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Oxygen Equilibrium and EPR Studies on α1β1 Hemoglobin Dimer

Balan Venkatesh[1], Gentaro Miyazaki[1], Kiyohiro Imai[2], Hideki Morimoto[1] and Hiroshi Hori[1,*]

[1]Division of Bioengineering, Graduate School of Engineering Science, Osaka University, Toyonaka, Osaka 560-8531; and [2]Department of Materials Chemistry, Faculty of Engineering Hosei University, Koganei, Tokyo 184-8584

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We undertook this project to clarify whether hemoglobin (Hb) dimers have a high affinity for oxygen and cooperativity. For this, we prepared stable Hb dimers by introducing the mutation Trp→Glu at β37 using our *Escherichia coli* expression system at the α1β2 interface of Hb, and analyzed their molecular properties. The mutant hybrid Hbs with a single oxygen binding site were prepared by substituting Mg(II) protoporphyrin for ferrous heme in either the α or β subunit, and the oxygen binding properties of the free dimers were investigated. Molecular weight determination of both the deoxy and CO forms showed all these molecules to be dimers in the absence of IHP at different protein concentrations. Oxygen equilibrium measurements showed high affinity and non-cooperative oxygen binding for all mutant Hb and hybrid Hb dimers. However, EPR results on the [α^{N}(Fe-NO)β^{M}(Mg)] hybrid showed some α1β1 interactions. These results provide some clues as to the properties of Hb dimers, which have not been studied extensively owing to practical difficulties in their preparation.

Key words: cooperativity, EPR, Hb dimers, hybrid hemoglobin, mutagenesis, oxygen binding.

Abbreviations: HbA, adult human hemoglobin; βW37E, recombinant hemoglobin with the β37 Trp→Glu mutation; PPIX, protoporphyrin IX; IHP, inositol hexakisphosphate, [α^{N}(Mg)β^{M}(Fe)], hybrid hemoglobin containing Mg(II) protoporphyrin IX in the native (N) α-subunit and ferrous protoheme in the mutant (M) β-subunit (βW37E); [α^{N}(Fe)β^{M}(Mg)], hybrid hemoglobin complementary to [α^{N}(Mg)β^{M}(Fe)]; XLHb, normal human Hb which has been cross linked between its two α99 lysine residues by reaction with bis (3,5-dibromosalicyl) fumarate.

For several decades it has been assumed that free hemoglobin (Hb) dimers have a high affinity for oxygen and show no co-operativity. Many studies in the past involving isolated α and β chains, as well as αβ dimers (using low concentrations of native Hb), demonstrated high ligand affinities without significant cooperativity (*1*, *2*). It is not easy to study the properties of free Hb dimers in their native form due to technical difficulties in their isolation and stabilization and dimers are susceptible to changes, which include the autoxidation of Hb (*3*). Only one report has appeared in the past in which deoxy-Hb dimers were stabilized by the addition of 0.9 M $MgCl_2$ to human des-Arg 141α-Hb (*4*). Another promising way to stabilize dimers in both the oxy and deoxy forms would be to replace some of the amino acids at the α1β2 interface using protein engineering (*5*–*7*).

The Hb tetramer can be regarded as an assembly of two αβ dimers joined via the α1β2 interface, which plays a major role in determining both the stability and ligand binding properties of the tetramer. There are two regions at the α1β2 interface, namely the hinge region (the region between the FG corner and C terminus of the α-subunit and the C helix of the β-subunit, which lies close to the rotation axes) and the switch region (the region between the FG corner and the C terminus of the β-subunit and the C helix of the α-subunit). One previous study indicated that a W37β mutation in the hinge region caused destabilization of the tetramer, which resulted in increased oxygen affinity (*6*). However, there have been few experiments performed on Hb dimers to check for high oxygen affinity and cooperativity.

In the present study, we adopted a W37→E mutation in the β chain to stabilize the αβ dimer (*6*), and prepared the αβ dimer in a stable form by site-directed mutagenesis and expression in *E. coli*. Further, we prepared hybrid Hbs with a single oxygen binding site by substituting Mg for Fe either in the α-subunit [α^{N}(Mg)β^{M}(Fe)] or the β-subunit [α^{N}(Fe)β^{M}(Mg)] to freeze one heme in the deoxy state and to ease the evaluation of the first oxygen binding constant, K_1 (which has not been easily determined before). Based on molecular weight determination and oxygen binding experiments, the dimer-tetramer equilibrium in these molecules is discussed. Further, the results of EPR studies of ^{14}NO-bound mutant Hb and mutant hybrid Hb are discussed.

MATERIALS AND METHODS

Preparation of Mutant β-Chain—The mutant β-chain (βW37E) was synthesized as reported earlier (*8*). The selected mutation was introduced into the human β-globin gene using an M13 phage vector (M13mp18-CIIFXβ) and *E. coli* cells (ST7-TG1). It is to be noted that valine-to-methionine substitution is necessitated by the difference in amino terminal processing in mammals and bacteria. Therefore, the β37 mutant also contains Met

*To whom correspondence should be addressed. Tel: +81-6-6850-6551, Fax: +81-6-6850-557, E-mail: hori@bpe.es.osaka-u.ac.jp

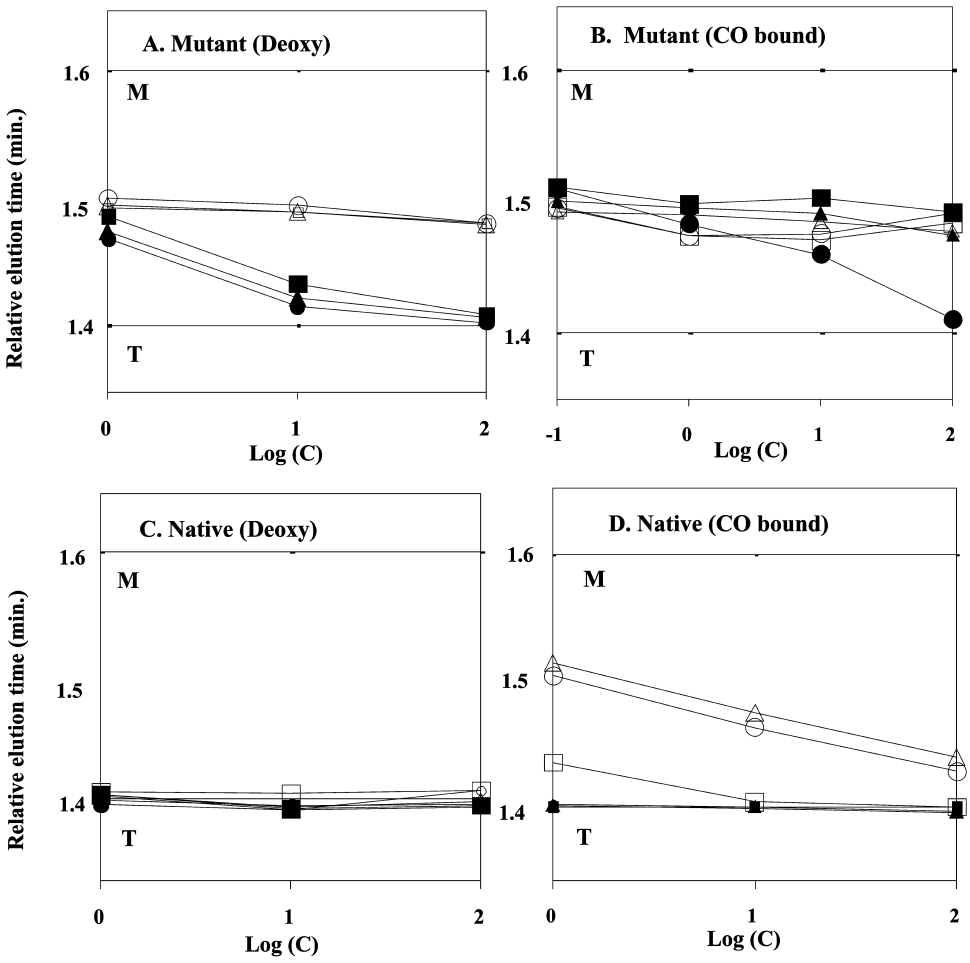


Fig. 1. **(Top) Plot of molecular weight analysis based on gel filtration experiments.** Here, the concentration (in μM) of native and βW37E mutant hemoglobins in logarithmic scale is plotted against relative elution time. The plots on the left show the deoxy samples; plots on the right show CO bound samples. Open symbols represent no IHP addition, while closed symbols represent samples containing 2 mM IHP. Standards, Mb (monomer, M) and XLHb (tetramer, T), were used as reference; open squres, native or mutant hemoglobin; open circles, native or βW37E mutant hybrid [α(Fe)β(Mg)]; open triangles, native or βW37E mutant hybrid [α(Mg)β(Fe)]. A and B represent mutant samples in the deoxy and CO-bound form, respectively; C and D represent native samples in the deoxy and CO-bound form, respectively.

substituting for Val-1 (βV1M). The mutation in the β-globin was confirmed by fast-atom-mass spectrometry on endo-peptidase digests. The expected molecular weight of mutant β (1M, 37E) is 15,842, while that of native β is 15867. We determined a molecular weight of 15,842, in agreement with the expected molecular weight.

Preparation of Mutant Hb and Its Hybrid Hb—Hb A was prepared by the method described in Ref. *9*, and isolated α and β chains were prepared by the one column method (*10*). Apo-α and apo-β chains were prepared by the acid-acetone method (*11*). Mg(II)-PPIX was prepared as described earlier (*12*). The α(Mg)-chain and mutant β(Mg)-chains were prepared according to the reported procedure (*13*). Mutant Hb was prepared by combining mutant apo β-globin with natural α-subunit [α^{N} (Fe)] to obtain a mutant semi Hb [α^{N}(Fe)β^{M} (–)], which was further purified (*8*). Hemin cyanide was added to this semi Hb at a molar ratio of 1:1.2, and the product was reduced with sodium dithionite under a carbon monoxide (CO) atmosphere, yielding mutant Hb [α^{N}(Fe-CO)β^{M}(Fe-CO)], which was further purified. For the preparation of mutant hybrids [α^{N}(Mg)β^{M}(Fe)], mutant Hb [α^{N}(Fe-CO)β^{M}(Fe-CO)] was treated with *p*-hydroxy mercuribenzoate and purified according to reported procedures (*14*) to yield α^{N}(Fe-CO) and β^{M}(Fe-CO). We removed CO from β^{M}(Fe-CO) by passing oxygen gas through the solution under illumination with a 200 W tungsten lamp for 30 min on ice, and the solution was then mixed with α^{N}(Mg) to give [α^{N}(Mg)β^{M}(Fe)]. The counter mutant hybrid [α^{N}(Fe)β^{M}(Mg)] was prepared by mixing β^{M}(Mg) with α^{N}(Fe). All samples were purified on ion-exchange columns (CM23 and DE23, Whattman) using 20 mM Tris/Cl, pH 7.2, as described previously (*13*). Purity was checked by isoelectric focusing.

Gel Filtration—Molecular weight determinations of the CO and deoxy derivatives of HbA, mutant Hb and mutant hybrid Hb were carried out at 25°C using Superdex S-200 (Pharmacia) packed in a 1 × 25 cm column connected to an HPLC pump (Shimadzu LC10AD). The elution patterns were monitored at 540 nm for the CO form and at 550 nm for the deoxy form (100 μM concentration), or at 420 nm for the CO form (10 to 0.1 μM concentrations). The column was calibrated with XLHb (*6*) and myoglobin (Sigma, MO, USA) using 50 mM Tris buffer containing 100 mM chloride, pH 7.4, at a flow rate of 0.25 ml/min. The buffer tank, HPLC pump and oxygen sensor (Iijima Electronic Corporation, Japan, Model MG 7G) were all kept inside an anaerobic glove bag in which the oxygen concentration was maintained below 0.001%. The spectra of the deoxy samples were recorded before and after passage through the column. Samples in the CO-bound form were diluted to final concentrations of 100, 10, 1, or 0.1 μM and mixed with blue dextran solution (10 mg/1 ml) to a final concentration of 0.1%. The difference in the elution time of an Hb sample to that of blue dextran was used to estimate the molecular weight of the

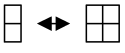
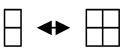
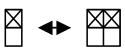
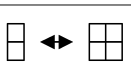
Samples	Deoxy condition		CO bound condition	
	+ IHP	- IHP	+ IHP	- IHP
[α^N(Fe)β^M(Fe)]				
[α^N(Fe)β^M(Fe)]				
[α^N(Mg)β^M(Fe)]				
HbA (Native)				
[α^N(Fe)β^N(Mg)]				
[α^N(Mg)β^N(Fe)]				

Fig. 2. **Schematic representation showing tetramer ↔ dimer equilibrium states.** Native and βW37E mutant Hb and hybrid Hb molecules at 100 μM concentration in both the deoxy and CO-bound forms, with and without 2 mM IHP are represented. The tetramer is designated as four square boxes joined together; the dimer is shown as two square boxes joined together. When a ligand is present in either the α or β chain, it is represented as a cross in the box.

sample. Deoxy Hb was examined by deaerating the sample in a rubber capped cuvette adaptor, and by the addition of 1 mM dithionite and deaerated blue dextran solution, and then injecting the solution onto the top of the column with a gas tight Hamilton syringe to a final concentration of 100, 10, or 1 μM. In the case of IHP-added samples, an elution buffer containing 2 mM IHP was used.

Oxygen-Binding Experiments—Oxygen equilibrium curves (OEC) were measured with an Imai apparatus and analyzed according to the Adair equation (*15*). The concentration of the Hb samples was 10 μM on a heme basis, and the temperature in the oxygenation cell was maintained at 25°C ± 0.05°C. The details of the buffers used are given in Table 1 as a footnote. To maintain the metHb content at minimum levels, an enzyme reducing system was added to each sample (*16*). The metHb content was estimated to be between 2 and 7%.

Electron Paramagnetic Resonance (EPR)—EPR for mutant Hb (βW37E) and mutant hybrids [α^N(Mg)β^M(Fe)], [α^N(Fe)β^M(Mg)] with nitric oxide (^{14}NO) bound to the ferrous heme was recorded at 77 K using a Varian E12 spectrometer operating at 9.36 GHz. The concentration of the samples was 100 μM in 50 mM Tris buffer containing 100 mM Cl^-, pH 7.4.

RESULTS AND DISCUSSION

The tetramer-dimer equilibrium of Hb is affected by the ligation state (*15*), protein concentration (*1*), and effectors such as IHP (*17*). In the present experiments, we tried to stabilize the dimer by introducing a Trp→Glu mutation at the β37 position. Further, hybrids with a single oxygen binding site were prepared by substituting Mg(II)PPIX for the proto heme in either the α-subunit [α^N(Mg)β^M(Fe)] or β-subunit [α^N(Fe)β^M(Mg)]. By using Mg(II) (the lowest affinity metal ion) instead of Fe(II), the error caused by the presence of metHb on the determination of K_1 value could be prevented.

Molecular weight analysis is based on the position of the eluted protein peak with respect to XLHb (tetramer) and Mb (monomer). The eluted sample is completely dimeric if the observed peak position is between the tetramer and monomer. If the peak position is between the tetramer and dimer, then we assume equilibrium between the tetrameric and dimeric forms. In the 100 μM concentration range, these mutant Hbs behave as dimers in both the deoxy and CO-bound forms in the absence of IHP (Fig. 1, A and B). In the presence of 2 mM IHP, there is an equilibrium between dimers and tetramers in all deoxy Hbs examined, while of the CO-bound forms, only [α^N(Fe)β^M(Mg)] shows an equilibrium between tetramers and dimmers, while the other two still behave as dimers (Fig. 1B). The native HbA and its Fe-Mg hybrid Hb behave as tetramers in the deoxy form in both the presence and absence of IHP, while the CO-bound form of these molecules behave as tetramers in the presence of IHP, but show an equilibrium between tetramers and dimers in the absence of IHP (Fig. 1, C and D). The tetramer ↔ dimer equilibrium states of the native and βW37E mutant Hb and hybrid Hb molecules at 100 μM concentration are shown in Fig. 2.

The EPR spectra of the ^{14}NO-bound native and mutant Hbs and hybrid Hbs are shown in Fig. 3. It is to be noted that the T- and R-quaternary states are by definition exclusively for tetrameric Hb, since they deal with the packing mode of two α1β1 dimers. In the case of native nitrosyl HbA, it is well known that the addition of IHP results in R→T transition, causing a weakening of the bond strength between the iron and heme linked histidine, and that an equilibrium is set up between the 5- and 6-coordinated hemes of the α-subunit within the Hb tetramer (*17–19*). The 5-coordinated ^{14}NO heme within the α-subunit shows characteristic triplet hyperfine

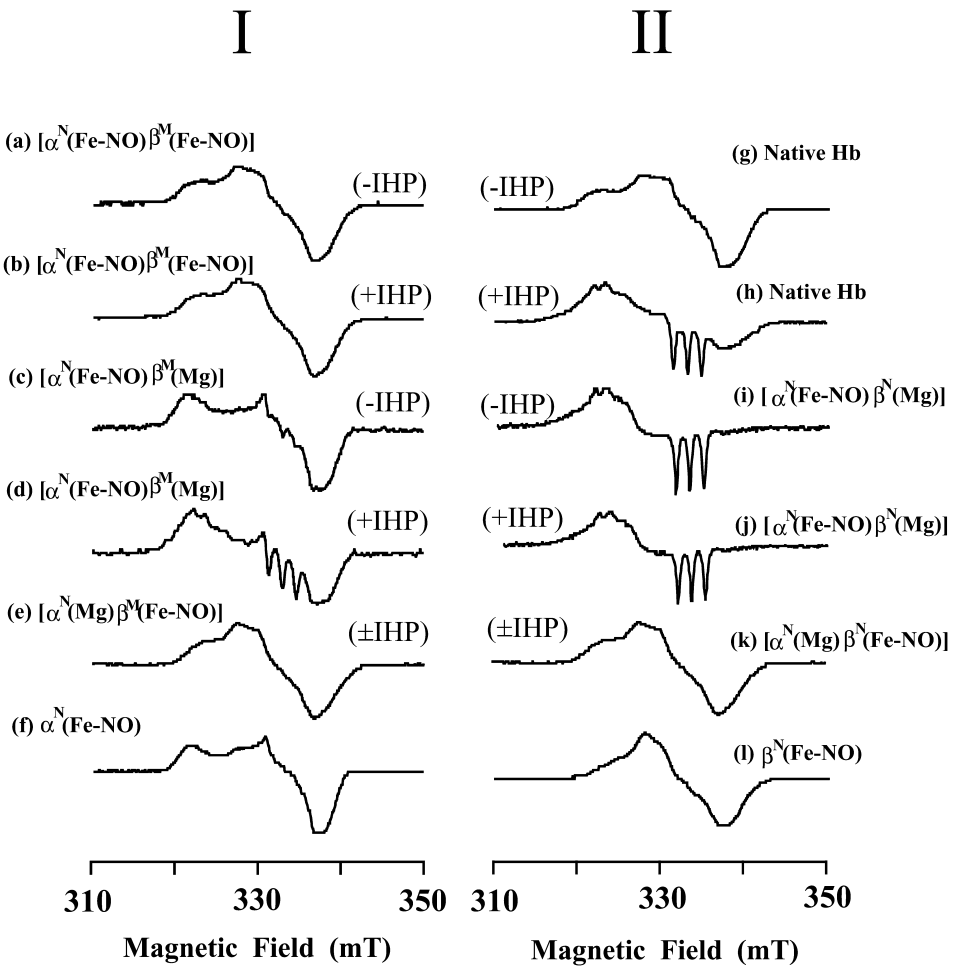


Fig. 3. **(Left, I) EPR spectra of mutant NO complexes of (a), [α^N(Fe-NO)b^M(Fe-NO)] without IHP; (b), [α^N(Fe-NO)β^M(Fe-NO)] with 2 mM IHP; (c), [α^N(Fe-NO)β^M(Mg)] without IHP; (d), [α^N(Fe-NO)β^M(Mg)] with 2 mM IHP; (e), [α^N(Mg)β^M(Fe-NO)] with and without 2 mM IHP; (f), isolated α^{NO} chains.** (Right, II) EPR spectra of native NO complexes (g), native Hb without IHP; (h), native Hb with 2 mM IHP; (i), [α^N(Fe-NO)β^N(Mg)] without IHP; (j), [α^N(Fe-NO)β^N(Mg)] in with 2 mM IHP; (k), [α^N(Mg)β^N(Fe-NO)] with and without 2 mM IHP; (l), isolated α^{NO} chains. Experimental conditions: 100 μM (heme) in 20 mM Tris buffer, pH 7.4.

structure, which serves as a T state marker. Such transitions are not possible in the case of [α^N(Fe-NO) β^N(Mg)] hybrid tetramers, since these molecules are already switched to the T-state. In the case of mutant nitrosyl Hb (βW37E), such an R → T-like transition induced by IHP does not occur. However, in the case of the [α^N(Fe-NO)β^M(Mg)] hybrid without IHP, a small degree of triplet hyperfine structure can be observed (Fig. 3c). This is clearly evident in comparison with the EPR spectrum of the mutant nitrosyl hybrid Hb in the absence of IHP (Fig. 3a). This could be due to the existence of a minor tetramer-dimer equilibrium or due to interaction between the α1 and β1 subunit. The EPR spectrum of the nitrosylated α subunit of [α^N(Fe-NO)β^M(Mg)] in the presence of IHP exhibits a mixture of the 5- and 6-coordinated ^{14}NO heme (Fig. 3d). It is very difficult to measure the molecular weight of NO bound samples. By comparing [α^N(Fe-NO)β^M(Mg)] with the CO form (Fig. 1), it may appear that there is an equilibrium between dimers and tetramers. However, allosteric effects are ligand-dependent, and it is not possible to compare directly data involving different ligands (that is, CO and NO).

Tetrameric [α^N(Mg)β^N(Fe)], [α^N(Fe)β^N(Mg)] hybrid Hbs have been observed to have the T-quaternary structures with extremely low oxygen affinities because the Mg ion is responsible for stabilizing the extreme T state (*13*). Thus we believed that having one Mg in either the α- or β-subunit of the dimeric Mg-Fe hybrids would produce low affinity states in these dimers if there is communication between the α1 and β1 subunits. However, we observed high oxygen affinity for all the hybrids. The results of OEC measurements on mutant Hb (βW37E) and mutant hybrids [α^N(Mg)β^M(Fe)], [α^N(Fe)β^M(Mg)] are summarized in Table 1 in comparison the data for native HbA, α- and β-chains. To summarize the oxygen binding data (Table 1), all the mutant Hb and hybrid Hbs in the absence of IHP showed high oxygen affinity with non-cooperativity. There is no systematic pH dependence in the P_{50} (or K_1) values. Upon the addition of IHP at pH 7.4, the oxygen affinity is dropped two-fold for Hb (βW37E) and three-fold for [α^N(Mg)β^M(Fe)]. This may be due to stabilization of the tetramer in the deoxy condition in the presence of IHP. It is to be noted that molecular weight analysis at 10 μM concentration (in order to reduce the tetramer contribution) showed all samples to be dimers in both the ligated and non-ligated forms in the absence of IHP, while in the presence of IHP, an equilibrium between tetramers and dimers was found for all samples except mutant Hb and hybrid [α^N(Mg)β^M(Fe)] in the ligated forms (Fig. 1). The possible role of MgPPIX in the β-subunit to stabilize the lower oxygen affinity state of [α^N(Fe)β^M(Mg)] cannot be ruled out. Also, the K_1 value of the dimer is two orders of magnitude greater than that of HbA in the tetrameric form, as shown in Table 1.

In conclusion, the results demonstrate that the βW37 position in the hinge region of the α1β2 interface of the

Table 1. **Oxygen equilibrium parameters for HbA, α-chain, β-chain, mutant Hb [α^N(Fe)β^M(Fe)] and mutant hybrids [α^N(Fe)β^M(Mg)], [α^N(Mg)β^M(Fe)].**

		no IHP added			+ 2 mM IHP			Reference
		P_{50}[a]	n_{max}[b]	K_1[c]	P_{50}[a]	n_{max}[b]	K_1[c]	
HbA (Native)	pH 7.4	3.6	2.6	0.022	44.0	2.5	0.005	(*1*)
α-chain	pH 7.4	0.56	1.0	1.79	0.59	1.0	1.70	this work
β-chain	pH 7.4	0.30	1.0	3.33	0.43	1.0	2.33	this work
[α^N(Fe)β^M(Fe)]	pH 6.4	0.82	1.0	1.22	1.6	1.2	0.63	this work
	pH 7.4	0.59	1.0	1.70	1.0	1.1	0.91	this work
	pH 8.4	0.66	1.0	1.52	0.68	1.0	1.47	this work
[α^N(Fe)β^M(Mg)]	pH 6.4	0.54	1.0	1.85	2.1	1.1	0.48	this work
	pH 7.4	0.50	1.1	2.0	1.1	1.1	0.91	this work
	pH 8.4	0.50	1.0	2.0	0.41	1.0	2.44	this work
[α^N(Mg)β^M(Fe)]	pH 6.4	1.1	1.0	0.91	6.5	1.0	0.15	this work
	pH 7.4	0.98	1.0	1.02	3.1	1.0	0.32	this work
	pH 8.4	0.72	1.0	1.39	0.84	1.0	1.19	this work
[α^N(Fe)β^N(Mg)]	pH 7.4	56.0	1.3	0.010	184.0	1.0	0.005	(*13*)
[α^N(Mg)β^N(Fe)]	pH 7.4	57.0	1.1	0.014	169.0	1.0	0.006	(*13*)

Experimental conditions: Hb concentration, 10 μM on heme basis, in 0.01 M Tris (pH 8.4) or 0.05 M bis-Tris (pH = 7.4 and 6.4) containing 0.1 M Cl^-; 25°C; [a]Partial pressure of oxygen at half saturation; [b]Hill's coefficient (maximum slope of the Hill plot); [c]Association equilibrium constant for the first binding oxygen ($mmHg^{-1}$).

Hb molecule exercises control over tetramer-dimer equilibrium, and that this interface plays a major role in determining both the stability and cooperative ligand binding properties of Hb. The present OEC results demonstrate that dimers possess a high affinity for oxygen; if there are interactions between the α and β subunits via the α1β1 (or α2β2) interface (in free Hb dimer), the oxygen affinity should be very weak. However, the EPR results for the [α^N(Fe-NO)β^M(Mg)] hybrid display some α1β1 interactions that cannot be simply ignored. A clearer picture will emerge when the molecular weight analysis of the NO bound hybrids are available. Our present results on dimers with two ligands bound and one ligand bound either in the α or β chain under moderate solution conditions is a first step toward gaining a clear understanding of the properties of Hb dimers.

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