# Determination of the Hemoglobin Surface Domains That React with Cytochrome $b_5$[†]

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ABSTRACT: We have compared the photoinitiated electron-transfer (ET) reaction between cytochrome $b_5$ ($b_5$) and zinc mesoporphyrin-substituted hemoglobin [(ZnM)Hb] and Hb variants in order to determine whether $b_5$ binds to the subunit surface of either or both Hb chains, or to sites which span the dimer−dimer interface. Because the dimer−dimer interface would be disrupted for monomers or $\alpha\beta$ dimers, we studied the reaction of $b_5$ with αZnM chains and (ZnM)Hb $\beta$W37E, which exists as $\alpha\beta$ dimers in solution. Triplet quenching titrations of the ZnHb proteins with $Fe^{3+}b_5$ show that the binding affinity and ET rate constants for the α-chains are the same when they are incorporated into a Hb tetramer or dimer, or exist as monomers. Likewise, the parameters for $\beta$-chains in tetramers and dimers differ minimally. In parallel, we have modified the surface of the Hb chains by neutralizing the heme propionates through the preparation of zinc deuterioporphyrin dimethyl ester hemoglobin, (ZnD-DME)Hb. The charge neutralization increases the ET rate constants 100-fold for the α-chains and 40-fold for the $\beta$-chains (but has has little effect on the affinity of either chain type for $b_5$, similar to earlier results for myoglobin). Together, these results indicate that $b_5$ binds to sites at the subunit surface of each chain rather than to sites which span the dimer−dimer interface. The charge-neutralization results further suggest that $b_5$ binds over a broad area of the subunit face, but reacts only in a minority population of binding geometries.

Ferrous hemoglobin ($Fe^{2+}$Hb) functions in oxygen transport, but under physiological conditions, the oxyHb and deoxyHb can be oxidized to inactive metHb ($Fe^{3+}$Hb). In the erythrocyte, the concentration of $Fe^{3+}$Hb is maintained at a low level by an electron-transport chain in which a soluble cytochrome $b_5$ ($b_5$) mediates electron transfer between the flavin-containing cytochrome $b_5$ reductase and $Fe^{3+}$Hb (*1*−*3*). In this report, we focus on the terminal electron-transfer (ET) step, in which $Fe^{2+}b_5$ is the electron donor to $Fe^{3+}$Hb (eq 1).

$$Fe^{2+}b_5 + Fe^{3+}Hb \rightarrow Fe^{3+}b_5 + Fe^{2+}Hb \qquad (1)$$

This reaction appears to involve the formation of a protein−protein complex between $Fe^{3+}$Hb and $Fe^{2+}b_5$ in which one molecule of $b_5$ binds to each subunit of the $\alpha_2\beta_2$ Hb tetramer (*4*−*7*). Here, we further examine the docking of $b_5$ to the Hb tetramer.

Previous work has studied the binding affinity ($K$) and ET rate constants ($k$) for the complex of Hb and $b_5$, using triplet quenching titrations of zinc-substituted hemoglobins by $Fe^{3+}b_5$ (*7*−*11*). Substitution of the heme (FeP) of one partner of an ET complex by a closed-shell metalloporphyrin (ZnP in the present case) offers a means of studying binding *and* intracomplex ET (*12*−*15*). The metalloporphyrin triplet state, $^3$ZnP, produced by laser-flash excitation is a strong reductant, and in a complex with an $Fe^{3+}$P quencher, its lifetime is decreased by long-range, intracomplex $^3$ZnP → $Fe^{3+}$P ET (eq 2).

$$^3ZnP + Fe^{3+}P \rightarrow ZnP^+ + Fe^{2+}P \qquad (2)$$

The resulting ET intermediate returns to the ground state by thermally activated ET from the $Fe^{2+}$P to the porphyrin-center $\pi$-cation radical, $ZnP^+$, according to eq 3.

$$ZnP^+ + Fe^{2+}P \rightarrow ZnP + Fe^{3+}P \qquad (3)$$

This metal-substitution approach is justified by experiments demonstrating that Hbs substituted with closed-shell metalloporphyrins are faithful analogues of deoxyHb. Thus, the crystal structure of MgHb in which all four heme groups were replaced by MgP is identical to that of the native protein (*16*), and thermodynamic studies show that the free energy of cooperativity for ZnHb is identical to that of FeHb (*17*, *18*).

Our recent study of the reaction of mixed-metal [ZnM, $Fe^{3+}(N_3^-)$] Hb hybrids and of fully substituted Zn-mesoporphyrin (ZnM)Hb with $Fe^{3+}b_5$ gave the reactivity and affinity constants for interactions of $b_5$ with the individual α- and $\beta$-chains of T-state Hb (*7*). These results were consistent with a docking model in which each subunit of the Hb tetramer binds a molecule of $b_5$ as depicted in Figure 1A,B. In this figure, the Hb tetramer is represented by the $\alpha_1\beta_1$ dimer (black) and the $\alpha_2\beta_2$ dimer (gray). It has been proposed that

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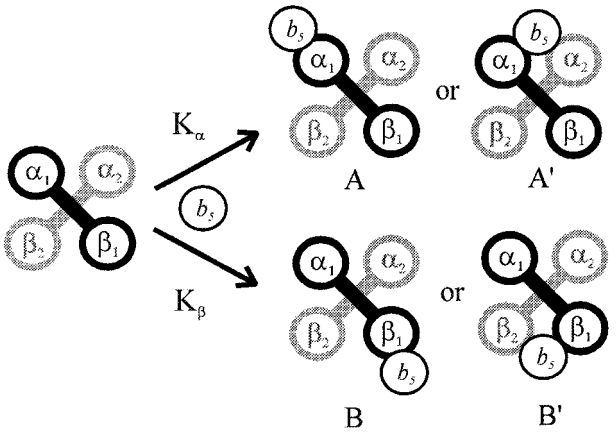


FIGURE 1: Model of $b_5$ binding to the α- and $\beta$-chains of Hb. The $\alpha_1\beta_1$ dimer is shown in black and the $\alpha_2\beta_2$ dimer in gray. Representation of a molecule of $b_5$ binding to an α-chain binding site (A), a $\beta$-chain binding site (B), an $\alpha_1\alpha_2$-site which spans the dimer−dimer interface (A′), and a $\beta_1\beta_2$-site which also spans the dimer−dimer interface (B′).

$b_5$ binds at the heme face of each chain, where acidic residues of $b_5$ interact with basic residue of Hb (*19*). The kinetic results, however, also permitted an alternative model in which $b_5$ reacts with a chain by binding at sites which span the dimer−dimer interfaces (*7*). A possible $\alpha_1\alpha_2$-site was noted in which Lys residues on both α-chains provide binding contacts as represented in Figure 1A′. A complementary $\beta_1\beta_2$-site was also considered in which Lys residues on both $\beta$-chains interact with $b_5$ as depicted in Figure 1B′, although this site appeared to be inconsistent with a study of Hb variants which indicates that Lys residues away from the dimer−dimer interface are involved in $b_5$ binding (*20*).

To determine whether $b_5$ binds to the subunit surface of either or both Hb chains (Figure 1A,B), or instead binds at one or more sites that span a dimer−dimer interface (Figure 1A′,B′), we here examine the photoinitiated ET reaction between $b_5$ and Zn-substituted Hb variants and compare the results to the reaction between $b_5$ and $ZnHbA_0$. Because the dimer−dimer interface would be disrupted for monomers or $\alpha\beta$ dimers, we have studied the reaction of $b_5$ with monomeric αZnM chains and with (ZnM)Hb $\beta$W37E. Characterization of Hb $\beta$W37E, which is altered at an important contact in the dimer−dimer interface, has shown that this variant is fully dissociated into $\alpha\beta$ dimers under the conditions employed here (*21*−*24*). In parallel, we have modified the surface of the Hb chains by neutralizing the heme propionates through the preparation of Zn deuterioporphyrin dimethyl ester Hb, (ZnD-DME)Hb. Such an electrostatic modification of Mb has been found to have a major influence on the ET reaction between $b_5$ and Mb (*25*), and analogous results are found here. Together, the two approaches resolve the nature of $b_5$ binding to Hb.

## EXPERIMENTAL PROCEDURES

*Materials.* $HbA_0$ was isolated from outdated whole blood as previously described (*26*, *27*). The trypsin-solubilized bovine cytochrome $b_5$ was prepared according to the method of Mauk and co-workers (*28*). Zn mesoporphyrin IX (ZnM) and Zn deuterioporphyrin IX dimethyl ester (ZnD-DME) were purchased from Porphyrin Products (Logan, UT). D-Glucose, glucose oxidase (Type VII-S from *Aspergillus niger*), bovine liver catalase (thymol free), and inositol hexaphosphoric acid (IHP) as the potassium salt were purchased from Sigma.

*Preparation of (ZnM)Hb, (ZnM)Hb βW37E, and (ZnD-DME)Hb.* We employed Zn mesoporphyrin (ZnM), rather than Zn protoporphyrin (ZnP), for spectroscopic reasons previously detailed (*7*). The wild-type (ZnM)Hb was prepared as published (*27*). The (ZnM)Hb $\beta$W37E was prepared by combining αZnM chains (*29*) with $\beta$W37E globin (*21*) and a 1.2-fold excess of ZnM. Following an overnight incubation period, the recombination mixture was passed through a G-25 column equilibrated with 15 mM Tris-HCl buffer at pH 8.0. The protein was loaded onto a DE 52 anion exchange column preequilibrated with 15 mM Tris-HCl buffer at pH 8.0. The excess αZnM chains which did not bind under these conditions were removed. A gradient generated from 100 mL of 15 mM Tris-HCl buffer at pH 8.0 to 100 mL of 100 mM Tris-HCl buffer at pH 8.0 was applied, and the (ZnM)Hb $\beta$W37E was eluted. This protein was then further purified by HPLC (Waters 650) using a TSK-based anion exchange column (TosoHaas, 21.5 mm × 15 cm, DEAE-5PW). A gradient of 60 min from 15 mM Tris-HCl at pH 8.0 to 15 mM BisTris-HCl at pH 7.0 at a flow of 5 mL/min was applied, and the (ZnM)Hb $\beta$W37E was eluted at 38.7 min. The absorbance was monitored at 280, 414, and 576 nm (Waters 490 D multichannel detector). The isolated protein was homogeneous by isoelectric focusing and stored in liquid nitrogen. (ZnM)Hb and (ZnM)Hb $\beta$W37E exhibit identical optical properties with a Soret maximum at 414 nm and peaks at 542 and 578 nm in the "α-$\beta$" region of the visible spectrum.

(ZnD-DME)Hb was prepared by the reconstitution of heme-free globin with ZnD-DME. The solubility of ZnD-DME is quite different from the acid form. Instead of dissolving the porphyrin in a minimal amount of 0.1 M KOH followed by a 10-fold dilution with water, the ZnD-DME (∼1.25 equiv) was dissolved in minimal volumes of warm (60 °C) methanol/DMSO. The porphyrin solution was heated at 60 °C to give deep pink solution, then cooled, and added dropwise to globin solution on ice [25 mM potassium phosphate (KPi) buffer at pH 6.5]. The reconstitution was monitored by UV−Vis spectroscopy (HP 8451A diode array spectrophotometer) and IEF (PhastGel IEF 3−9). Additional porphyrin was added (total ∼3 equiv) until there was no free globin present. The reconstitution mixture was filtered to remove any precipitate.

The reconstitution mixture was loaded onto a Sephadex G-25 (fine) column equilibrated with 25 mM KPi at pH 7 to remove excess porphyrin. The protein was further purified by HPLC. The reconstitution mixture was loaded onto a TSK-based cation exchange column (Beckman, 21.5 mm × 15 cm, SP-5PW) that had been preequilibrated with 25 mM KPi at pH 7 and was eluted with a two-step linear gradient. The first step was a 25 min linear gradient from the equilibration buffer to 25 mM $K_2HPO_4$; the second step was a 70 min linear gradient to 70% 25 mM $K_3PO_4$, at a flow rate of 5 mL/min. The absorbance was monitored at 280, 408, and 542 nm. The isolated (ZnD-DME)Hb was homogeneous by isoelectric focusing and was stored in liquid nitrogen. The p*I* of (ZnD-DME)Hb is shifted from that of (ZnM)Hb, which is isoelectric with Fe(CO)Hb (p*I* = 7.2),

to a value slightly more basic than apoHb (p$I$ = 7.8) (*30*). The direction of this shift is consistent with the neutralization of the propionate charges. The spectrum of the reconstituted (ZnD-DME)Hb has a sharp Soret maximum at 412 nm and peaks at 542 and 578 nm, identical to (ZnD)Hb.

Anaerobic samples for the kinetic measurements were prepared in Pyrex cuvettes containing 2 mL of nitrogen-purged 10 mM KPi buffer. IHP concentration was 0.05 mM, when used. The oxygen scavenging system of 5 mM D-glucose, 25 $\mu$g/mL thymol-free catalase from bovine liver, and 100 $\mu$g/mL glucose oxidase was used (*31*). The protein stock solutions were exchanged into 10 mM KPi buffer using Centricon-10 microconcentrators (Amicon) and deaerated with nitrogen prior to addition to the sample. Hb concentrations were typically about 5 $\mu$M in subunits. For the titrations, aliquots of a ~1 mM stock solution of $Fe^{3+}b_5$ ($\epsilon_{413}$ = 117 $mM^{-1}$ $cm^{-1}$) (*32*) were added to the Hb solution.

The triplet decay kinetics of (ZnM)Hb, (ZnM)Hb $\beta$W37E, and (ZnD-DME)Hb are influenced by energy-transfer reactions between the chains. As first shown for (ZnP)Hb, at high laser powers the early-time kinetics are multiphasic because of triplet−triplet energy transfer within the Hb tetramer (*33*). As the excitation power is lowered, this phenomenon is suppressed, and the triplet decay becomes nearly exponential at excitation powers of $\lesssim$1 mJ/pulse. However, the energy-transfer contribution also decays quickly, and for the triplet quenching experiments, the kinetics at high power match those at low power after $t \geq 1$ ms. Hence, we often collected the data at high power, discarded the data for $t \lesssim 1$ ms, and fit only the later-time data. The dissociation constant for Hb $\beta$W37E indicates that at micromolar concentrations, 97% of the protein exists as $\alpha\beta$ dimers (*22*), and Zn substitution does not alter the Hb dimer−tetramer equilibrium (*17*). To confirm the behavior of (ZnM)Hb $\beta$W37E, we examined the intratetramer energy-transfer reaction which is maximally sensitive to the $\alpha_1 \rightarrow \beta_2$ triplet transfer and found that dissociation into dimers in fact eliminates this process (*34*).

*Laser Flash Photolysis.* Photoexcitation was achieved with a Q-switched, frequency-doubled Nd:YAG YG660A laser (Continuum, 7 ns pulse width, $\lambda$ = 532 nm). Transients were collected with an analyzing beam, digitized with a LeCroy model 9310 digitizer, transferred to an IBM-compatible computer, and fit with the Marquardt nonlinear least-squares algorithm (*11*). The incident laser power was varied with a high-power variable beam splitter (CVI Corp.) and measured with a power meter (Scientech model 372). Emission data were collected in situ using a fiber optic cable.

## RESULTS

*Reaction of $Fe^{3+}b_5$ with (ZnM)Hb Tetramers, (ZnM)Hb $\beta$W37E Dimers, and $\alpha$ZnM Monomers.* Figure 2A shows the time-resolved triplet decay of (ZnM)Hb in the absence of $b_5$. The decays of the two chain types within the tetramers are not resolved, and the decay is exponential with a rate constant $k^d = 42 \pm 2$ $s^{-1}$. The trace for (ZnM)Hb $\beta$W37E is indistinguishable ($k^d = 38 \pm 2$ $s^{-1}$; not shown), as is that for the $\alpha$ZnM monomers ($k^d = 46 \pm 2$ $s^{-1}$; not shown).

When $Fe^{3+}b_5$ is added to a solution of $\alpha$ZnM monomers, the triplet decay remains exponential while $^3ZnM \rightarrow Fe^{3+}b_5$ interprotein ET increases the triplet decay constant. For example, in the presence of a 3-fold excess of $Fe^{3+}b_5$, the decay rate constant for the $\alpha$ZnM monomers becomes $k_\alpha^{obs}$ = 112 $s^{-1}$ (Figure 2D). This behavior indicates that the exchange between the [Hb, $b_5$] complex and its components is fast ($k_{off} \gg k_{obs}$). A similar effect occurs upon the addition of $Fe^{3+}b_5$ to a solution of either (ZnM)Hb or (ZnM)Hb $\beta$W37E. However, the triplet decay for (ZnM)Hb (Figure 2C) or (ZnM)Hb $\beta$W37E (Figure 2B) becomes biexponential (eq 4), with the larger decay constant, $k_\alpha^{obs}$, corresponding to that of the $\alpha$-chains and the smaller, $k_\beta^{obs}$, to that of the $\beta$-chains, as shown earlier by a self-consistent study of the reactions of $Fe^{3+}b_5$ with the [ZnM, Fe] Hb hybrids and the fully substituted (ZnM)Hb (*7*).

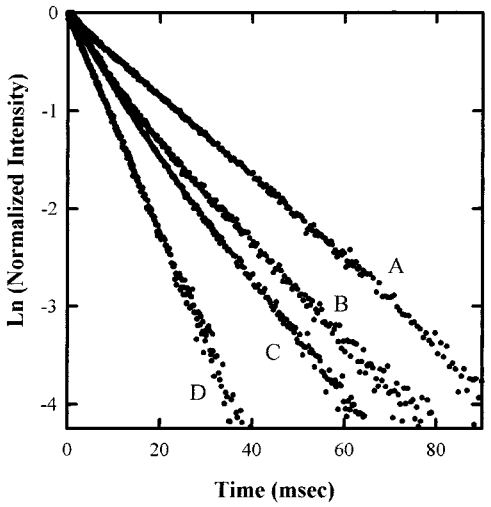


FIGURE 2: Semilog plot of the triplet decay traces for 5 $\mu$M (ZnM)-Hb, (ZnM)Hb $\beta$W37E, and $\alpha$ZnM monomers in the presence of a 3-fold excess of $Fe^{3+}b_5$. (A) (ZnM)Hb without $Fe^{3+}b_5$, $k^d = 42 \pm 2$ $s^{-1}$; (B) (ZnM)Hb $\beta$W37E with $Fe^{3+}b_5$, $k_\alpha^{obs}$ = 115 $s^{-1}$ and $k_\beta^{obs}$ = 45 $s^{-1}$; (C) (ZnM)Hb with $Fe^{3+}b_5$, $k_\alpha^{obs}$ = 115 $s^{-1}$ and $k_\beta^{obs}$ = 57 $s^{-1}$; (D) $\alpha$ZnM monomers with $Fe^{3+}b_5$, $k_\alpha^{obs}$ = 112 $s^{-1}$. Conditions: 10 mM KPi, 0.05 mM IHP, pH 7.0, for (ZnM)Hb and 10 mM KPi, pH 7.0, for the (ZnM)Hb $\beta$W37E and $\alpha$ZnM monomers, all at 20 °C.

$$\Delta A = A_o\left(\frac{e^{-k_\alpha^{obs}t} + e^{-k_\beta^{obs}t}}{2}\right) \quad (4)$$

To measure the reaction of $b_5$ to the tetramer, dimer, and monomer forms of Hb, the (ZnM)Hb, (ZnM)Hb $\beta$W37E, and $\alpha$ZnM monomers, respectively, were titrated with $Fe^{3+}b_5$. Figure 3 presents the [$Fe^{3+}b_5$] dependence of the quenching constants ($\Delta k_i = k_i^{obs} - k^d$, $i = \alpha, \beta$) obtained from titrations at pH 7, and clearly shows that the quenching of the $\alpha$ZnM chains is unaffected by incorporation into either dimers or tetramers. Comparison of Figure 3A and Figure 3B shows that the quenching of the $\alpha$ZnM chains is appreciably greater than that of the $\beta$ZnM chains, while Figure 3B shows that there is less quenching for the $\beta$ZnM chains in the dimers of (ZnM)Hb $\beta$W37E than in the tetrameric (ZnM)Hb. These titration profiles exhibit mild curvature, indicating $b_5$ binds weakly under these conditions.

The titration profile for the $\alpha$ZnM monomers shown in Figure 3A is overlayed with the fit to a simple binding isotherm in which the fraction of $\alpha$ZnM monomers that reside within a $b_5$ complex, $f_\alpha$, is given by eq 5:

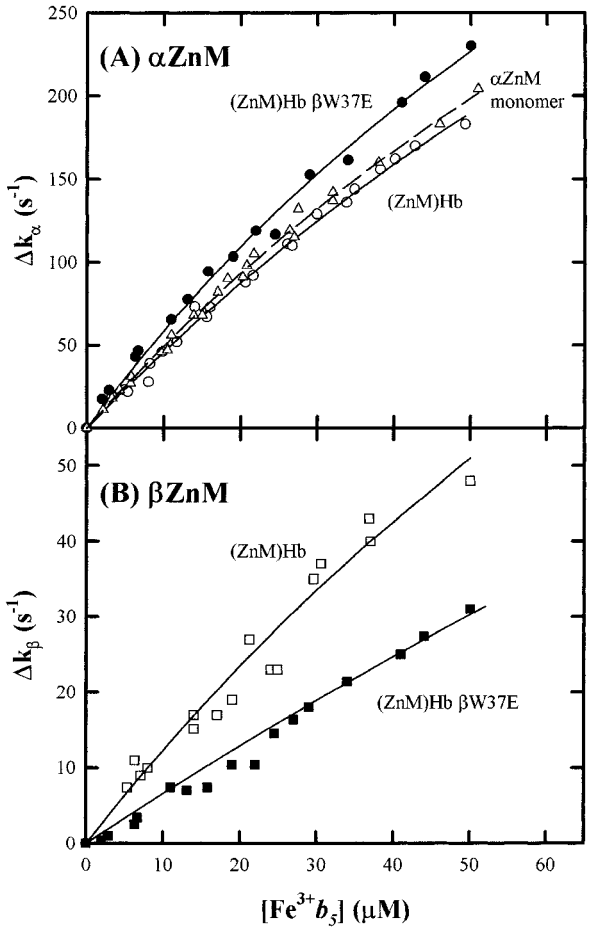


FIGURE 3: Triplet quenching of 5 $\mu$M (ZnM)Hb, (ZnM)Hb $\beta$W37E, and αZnM monomers by $Fe^{3+}b_5$ at pH 7.0. The lines are fits to the binding model described in the text with the fitting parameters summarized in Table 1. (A) Titration profiles for the αZnM chain in (ZnM)Hb (open circles), (ZnM)Hb $\beta$W37E (filled circles), and αZnM monomers (open triangles). (B) Titration profiles for the $\beta$ZnM chain in (ZnM)Hb (open squares) and (ZnM)Hb $\beta$W37E (filled squares). Conditions: 10 mM KPi, 0.05 mM IHP, pH 7.0, for (ZnM)Hb and 10 mM KPi, pH 7.0, for (ZnM)Hb $\beta$W37E and αZnM monomers, all at 20 °C.

$$f_\alpha = \frac{1/K_\alpha + [D]_o + [A]_o - \sqrt{(1/K_\alpha + [D]_o + [A]_o)^2 - 4[D]_o[A]_o}}{2[D]_o} \quad (5)$$

where $K_\alpha$ is the binding constant, $[D]_o$ is the initial concentration of αZnM monomers, and $[A]_o$ is the initial concentration of $b_5$. In the fast-exchange limit that describes these experiments, the measured quenching for the αZnM monomers ($\Delta k_\alpha = k_\alpha^{obs} - k^d$), at any point of a titration, is a product of the ET rate constant ($k_\alpha$) and the fraction αZnM monomers bound to $b_5$ ($f_\alpha$) (eq 5), and the fit gives both $K_\alpha$ and $k_\alpha$ (Table 1).

The lines in Figure 3 through the data for the $Fe^{3+}b_5$ titrations of tetrameric (ZnM)Hb and dimeric (ZnM)Hb $\beta$W37E are fits to a binding model in which each α- or $\beta$-chain within the $\alpha_2\beta_2$ Hb tetramer for (ZnM)Hb, or within the $\alpha\beta$ dimer for (ZnM)Hb $\beta$W37E, reacts *independently* with a $b_5$ bound at a site that is characteristic of that chain type (Figure 1). The equations which describe the observed quenching during the course of a titration were previously described (*7*). This model leads to expressions similar to eq 5. The measured quenching for a given type of ZnM-substituted chain ($\Delta k_i = k_i^{obs} - k^d$, $i = \alpha, \beta$) is a product of the ET rate constant for that chain ($k_\alpha$, $k_\beta$) and the fraction of that chain that has $b_5$ bound at its interacting site ($f_\alpha$, $f_\beta$) (eq 11 in ref *7*); the fits to $\Delta k_i$ for a titration give $K_i$ and $k_i$. The resulting binding constants and the ET rate constants obtained from titration profiles of the αZnM monomers, (ZnM)Hb, and (ZnM)Hb $\beta$W37E (Figure 3) are summarized in Table 1.

Table 1: Association Constants ($K_\alpha$, $K_\beta$) and ET Rate Constants ($k_\alpha$, $k_\beta$) of the Complexes of (ZnM)Hb, (ZnM)Hb $\beta$W37E, and αZnM Monomers with $Fe^{3+}b_5$[a]

| protein | $K_\alpha$ ($\times 10^3$ $M^{-1}$) | $k_\alpha$ ($s^{-1}$) | $K_\beta$ ($\times 10^3$ $M^{-1}$) | $k_\beta$ ($s^{-1}$) | $K_\beta \cdot k_\beta$ ($\times 10^6$ $M^{-1}$ $s^{-1}$) |
|---|---|---|---|---|---|
| (ZnM)Hb | $5.9 \pm 0.8$ | $850 \pm 90$ | $5.9 \pm 2.6$ | $230 \pm 80$ | 1.4 |
| (ZnM)Hb $\beta$W37E | $8.3 \pm 1.1$ | $790 \pm 80$ | − | − | 0.7 |
| αZnM monomers | $7.0 \pm 0.9$ | $780 \pm 80$ | − | − | − |

[a] Conditions: 10 mM KPi, 0.05 mM IHP, pH 7.0, for (ZnM)Hb, and 10 mM KPi, pH 7.0, for (ZnM)Hb $\beta$W96E and αZnB monomers, at 20 °C.

For (ZnM)Hb, the two chain types have the same affinity for $b_5$ ($K_\alpha \sim K_\beta \sim 5.9 \times 10^3$ $M^{-1}$), while the rate constant for the α-chains is ~4-fold greater than for the $\beta$-chains ($k_\alpha = 850 \pm 90$ $s^{-1}$; $k_\beta = 230 \pm 80$ $s^{-1}$). The $b_5$ binding affinity and ET rate constant for the α-chains are *independent* of whether they are incorporated in a tetramer, (ZnM)Hb, in an $\alpha\beta$ dimer, (ZnM)Hb $\beta$W37E, or exist as monomers: $K_\alpha \sim 7.0 \times 10^3$ $M^{-1}$ and $k_\alpha \sim 800$ $s^{-1}$. The titration profile for the $\beta$ZnM chains in (ZnM)Hb $\beta$W37E is linear, and a range of binding and rate constants can describe these data. Thus, for $\beta$-chains, it is only valid to compare the product of the binding constant and the ET rate constant ($K_\beta \cdot k_\beta$). This product is only about 2-fold larger for the $\beta$ZnM chains in the tetrameric (ZnM)Hb than in the dimeric (ZnM)Hb $\beta$W37E (Table 1).

*Interaction of (ZnD-DME)Hb with $Fe^{3+}b_5$.* Neutralization of the heme propionates in (ZnD-DME)Hb causes a dramatic increase in the ET quenching by $Fe^{3+}b_5$, as shown (Figure 4) by a comparison of the triplet decays of (ZnM)Hb (B) and (ZnD-DME)Hb (C) in the presence of ca. 3.5-fold excess of $Fe^{3+}b_5$. For the traces in Figure 4, the decay constant for the α-chains in (ZnD-DME)Hb is $1.9 \times 10^4$ $s^{-1}$ while that for (ZnM)Hb is only 450 $s^{-1}$; the decay constant for the $\beta$-chains in (ZnD-DME)Hb is $4.5 \times 10^3$ $s^{-1}$ while that for (ZnM)Hb is only 115 $s^{-1}$ (*35*). The inset of Figure 4 shows the time course of the ET intermediate (I) as monitored at a triplet isosbestic point ($\lambda = 563$ nm); the signal rises with the same rate as the decay of the triplet (C), confirming that the observed quenching is indeed involves the $^3$ZnD-DME → $Fe^{3+}$P ET reaction.

To measure the binding of $b_5$ to (ZnD-DME)Hb, a solution of (ZnD-DME)Hb was titrated with $Fe^{3+}b_5$. Figure 5 shows the $[Fe^{3+}b_5]$ dependence of the quenching rate constants ($\Delta k_i = k_i^{obs} - k^d$, $i = \alpha, \beta$) obtained from the titration of (ZnD-DME)Hb with $Fe^{3+}b_5$ at pH 6.0. The lines are fits to the binding model previously described (*7*); the parameters are given in Table 2 along with those of (ZnM)Hb at these conditions. For the α-chains, the binding constant is only about 2-fold smaller for (ZnD-DME)Hb than for (ZnM)Hb;

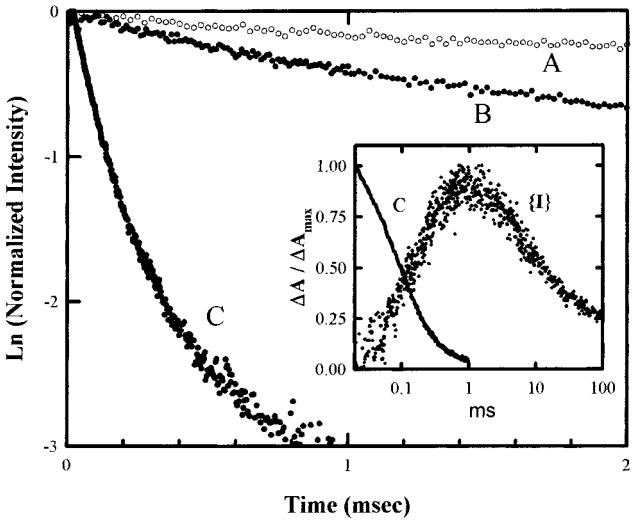


FIGURE 4: Comparison of the $Fe^{3+}b_5$ triplet quenching of (ZnM)-Hb and (ZnD-DME)Hb. (A) (ZnM)Hb without $Fe^{3+}b_5$; (B) (ZnM)-Hb with 17 $\mu$M $Fe^{3+}b_5$; (C) (ZnD-DME)Hb with 18 $\mu$M $Fe^{3+}b_5$. Conditions: 10 mM KPi, 0.05 mM IHP, pH 6.0, at 20 °C. Inset: Time courses of the (ZnD-DME)Hb triplet (C) recorded at 440 nm and the ET intermediate ({I}) recorded at 563 nm. Conditions: 5 $\mu$M (ZnD-DME)Hb and 18 $\mu$M $Fe^{3+}b_5$; 10 mM KPi, 0.050 mM IHP, pH 6.0, at 20 °C.

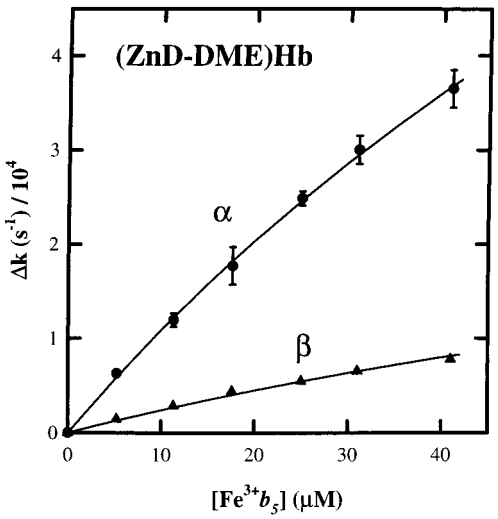


FIGURE 5: Triplet quenching of 5 $\mu$M (ZnD-DME)Hb by $Fe^{3+}b_5$ at pH 6.0. Titration profiles for the αZnD-DME chains (circles) and the $\beta$ZnD-DME chains (triangles). The solid lines are fits to the binding model described in the text with the fitting parameters given in Table 2. Conditions: 10 mM KPi, 0.050 mM IHP, pH 6.0, at 20 °C.

Table 2: Association Constants ($K_\alpha$, $K_\beta$) and ET Rate Constants ($k_\alpha$, $k_\beta$) of the Complexes of (ZnM)Hb and (ZnD-DME)Hb with $Fe^{3+}b_5$[a]

| protein | $K_\alpha$ ($\times 10^3$ $M^{-1}$) | $k_\alpha$ ($s^{-1}$) | $K_\beta$ ($\times 10^3$ $M^{-1}$) | $k_\beta$ ($s^{-1}$) |
|---|---|---|---|---|
| (ZnM)Hb | $19 \pm 2$ | $1500 \pm 80$ | $4.9 \pm 3.6$ | $850 \pm 90$ |
| (ZnD-DME)Hb | $7.9 \pm 1.0$ | $(1.5 \pm 0.2) \times 10^5$ | $7.8 \pm 3.2$ | $(3.4 \pm 1.1) \times 10^4$ |

[a] Conditions: 10 mM KPi, 0.05 mM IHP, pH 6.0, at 20 °C.

for the $\beta$-chains, the binding constant is the same for the two proteins, within experimental error. However, the ET rate constant of the α-chains in (ZnD-DME)Hb is 100-fold larger than that of (ZnM)Hb, and that for the $\beta$-chains is 40-fold larger than that of (ZnM)Hb.

## DISCUSSION

To determine whether $b_5$ binds to the subunit surface of either or both Hb chains, or instead binds at one or more sites that span the dimer−dimer interface, we have examined the reaction of $b_5$ with tetrameric, dimeric, and monomeric Zn-substituted Hbs, and also have examined the surface-charge 'mutant', (ZnD-DME)Hb, where the heme propionates have been neutralized by esterification.

The triplet quenching of (ZnM)Hb and αZnM monomers by $Fe^{3+}b_5$ was used to measure the binding affinity ($K$) and intracomplex ET rate constants ($k$) for the reaction of $b_5$ with the tetramer and monomer forms of Hb, respectively. To study the reaction of $b_5$ with $\alpha\beta$ dimers, we prepared (ZnM)-Hb $\beta$W37E. Direct measurement of the dimer−tetramer equilibrium constant (tetramer formation) for Hb $\beta$W37E at pH 7.4 gives ${}^0K_2 = 7.2 \times 10^3$ $M^{-1}$ as compared to ${}^0K_2 = 4.4 \times 10^{10}$ $M^{-1}$ for $HbA_0$, and shows that Zn substitution does not alter the dimer−tetramer equilibrium (*17*, *18*). Direct calculation then predicts that, at pH 7.4 and for the micromolar concentrations employed here, ∼97% of Hb $\beta$W37E exists as $\alpha\beta$ dimers (*22*). CO combination experiments confirm that Hb $\beta$W37E is almost exclusively $\alpha\beta$ dimers at pH 7, but indicate that it is a mixture of dimers and tetramers at pH 6 (*21*). We thus performed measurements at pH 7 as a compromise between the conditions for optimal reactivity, which increases as the pH is lowered (*7*), and those for optimal dimer formation, which increases with pH.

The triplet quenching titration profiles of (ZnM)Hb, (ZnM)Hb $\beta$W37E, and αZnM monomers have been fit with a model in which a single site associated with each chain reacts with $b_5$, but with different affinities and different intracomplex ET rate constants for the reactions with the α-chains and the $\beta$-chains (*7*). The $b_5$ binding affinities and ET rate constant for the α-chains are independent of whether they are incorporated in a tetramer, (ZnM)Hb, in an $\alpha\beta$ dimer, (ZnM)Hb $\beta$W37E, or exist as monomers: $K_\alpha \sim 7.0 \times 10^3$ $M^{-1}$ and $k_\alpha \sim 800$ $s^{-1}$. Similarly, the product of the binding and ET rate constants ($K_\beta \cdot k_\beta$) for $b_5$ binding to $\beta$-chains in tetrameric (ZnM)Hb is only slightly larger, about 2-fold, than that for the dimeric (ZnM)Hb $\beta$W37E. Clearly, an interface binding site would be abolished upon dissociation of tetramers into dimers.

In contrast, our modification of the subunit surfaces, through the preparation of (ZnD-DME)Hb in which the negative charges of the heme propionate groups are neutralized, enhanced the triplet quenching of $Fe^{3+}b_5$ ∼100-fold for both chain types of (ZnD-DME)Hb. A similar result was found when surface charges on the $\beta$-chain were mutagenically reversed (*20*). Together, these results establish that $b_5$ binds to the "faces" of the individual α- and $\beta$-chains (Figure 1A,B) and does not span a dimer−dimer interface as depicted in Figure 1A′,B′.

The effects on interprotein ET, caused by neutralization of the heme propionates are quite dramatic. At pH 6.0, $b_5$ binds to α- and $\beta$-chains of (ZnD-DME)Hb and (ZnM)Hb with minimal differences in affinity. In contrast, charge neutralization caused the ET rate constants to increase 100-fold for the α-chains and 40-fold for the $\beta$-chains. Similar findings have been reported for (ZnD-DME)Mb (25), and we apply the interpretation for Mb to the Hb chains, as well. In this interpretation, the overall binding constant for $b_5$ is

not affected by the neutralization of the Hb(Mb) heme propionates because $b_5$ binds over a broad region, with a wide distribution of rapidly interconverting binding configurations. The binding constant is mostly determined by conformations where $b_5$ does not interact strongly with the propionates of Hb (or Mb), and with relatively small ET rate constants. The neutralization of the Hb(Mb) negative charges instead changes the *distribution* of binding conformations, resulting in a large *percentage* increase in the population of a *minority* set of conformations which involve the propionates and have a large ET rate constant. As a result, the overall binding constant changes minimally, yet the ET rate constant increases sharply. The contribution of these favorable conformations remains small, so that the overall binding constant changes minimally.

*Summary.* We have compared the photoinitiated ET reaction between $b_5$ and Zn-substituted Hb chains, dimers, and tetramers in order to determine whether $b_5$ binds to the subunit surface of either or both Hb chains or to sites which span the dimer−dimer interface. Together, these results show that the reaction of $b_5$ with Hb occurs at sites on the face of a subunit, rather than at sites which span the dimer−dimer interface. The charge-neutralization results further suggest that $b_5$ binds over a broad area of the subunit face, but reacts only in a minority population of binding geometries. The present study further suggests that α-chains are a particularly attractive vehicle for future studies of interprotein recognition and ET with $b_5$. They are monomeric, like Mb (25), and thus amenable to detailed theoretical and experimental investigation. However, the binding of $b_5$ to α-chains is tighter than to Mb, such that it is possible to determine $K$ and $k$ independently for α-chains, whereas only the product has been determined for Mb.

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