of the two interface mutations are roughly additive in the double mutant. Dramatic decreases in geminate rebinding have been observed in rHb ( $\alpha$ V1M/ $\beta$ V1M/ $\alpha$ L29F) and rHb ( $\alpha$ V1M/ $\beta$ V1M/ $\alpha$ L29W) (42), and it was expected that these substitutions in the distal-heme binding pocket would cause the same effect in the triple mutants. However, the observed reductions in CO geminate recombination to < 10% is more severe than was found in the single  $\alpha$ L29F and  $\alpha$ L29W mutants and reflects the decrease in iron reactivity due to the greater T-state character caused by the interface mutants. The allosteric transition in Hb has a pronounced effect on the CO geminate reaction reducing the population of rebinding from ~40% in the R-quaternary structure to < 1 % for the T-state (47).

# Measurement of Oxygen Equilibrium Curves

The partial pressure of  $O_2$  at 50% saturation ( $P_{50}$ ) and the slope of the Hill plot at 50% saturation, the Hill coefficient ( $n_{50}$ ), are reported in Table 2. These empirical parameters are independent of any allosteric model and provide insight to the effects of mutations on  $O_2$  equilibrium binding parameters.

OECs were measured in 0.1 M sodium phosphate buffer at pH 7.0 at 20 °C, which matches conditions of the kinetic experiments, but differs in temperature, pH or both from previously reported binding curves for some of these rHbs. Wiltrout and coworkers reported P<sub>50</sub> values of 15, 10.9, and 40 Torr for Hb A, rHb ( $\alpha$ L29F) and rHb ( $\alpha$ L29W) at pH values of 7.0, 6.8 and 7.13, respectively at 29 °C (35). Their results match the trend seen in Table 2 and indicate a roughly uniform ~2-fold change in P<sub>50</sub> due to the 9 °C difference between experimental conditions. Tsai and Ho report  $P_{50}$  values of 8.0, 12.8, 24.5 and 48.8 for Hb A, rHb ( $\alpha$ V96W), rHb ( $\beta$ N108K) and rHb ( $\alpha$ V96W/ $\beta$ N108K), respectively, at pH 7.4 and 29 °C (51). This trend of decreasing O<sub>2</sub> affinity is reproduced at pH 7.0 and 20 °C. The effects of pH and temperature on P<sub>50</sub> appear to compensate each other in these studies, leading to exact matches for the P<sub>50</sub> values of Hb A and rHb ( $\alpha$ V96W) and to close agreement for the rHb ( $\beta$ N108K). The exact compensation between the effects of the pH and temperature is again seen when comparing our results to those of Jeong and coworkers (28). Their P<sub>50</sub> values of 8.0, 4.0, 38 and 22 Torr for Hb A, rHb (αL29F), rHb (αV96W/βN108K) and rHb (αL29F/αV96W/βN108K), respectively, measured at pH 7.4 and 29 °C exactly match our results which were recorded at pH 7.0 and 20  $^{\circ}$ C.

The equilibrium  $O_2$  binding curve for rHb ( $\alpha$ L29W/ $\alpha$ V96W/ $\beta$ N108K) is difficult to analyze due to: (i) its very low affinity and (ii) its tendency to oxidize during the course of the experiment. Oxidation was estimated by spectral analysis to be < 5% for the other Hbs, but was as high as 9% for rHb ( $\beta$ L29W/ $\beta$ V96W/ $\beta$ N108K).

A representative equilibrium oxygen-binding dataset for rHb ( $\alpha L29W/\alpha V96W/\beta N108K$ ) is shown in Figure 4B along with a binding curve for Hb A collected on the same day. The upper asymptote for the rHb ( $\alpha L29W/\alpha V96W/\beta N108K$ ) curve is undefined and the lower asymptote deviates from that of Hb A. The discrepancy between Hb A and the mutant Hb at low  $O_2$  tensions could be due to oxidation. To account for this oxidation, an asymptotic value for the absorbance of the deoxygenated species was used, which is the average of the deoxy-Hb A lower limit and the lowest mutant deoxy-Hb absorbance value. The upper asymptotic value for the absorbance of fully oxygenated Hb A was used for the mutant protein. These scaling procedures allow estimation of the  $P_{50}$  of rHb ( $\alpha L29W/\alpha V96W/\beta N108K$ ) as  $120 \pm 10$  Torr in three trials. In addition to altering the absorbance values, oxidation is expected to increase the apparent  $O_2$  affinity of the Hb, suggesting that the  $O_2$  affinity of rHb ( $\alpha L29W/\alpha V96W/\beta N108K$ ) may be even lower. Regardless of the exact  $P_{50}$  determination, it is clear that these three mutations combine to create a recombinant Hb with dramatically reduced  $O_2$  affinity.

Oxygen affinity is critical in the rational design of a HBOC. The single-mutant Hbs affect  $O_2$  affinity either directly, by interfering with ligand binding at the iron atom, or indirectly by altering allostery in the tetramer. The current studies of the combined effects of mutations, together with previous reports reveal a relatively simple dependence of  $P_{50}$  in the multiple mutant on the  $P_{50}$  of each single mutant according to the relationship:

predicted 
$$P_{50\text{mutations}1+2+3...} = P_{50\text{HbA}} * \frac{P_{50\text{mutation}1}}{P_{50\text{HbA}}} * \frac{P_{50\text{mutation}2}}{P_{50\text{HbA}}} * \frac{P_{50\text{mutation}3}}{P_{50\text{HbA}}} ...$$
 (2)

The effect of each single mutation is expressed as the quotient of the  $P_{50}$  value of that single mutant rHb,  $P_{50\text{mutation}}$ , divided by the  $P_{50}$  value of Hb A in the same conditions. The product of these proportionality factors and of the  $P_{50}$  of Hb A, yields the predicted  $P_{50}$  value of a multiply-mutated hemoglobin containing each of the single mutations, predicted  $P_{50\text{mutations}1+2+3...}$  A plot of the predicted versus measured  $P_{50}$  of double- and triple-mutant Hbs is shown in Figure 5. This simple linear relationship is expected to be an oversimplification, and there are relatively few rHbs for which both the single and multiple mutants have been measured. However, the correlation is striking and the prediction of the ultra-large  $P_{50}$  value of the rHb ( $\alpha$ L29W/ $\alpha$ V96W/ $\beta$ N108K) triple mutant is remarkably good. These multiple mutants contain substitutions at different regions of the protein, minimizing the potential for direct interference or overlapping effects of mutation. The agreement between the available data points and the line y = x suggests that this relationship may be useful, as a first approximation, for predicting  $P_{50}$  values of new multiple mutant Hbs based on the effects of the single mutations. Deviation from the predicted value would indicate a more complicated interaction between the single mutations.

### <sup>1</sup>H-NMR Spectra

<sup>1</sup>H-NMR spectra were collected for samples in the same buffer conditions as were used for kinetic and equilibrium studies, 0.1 M sodium phosphate buffer at pH 7.0 and 20 °C. Figure 6 shows regions of the HbCO spectra featuring exchangeable and ring-current shifted resonances with selected marker peaks labeled. The  $\alpha_1\beta_1$  interface was monitored by NεH resonances of  $\alpha_122$ His and  $\alpha_103$ His side-chains, which arise shifted 12.9 and 12.1 ppm from DDS, respectively (52). These two His side-chains span the  $\alpha_1\beta_1$  interface to contact β35Y and β131N, respectively and produce markers conserved in both liganded- and deoxy-spectra (19). The  $\alpha_1\beta_2$  interface was monitored by the characteristic R-quaternary state marker at 10.7 ppm. This marker arises from the NεH of the β37W side chain and moves from ~10.7 ppm in liganded Hb to ~11.0 ppm in deoxy Hb (53). The β37W side chain is located within the hinge region of the  $\alpha_1\beta_2$  interface, and forms a H-bond with  $\alpha_1\beta_2$  hotolysis, early in the R→T transition (54). Noble and coworkers systematically replaced over 20 interfacial

side chains with Ala and Gly and suggested that the  $\beta 37W$ - $\alpha 140Y$  interaction is the "keystone" of the network stabilizing the T-state conformation (55). The distal-pocket environment was tracked by the position of the ring-current shifted  $\alpha 62V$  and  $\beta 67V$  methyl resonances at -1.8 ppm (56). The distal valine side-chains approach within 4 Å of the bound  $O_2$  according to a recent x-ray crystal structure determination (pdb accession code 2DN1), and is a sensitive probe of the distal-pocket region comprising the  $O_2$  binding site (57). A resonance at -1.1 ppm arises from the  $\beta 141L$  residue (58). This side-chain is located in the proximal portion of the heme pocket,  $\sim 4.5$  Å from the proximal histidine, and adjacent to the organic phosphate binding site of the  $\beta \beta$  cleft. Marker proton resonances of Hb A are labeled in Figure 6.

Spectra of rHb ( $\alpha$ L29F) and rHb ( $\alpha$ L29W) are unchanged from HbCO A control for markers in the  $\alpha_1\beta_1$  and  $\alpha_1\beta_2$  interfaces, but show clear shifts in the resonance for  $\alpha$ 62V, indicating a perturbation to the structure of the  $\alpha$ -chain distal-heme pocket environment. These results indicate that the structural consequence of these mutations is local in nature, with the substituted side-chains altering the resonance of the nearby distal valine side-chain, without extending conformational changes to either  $\alpha_1\beta_1$  or  $\alpha_1\beta_2$  interface, or the  $\beta$ -subunit heme-pocket in the R-state quaternary structure. The large ring-current shift in the mutated  $\alpha$  (L29F) subunit suggests, unexpectedly, that the  $\alpha$  Phe(B10) side-chain causes the C $\gamma_2$  atom of Val62 to move toward the center of the porphyrin ring. Both Hbs containing the  $\alpha$ L29F substitution exhibit this upfield shift of for the  $\alpha$ 62V C $\gamma_2$  resonance. The opposite effect is observed in the Hbs containing  $\alpha$ L29W substitutions where the  $\alpha$ 62V C $\gamma_2$  resonance shows a downfield shift of roughly 0.5 ppm, indicating that the valine side-chain is pushed away from the center of the porphyrin ring.

These findings match the trend for aromatic substitutions at helical position B10 reported previously (28,35). Such a direct change to the ligand-binding site is not surprising considering the large sizes of the aromatic side chains and the proximity of the  $\alpha$ 29L position to both  $\alpha$ 62V and the bound ligand. The shifts in the resonance of the  $\alpha$ 62V marker in these single mutants are conserved in rHb ( $\alpha$ L29F/ $\alpha$ V96W/ $\beta$ N108K) and rHb ( $\alpha$ L29W/ $\alpha$ V96W/ $\beta$ N108K).

The  $\alpha$ V96W substitution causes three subtle changes in the  $^1H$ -NMR spectrum of the rHbCO. In the  $\alpha_1\beta_2$ -interface region, the resonance for the  $\beta$ 37W side-chain is shifted 0.1 ppm downfield, indicating an alteration to the local environment of the  $\beta$ 37W side-chain within the  $\alpha_1\beta_2$  interface. This change is accompanied by small shifts of the  $\beta$ 67V and  $\beta$ 141L resonances, indicating a change in the heme-pocket of the  $\beta$ -chain and showing that this mutation acts across the ( $\alpha_1\beta_2$  interface to alter the heme-pocket structure of the wild-type partner subunit. A small shift of the  $\alpha$ 62V marker resonance seems to indicate a perturbation of the  $\alpha$ -heme pocket environment, as well. Both markers in the  $\alpha_1\beta_1$  interface for rHb ( $\alpha$ V96W) are unchanged from those of wild-type Hb A.

We have reported a temperature- and allosteric-effector-dependent appearance of a resonance peak at ~14.2 ppm in the  $^1H$ -NMR spectrum of rHb ( $\alpha V96W$ ) (26). This peak originates from the H-bond between  $\alpha 42Y$  and  $\beta 99D$  in the  $\alpha_1\beta_2$  interface of the unliganded T-state molecule, and its emergence reflects a shift in the quaternary structure of the Hb molecule (59). The presence of both the 14-ppm T-state marker and the 10.7-ppm R-state marker indicates an intermediate quaternary state. We have further reported that IHP binding causes an increase in the strength of the T-state marker signal and loss of the R-state marker, indicating that rHb ( $\alpha V96W$ ) has a propensity to adopt the T-state quaternary structure even while fully saturated with CO (26). Thus, the structural perturbations noted in Figure 6 indicate a population, which is not uniformly in the normal R-state quaternary structure, but is tending towards the T-state structure.

The  $\beta N108K$  substitution clearly and dominantly perturbs the  $\alpha_1\beta_1$  interface, as seen in the  $\sim$ 0.25 ppm upfield shift of the  $\alpha103H$  marker. Figure 6 shows that this feature is conserved in each mutant containing the  $\beta N108K$  mutation. Acharya and coworkers found a similar perturbation in  $\alpha_1\beta_1$  interface of Tg-Hb Presbyterian, which is Hb containing the  $\beta N108K$  substitution, and expressed in a transgenic pig (20). Alteration of the  $\alpha103H$  marker has been reported for rHb ( $\beta N108Q$ ), rHb ( $\beta N108R$ ), and rHb ( $\beta N108E$ ) indicating a role for the naturally occurring Asn side chain in the structure of the normal R-state  $\alpha_1\beta_1$  dimers, and demonstrating the dominant nature of this mutation on the structure of the protein (51). In addition to the shift in the  $\alpha103H$  resonance, the  $\beta N108K$  substitution induces a change in the characteristic R-quaternary state marker at 10.7 ppm, which is associated with  $\beta37W$  in the  $\alpha_1\beta_2$  interface of HbCO A. In rHb ( $\beta N108K$ ), this peak appears to have shifted upfield, indicating a marked change in the environment of  $\beta37W$  in the  $\alpha_1\beta_2$  interface, implying communication between the two major dimer interfaces in the tetrameric Hb A.

Thirdly, there is a small, downfield shift of the  $\beta141L$  resonance in the ring-current shifted region for the  $\beta N108K$  substitution, indicating a structural perturbation in the vicinity of the proximal side of the heme group. This structural feature exactly matches that seen in the rHb ( $\alpha V96W$ ) spectrum for the  $\beta141L$  marker of the  $\beta$ -subunit proximal heme-pocket area. Like the  $\alpha V96W$  mutation, the  $\beta N108K$  substitution also increases the population of the T-state conformation at low temperatures and in the presence of the allosteric effector IHP, as indicated by the emergence of the resonance at 14.2 ppm (19,20). It appears that the structural changes resulting from the  $\beta N108K$  substitution shown in Figure 6 propagate from the site of the mutation in the  $\alpha_1\beta_1$  interface to both the  $\alpha_1\beta_2$  interface and heme-pocket regions of the liganded molecule, promoting conversion to the T-state structure. Again, this propagation is expected for an "allosteric" mutation.

The rHbs containing both distal-pocket and interface mutations reflect the cumulative structural effects of their constituent single mutations. The two  $\alpha_1\beta_1$ -interface markers of rHb ( $\alpha$ V96W/  $\beta$ N108K), rHb ( $\alpha$ L29F/ $\alpha$ V96W/ $\beta$ N108K), and rHb ( $\alpha$ L29W/ $\alpha$ V96W/ $\beta$ N108K) contain structural perturbations seen for the rHb ( $\beta$ N108K). In addition, small but distinct peaks are apparent at ~14 ppm in the spectra of rHb ( $\alpha$ V96W/ $\beta$ N108K) and rHb ( $\alpha$ L29F/ $\alpha$ V96W/ $\beta$ 108K) and at ~11.2 ppm in the spectra of rHb ( $\alpha$ L29F/ $\alpha$ V96W/ $\beta$ N108K) and rHb ( $\alpha$ L29W/ $\alpha$ V96W/  $\beta$ N108K). An additive effect of the  $\alpha$ V96W and  $\beta$ N108K substitutions on the strength of the resonance at 14.2 ppm was noted by Tsai and coworkers, and appears to underlie the appearance of that signal in our spectrum (19). The peak evident at 11.2 ppm arises from the  $\alpha$ 94D- $\beta$ 37W H-bond and, like the 14-ppm marker, is also in the region of a characteristic T-state quaternary structural marker. Emergence of these resonances indicates the presence of T-state character in the CO saturated tetramer of all three multiple mutants. This conclusion is supported by the waning of the characteristic R-state marker associated with β37W in the three multiple mutants, indicating that the ligand-bound forms of these multiple mutant molecules are not in a normal R-state conformation in 0.1 M phosphate buffer at pH 7.0 and 20 °C. The heme-pocket regions of these three proteins also shows the additive effects of the single mutations. The 0.1-ppm downfield shift of the  $\beta$ 141L resonance shared in the spectra of rHb ( $\alpha$ V96W) and rHb ( $\beta$ N108K) is conserved in all three multiple mutants. The pattern of migration of the  $\alpha$ 62V resonance in rHbs containing αL29F and αL29W substitutions is also conserved. The distal pocket marker resonance for αV62 Cγ<sub>2</sub> shifts upfield by ~0.25 ppm in both αL29F containing mutants, and downfield by 0.45 and 0.55 ppm for all the αL29W containing mutants.

## DISCUSSION

In general, the mutations  $\alpha$ L29F,  $\alpha$ L29W,  $\alpha$ V96W, and  $\beta$ N108K can be thought of as producing either direct, local structural and functional changes at the ligand-binding site, which alter the intrinsic properties of that subunit, or of producing indirect, global effects in the tetramer that

promote changes from the R- to T-like states. The simplest hypothesis is that the B10 mutations affect only the distal-pocket and that the  $\alpha_1\beta_1$ - and  $\alpha_1\beta_2$ -interface mutations affect only the equilibrium between the R- and T-quaternary states. The purpose of this work is to examine that hypothesis and verify whether these effects are additive in multiple mutants that could be potential thirdgeneration blood substitute prototypes.

### **Distal Pocket Substitutions**

Placement of Phe or Trp at position B10 of the α-subunit produced dramatic 40- and 330-fold decreases, respectively, in the rate constant for bimolecular  $O_2$  binding to  $\alpha$ -subunits after partial photolysis. In contrast, the rate constants for the wild-type  $\beta$ -subunits are, as expected, relatively unperturbed. These decreases in k'<sub>O2</sub> are similar to those observed for the same mutations in sperm whale Mb and are unlikely to be due to changes in the population of the R- and T-states given that T-state rate constants for Hb A are only ~ 5- to 15-fold smaller than those of the R-state. The Phe (B10) substitution also produces a marked decrease in k<sub>O2</sub>, but not k<sub>CO</sub>, indicating a specific electrostatic interaction of bound O<sub>2</sub> with the positive edges of the phenyl-ring multipole. Similar favorable interactions for the Phe (B10) mutation are seen in recombinant Mb (L29F) (30). In contrast to Phe (B10), the large size of the indole ring in the Trp (B10) mutant markedly hinders the binding of all ligands due to unfavorable steric interactions, and this effect dominates, causing marked decreases in O2 affinity for all Trp (B10) mutants, including both Hb subunits and all Mbs that have been investigated. Thus, there is a balance between favorable electrostatic and unfavorable steric effects for aromatic substitutions at the B10 position. The former results in higher O<sub>2</sub> affinity for the αL29F subunit, but the latter results in a much lower O<sub>2</sub> affinity for the αL29W subunit. Thus, O<sub>2</sub> affinity can be manipulated independently of the O2 association or NO dioxygenation rate constant, both of which decrease due to filling the distal pocket with the large aromatic side chains. The distalpocket structures of rHb (αL29F) and rHb (αL29W) are clearly perturbed, as observed by changes in the  $\alpha$ V62 C $\gamma_2$  resonance in the ring-current shifted region (Figure 6). Changes in the extent of the geminate recombination further indicate that these substitutions modify the structure of the active site. Thus, in both rHbs, the intrinsic O<sub>2</sub> affinity of the altered subunit is changed markedly, but there is no effect on the unmodified  $\beta$ -subunit or on the R-state quaternary structure of the tetramer. The ability to produce changes in the intrinsic affinity of different subunits has the potential to lower the cooperativity as measured by the Hill coefficient (n<sub>50</sub>). Significant subunit heterogeneity is expected to reduce the apparent cooperativity as measured by n<sub>50</sub> (60,61). NMR studies have detected subunit heterogeneity of Hb A for the first step of O<sub>2</sub> binding in the presence of organic phosphate (62). Table 2 shows that reductions in the  $n_{50}$  values are observed for rHbs containing  $\alpha$ L29F and  $\alpha$ L29W substitutions, as would be expected if the  $\alpha$ -subunit has much higher and much lower  $O_2$  affinity, respectively, than its partner β-subunit.

# **Interface Mutations**

The  $\beta N108K$  substitution in the  $\alpha_1\beta_1$  interface produces a small slow phase for both  $O_2$  and CO rebinding after partial photolysis (~25% of the total amplitude). The rate constant for this slow phase matches reported values for the first step of the ligand binding to the native Hb A tetramers, which are considered to be the T-state rate constants for  $O_2$  and CO binding. This lowered rate constant does not appear to be due to a change in the intrinsic reactivity of the mutated subunit, as was seen for the B10 mutants, but instead appears to be due to a shift toward the T-quaternary state. The relatively large distances, 15 and 21 Å, between the  $\alpha$ -carbon of the  $\beta N108K$  amino acid and the  $\beta$ - and  $\alpha$ -subunit iron atoms, respectively, seems to preclude a simple direct effect. The agreement between the values of the faster and slower apparent rate constants and those reported for the R- and T-state Hb A suggests that the partially liganded (Hb<sub>4</sub>X<sub>3</sub>) form of the mutant rHb is comprised of populations of the R- and T-type conformational states, which are not seen in the Hb A control.

Results of the  $^1\text{H-NMR}$  measurements and geminate recombination studies show that the  $\beta N108K$  substitution produces spectral changes indicative of a change in the population of the quaternary structures with only small alterations in the heme-pocket compared to the  $\alpha L29F$  and  $\alpha L29W$  mutations. Thus, as expected, the  $\beta N108K$   $\alpha_1\beta_1$  mutation acts indirectly through global structural changes in the tetramer, to produce less reactive T-like active sites for slower ligand association, more rapid dissociation, and less geminate rebinding. Supporting this interpretation is our NMR evidence that fully liganded rHb ( $\beta N108K$ ) and rHb ( $\alpha V96W$ ) convert to the T-state like structure in response to lowered temperatures and upon addition of the allosteric effector IHP(19,20,26).

The  $\alpha$ V96W mutation produces changes that are similar to, but less dramatic than those of the  $\beta$ N108K replacement. Bimolecular association of  $O_2$  with  $Hb_4(O_2)_3$  occurs in two phases, with the slower phase accounting for only 10% of the observed absorbance change, but at a rate similar to that expected for the T-state hemoglobin. This mutation shares with rHb ( $\beta$ N108K): (i) the ability to switch quaternary forms without changing ligation state; (ii) perturbation of the proximal heme-pocket marker at -1.2 ppm; and (iii) lowered  $O_2$  affinity. Unlike rHb ( $\beta$ N108K), rHb ( $\alpha$ V96W) shows no changes for the  $\alpha_1\beta_1$ -interface  $^1$ H-NMR markers. Thus, a perturbed  $\alpha_1\beta_1$  interface is not necessary for the ability to switch quaternary forms independent of the ligation state. However, the correlation between the shifts in the -1.2-ppm resonance raises the possibility that perturbation of the  $\beta$ 141L marker is linked to alterations at the  $\alpha_1\beta_1$  interface.

#### **Effects of Mutations on the Allosteric Transition**

As described above, both rHb ( $\beta$ N108K) and rHb ( $\alpha$ V96W) can convert to the T-state in fully liganded tetramers at lowered temperatures and upon addition of the allosteric effector IHP (19,20,26). The ability to switch quaternary conformation, even while fully saturated, is increased when these two mutations are combined in rHb ( $\alpha$ V96W/ $\beta$ N108K) (51). This propensity is reflected in the  $^1$ H-NMR spectra in Figure 6, which show increasing perturbation from the native R-state-like structure according to the trend:  $\alpha$ V96W/ $\beta$ N108K >  $\beta$ N108K >  $\alpha$ V96W. Table 2 shows that P<sub>50</sub> follows the same trend, indicating, as expected, that lowered O<sub>2</sub> affinity coincides with increased ability to access the T-state conformation. This trend is evident in the cooperativity of O<sub>2</sub> binding, measured as the Hill coefficient (n<sub>50</sub>). The value of n<sub>50</sub> diminishes as the molecule exhibits greater T-state character in the presence of saturating ligand concentrations. Also, in the partial photolysis ligand rebinding measurements, the increases in amplitude of the slow bimolecular T-state phases and the decreases in the extent of geminate rebinding for O<sub>2</sub> and CO follow the same trend.

The molecular code model developed by Ackers and coworkers describes  $O_2$  binding to Hb in terms of a series of intermediate steps involving intra-dimer cooperativity and a role for the  $\alpha_1\beta_1$  interface in influencing the overall  $O_2$  binding reaction (14,15). Our results for rHb ( $\beta N108K$ ) show that the perturbation of the  $\alpha_1\beta_1$  interface can affect  $O_2$  binding by acting upon the allosteric transition, supporting the idea of an important role of this interface in the overall reaction. In addition, the  $^1H$ -NMR spectra of all rHbs containing the  $\beta N108K$  substitution display a shift in the resonance at -1.2 ppm, indicating that the liganded structure of these mutant Hbs differs from the normal R-state structure of Hb A. However, the most important result is that the effects of the  $\alpha_1\beta_1$ - and  $\alpha_1$ - $\beta_2$ -interface mutants are additive, a result that is important for engineering ligand-binding properties in Hb-based blood substitutes.

#### **Blood Substitute Design**

The appropriate  $O_2$  affinity for an Hb-based  $O_2$  carrier (HBOC) remains controversial. Hb A serves as an  $O_2$  delivery agent, with its transport capabilities maximal at its  $P_{50}$ , which is ~28 Torr in blood. Normal capillaries have  $P_{O2}$  of ~20 to 30 Torr (33,34), supporting the view that

as free  $O_2$  diffuses from the capillary, it is quickly replenished from the store of HbO $_2$  within RBCs. A high-affinity rHb ( $P_{50} < 10$  Torr) will remain saturated at these normal  $P_{O2}$  values. Conversely, a low-affinity rHb, such as rHb ( $\alpha L29W/\alpha V96W/\beta N108K$ ) may not fully saturate in the lung and will start to desaturate at higher  $P_{02}$  values similar to those found in arterioles and large arteries, resulting in premature  $O_2$  delivery. Thus, for Hb in RBCs, a  $P_{50}$  of 20 to 30 Torr is optimal and most workers feel that the same logic applies to extracellular HBOCs. However, this view has been challenged (62). Extracellular HBOCs have no unstirred layers surrounding them, permeate the cell-free plasma layer lining the vessel walls of arteries and arterioles, and as result, deliver oxygen two to three times more efficiently on an iron basis than RBCs alone (23). To prevent premature and excessive  $O_2$  delivery by HBOCs, Cole and coworkers suggest that a  $P_{50} < 15$  Torr is optimal for HBOC (25). Otherwise the excess  $O_2$  delivery will cause overcompensating auto-regulatory responses that will cause vasoconstriction. These workers have argued that the hypertensive side effect of all first generation HBOCs is due to this problem of too much  $O_2$  delivery.

However, Doherty and coworkers examined the hypertensive side-effect of a large number of rHb mutants that had been well characterized with respect to  $O_2$  binding and NO scavenging, including *in-vitro* measures of the rate constants for NO dioxygenation. They showed a striking correlation between  $k'_{No.ox}$  (from 2 to  $70 \, \mu M^{-1} s^{-1}$ ) and change in mean arterial pressure ( $\Delta$ MAP) in 10% top-load, rat model. On the other hand, there was no correlation between  $P_{50}$  and  $\Delta$ MAP for a set of rHbs with  $P_{50}$  values ranging from 3 to 50 Torr (32,64). These findings strongly suggest that NO scavenging is the underlying cause of the hypertensive effect.

Our long-term goal is to limit the rate of NO dioxygenation through mutation of the distal-pocket with large amino acid side-chains, while adjusting  $P_{50}$  values to a range of 20 to 30 Torr for optimal  $O_2$  transport in a normal capillary. In previous work, Olson, Doyle, Lemon, and others have shown that replacement of Leu (B10) and Val (E11) with Phe and Trp can markedly reduce the rate of NO dioxygenation (29,32,64). In this work, we have shown that  $O_2$  affinity is profoundly altered by the Phe (B10) and Trp (B10) mutations in the  $\alpha$ -subunits, but through adjustments to  $\alpha_1\beta_1$  and  $\alpha_1\beta_2$  interfaces that alter the allosteric equilibrium, the  $P_{50}$  values of the distal-pocket mutant can be re-adjusted to more favorable values, particularly in the case of the  $\alpha$ L29F mutant.

Cooperativity in O<sub>2</sub> binding is decreased in the mutant rHbs in the order Hb A > rHb ( $\alpha$ L29F)  $\approx$  rHb ( $\alpha$ V96W)  $\approx$  rHb ( $\beta$ N108K) > rHb ( $\alpha$ L29W) > rHb ( $\alpha$ V96W/ $\beta$ 108K) > rHb ( $\alpha$ L29F/  $\alpha V96W/\beta N108K) > rHb$  ( $\alpha L29W/\alpha V96W/\beta N108K). There are two trends within this overall$ variation. First, in comparing the distal-pocket mutants rHb ( $\alpha$ L29F) and rHb ( $\alpha$ L29W), the rHb with the greater subunit difference in the O<sub>2</sub> affinity, rHb (αL29W), also exhibits the largest decrease in the n<sub>50</sub> value. In this mutant, there is an ordered addition of ligand, with O<sub>2</sub> binding first to T-state  $\beta$ -subunits and then to the R-state rHb  $\alpha(L29W)$  subunits that have roughly the same low affinity due to steric hindrance by the large indole ring adjacent to the bound ligand. Second, the rHbs showing the greatest manifestation of the T-state functional and structural traits, even when liganded, also exhibit low n<sub>50</sub> values because little switching to the highaffinity state occurs until after all four ligands have been bound. In principle, high cooperativity is desired in a HBOC in order to provide more efficient O<sub>2</sub> delivery over small changes in the P<sub>O2</sub> values. Our mutants show that the cooperativity is reduced by either markedly increasing or decreasing the affinity of one subunit versus the other causing ordered addition of O<sub>2</sub> with little change in the affinity even with a quaternary transition or by inhibiting the allosteric transition from low- to high-affinity quaternary structure.

In the triple mutant rHb ( $\alpha$ L29F/ $\alpha$ V96W/ $\beta$ N108K), inhibition of the T to R transition by the two interface mutations compensates for the intrinsic increase in  $O_2$  affinity caused by the  $\beta$ L29F substitution. The resultant triple mutant has a moderate overall affinity, low rates of

auto-oxidation, and presumably low rates of NO scavenging by the  $\alpha$ -subunit. Thus, it serves as a promising prototype HBOC molecule. In contrast, the triple mutant with the  $\alpha$ Trp (B10) replacement, rHb ( $\alpha$ L29W/ $\alpha$ V96W/ $\beta$ N108K), starts with an intrinsically low affinity of the  $\alpha$ -subunits and when combined with the interface mutations, results in a molecule that cannot be saturated in air. Perhaps the most remarkable result of this study is that the effects of the single distal-pocket and interface mutations are additive and their individual properties can be used to predict those of the multiple mutants. These correlations are highly encouraging for using rational protein engineering and our library of single-point mutations to design safer, more efficient, and more stable HBOCs.

## Abbreviations used

Hb, hemoglobin
Mb, myoglobin
rHb, recombinant hemoglobin
rMb, recombinant myoglobin
D<sub>2</sub>O, deuterium oxide
ns, nanosecond
OEC, oxygen equilibrium curve
2,3-BPG, 2,3 bisphosphoglycerate
HBOC, hemoglobin-based oxygen carrier
IHP, inositol hexaphosphate
DSS, 2,2-Dimethyl-2-silapentane-5-sulfonic acid
ΔMAP, change in mean arterial pressure

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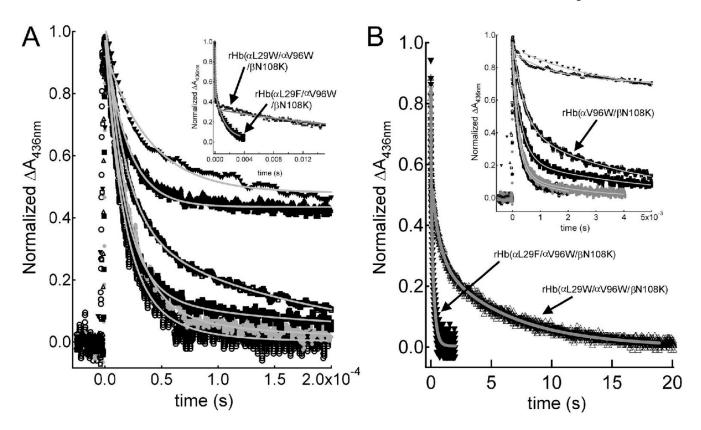


Figure 1. Representative time courses of ligand rebinding following partial (10%) photolysis. Lines indicate fits to one- or two-exponential functions. **A**, Rebinding of 100%  $O_2$  to native Hb A (•), rHb (αV96W) (•), rHb (βN108K) (•), rHb (αV96W/βN108K) (□), rHb (αL29F/αV96W/βN108K) ( $\blacktriangledown$ ), and rHb (αL29W/αV96W/βN108K) (Δ). Inset, rebinding on longer time scale shows the slower phase of the triple mutant rHbs. **B**, Rebinding of 100% CO to the same Hbs reveals similar behaviors. Experiments were performed in 0.1 M sodium phosphate buffer at pH 7.0 and 20 °C.

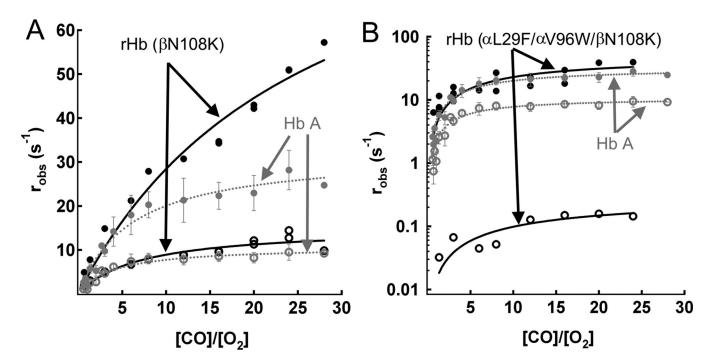


Figure 2. Fits of Equation 1 to observed rates  $(r_{obs})$  of  $O_2$  replacement for two representative rHbs. Fast ( ) and slow ( ) phases of  $O_2$  dissociation from Hb A are compared to:  $\mathbf{A}$ , rHb  $(\beta N108K)$  which exhibits a faster fast phase, and  $\mathbf{B}$ , rHb  $(\alpha L29F/\alpha V96W/\beta N108K)$  which exhibits a markedly slower slow phase. A log scale for the abscissa was employed in panel  $\mathbf{B}$ . Experiments were performed in 0.1 M sodium phosphate buffer at pH 7.0 and 20 °C.

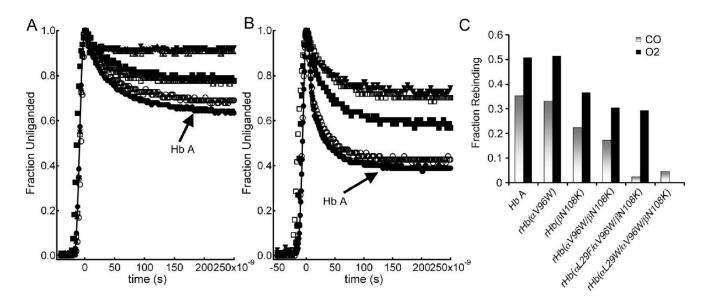


Figure 3. Representative time courses of geminate recombination following flash photolysis. **A**, Rebinding of 100% CO to native Hb A ( $\bullet$ ), rHb ( $\alpha$ V96W) ( $\circ$ ), rHb ( $\beta$ N108K) ( $\blacksquare$ ), rHb ( $\alpha$ V96W/ $\beta$ N108K) ( $\square$ ), rHb ( $\alpha$ L29F/ $\alpha$ V96W/ $\beta$ N108K) ( $\triangledown$ ), and rHb ( $\alpha$ L29W/ $\alpha$ V96W/ $\beta$ N108K) ( $\triangle$ ). **B**, Rebinding of 100% O<sub>2</sub> to the same Hbs.C, Fraction of O<sub>2</sub> and CO geminate recombination observed in each mutant is shown. The O<sub>2</sub> experiment using rHb ( $\alpha$ L29W/ $\alpha$ V96W/ $\beta$ N108K) is excluded due to oxidation and hemichrome formation within the sample. Experiments were performed in 0.1 M sodium phosphate buffer at pH 7.0 and room temperature.

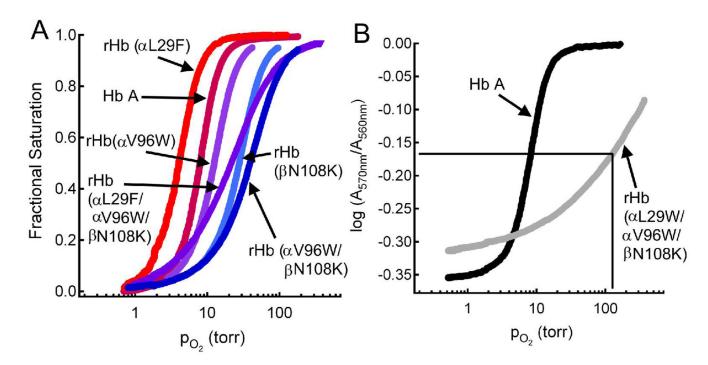


Figure 4. Oxygen dissociation curves.  $P_{50}$  and the Hill coefficient  $(n_{50})$  values were calculated from curves of: **A**, Hb A ( ), rHb (αL29F) ( ), rHb (αV96W) ( ), rHb (βN108K) ( ), rHb (αV96W/βN108K) ( ), and rHb (αL29F/αV96W/βN108K) ( ). B, Oxidation of the sample complicated the analysis of rHb (αL29W/αV96W/βN108K), so a raw data-set of the  $O_2$  equilibrium binding measurement is presented together with Hb A, with the estimation of  $P_{50}$  shown by lines. Experiments were performed in 0.1 M sodium phosphate buffer at pH 7.0 and 20 °C.

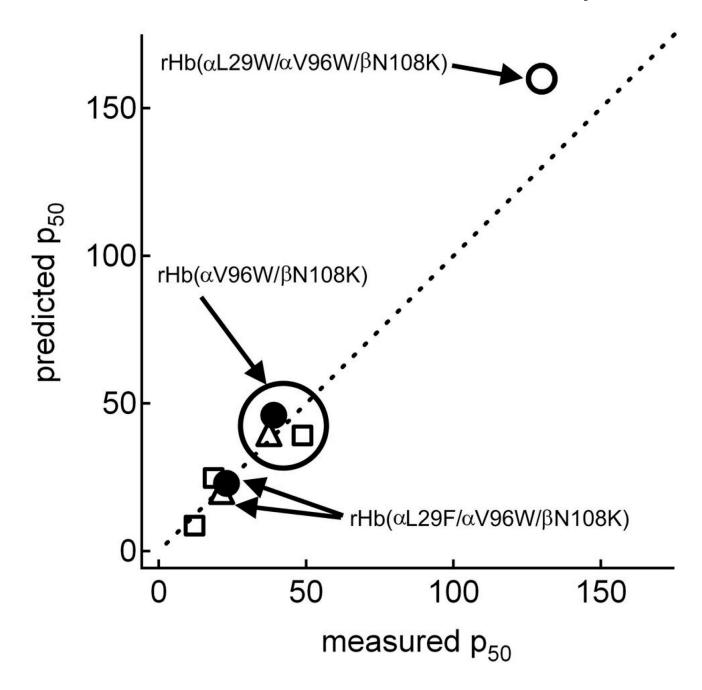


Figure 5. Prediction of  $P_{50}$  values. Measured  $P_{50}$  values for several multiple mutant Hbs are plotted against those predicted by Equation 1. The line y=x is provided. Three of the data points refer to separate reports on the same rHb, as indicated. Data cited are from Jeong and coworkers ( $\Delta$ ) (28) and Tsai and Ho ( $\Box$ ) (51). The rHb ( $\alpha$ L29W/ $\alpha$ V96W/ $\beta$ N108K) point is expected to be an underestimate of the true  $P_{50}$  due to oxidation in the sample during the course of the measurement.

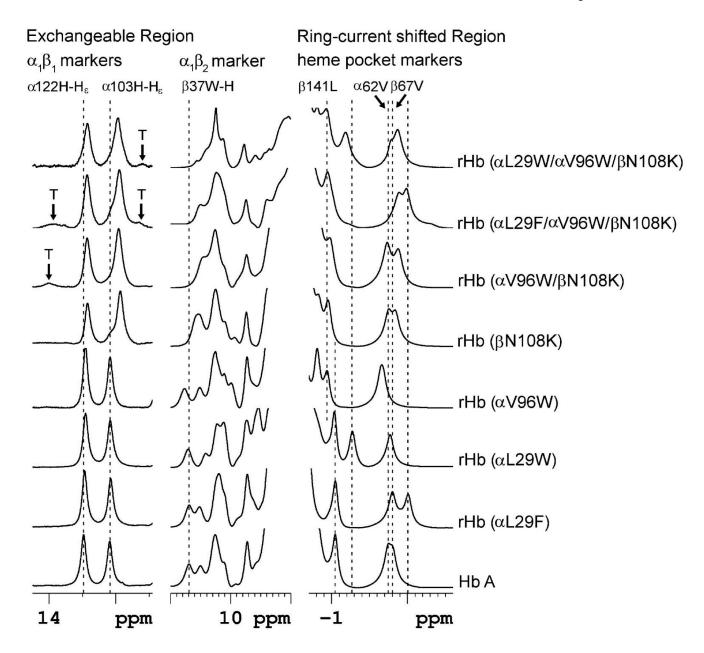


Figure 6.  $^1\text{H-NMR}$  spectra of Hbs in 100% CO gas. Hb solutions of 60 mg/mL in 0.1 M phosphate buffer at pH 7.0 were measured at 20 °C. Positions of marker peaks in the heme pocket,  $\alpha_1\beta_1$  interface and  $\alpha_1\beta_2$  interface are indicated. Positions of marker peaks characteristic of the deoxy structure of hemoglobin are labeled T and these markers monitor the  $\alpha_1\beta_2$  interface.

NIH-PA Author Manuscript **Table 1** Ligand binding parameters. Changes of 5- and 100-fold from native Hb A are indicated in bold and bold italics.<sup>a</sup> NIH-PA Author Manuscript NIH-PA Author Manuscript

			${ m O_2}$ Binding	ling				CO Binding	Bu	
	K'O2, slow	k'02, fast	Aslow	Afast	k <sub>02, slow</sub>	k <sub>O2,</sub> fast	k'CO, slow	K'CO, fast	Aslow	$\mathbf{A}_{\mathrm{fast}}$
	$\mu M^{-1}  s^{-1}$	μM <sup>-1</sup> s <sup>-1</sup>			s <sub>-1</sub>	s-1	$\mu M^{-1} s^{-1}$	$\mu M^{-1}  s^{-1}$		
Hb A 'R-state' (44)	36	9/			16	32	6.0	7.4		
Hb A 'R-state' (45)	28	100			12	22	2.9	7.1		
Hb A	72	73	0.51	0.49	11	32	2.4	7.1	0.48	0.52
rHb (αL29F)	89.0	55	0.47	0.53	0.16	33	0.020	5.5	0.71	0.29
rHb (αL29W)	0.083	99	0.57	0.43	86.0	25	0.0037	5.4	0.64	0.36
rHb ( $\alpha$ V96W)	10	55	0.09	0.91	14	49	1.2	5.7	0.35	0.65
rHb (βN108K)	4.3	54	0.24	0.76	15	100	0.34	3.2	0.23	0.77
rHb ( $\alpha$ V96W/ $\beta$ N108K)	4.2	43	0.36	0.64	20	120	0.43	2.6	0.39	0.61
rHb (αL29F/αV96W/βN108K)	0.74	26	0.49	0.51	0.30	48	0.0062	0.084	0.55	0.45
rHb (αL29W/αV96W/βN108K)	0.035	40	0.43	0.57	2.2	39	0.00024	0.063	0.46	0.54
Hb A 'T-state' (45)	5.6	6.7			430	029	0.16	0.07		
Hb A 'T-state' (46)	8.8	7.5			2000	2000	0.15	0.15		

 $^{a}\mathrm{Conditions:~0.1~M}$  sodium phosphate buffer pH 7.0,20  $^{\circ}\mathrm{C.}$ 

Hemoglobin	p <sub>50</sub> (Torr)	n <sub>50</sub>
	_	
Hb A	8.0	3.0
rHb (αL29F)	4.0	2.6
rHb (αL29W)	28	2.1
rHb (αV96W)	13	2.5
rHb(βN108K)	28	2.4
$rHb(\alpha V96W/\beta N108K)$	39	1.8
rHb ( $\alpha$ L29F/ $\alpha$ V96W/ $\beta$ N108K)	23	1.3
rHb ( $\alpha$ L29W/ $\alpha$ V96W/ $\beta$ N108K)	120	0.9

 $<sup>^</sup>a\mathrm{Conditions:~0.1~M}$  sodium phosphate buffer pH 7.0,20 °C.