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Determination of Oxygen Binding Properties of the Individual Subunits of Intact Human Adult Hemoglobin

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We determined the oxygen (O_2) binding properties of the individual subunits of intact human adult hemoglobin (Hb A) through the combined use of ^{1}H NMR and conventional spectrophotometric methods. We found that the O_2 affinities of the α and β subunits of Hb A are almost indistinguishable in the absence of an allosteric effector, inositol hexaphosphate (IHP), and lower by factors of ≈8.4 and ≈10, respectively, in the presence of IHP at pH 7.40, as judged from the estimated P_{50} values, which represent the partial O_2 pressure required to achieve 50% oxygenation of the individual subunits. These results demonstrated that the binding of IHP to the protein resulted in remarkable enhancement of the degree of nonequivalence in functional properties between the constituent subunits, which could be relevant to its cooperative ligand binding. This finding provides novel insights for elucidation of the molecular mechanisms responsible for the cooperativity in this allosteric protein.

Human adult hemoglobin (Hb A), a tetrameric oxygen (O_2)-transport hemoprotein, has served as an excellent model for investigating the structure–function relationships in allosteric proteins. Hb A consists of two α subunits of 141 amino acid residues and two β subunits of 146 residues, respectively.[1] The structures of the two types of subunits are quite similar to each other,[2] and each of the subunits contains one heme (Fe–protoporphyrin IX complex) as a prosthetic group, to which O_2 binds. Hence Hb A can bind up to four O_2 molecules. In the course of stepwise oxygenation of the protein, the subunits cooperate with each other so that oxygenation of one of them results in enhancement of the O_2 affinities of the others. This is known as cooperative O_2 binding, and is essential for efficient O_2 transport by the protein. Despite enormous efforts to characterize the cooperative O_2 binding of the protein,[1–13] the molecular mechanism has remained largely to be elucidated. One of the difficulties in Hb A studies is the lack of resolution as to the characterization of functional properties of the protein. Generally, the O_2 binding properties of the protein are characterized spectrophotometrically,[14] because of the high sensitivity of absorption spectra to the heme Fe coordination state.[15] However, the resolution of the spectra is not high enough to differentiate the heme environments of the individual subunits of the protein, and hence the O_2 affinities of the individual subunits of intact Hb A have never been quantitatively determined, although some attempts have been made.[16]

In this study, we determined the O_2 binding properties of the individual subunits of intact Hb A from the degrees of oxygenation of the individual subunits of the protein (Φ_i, i = α or β for the corresponding subunit), obtained through analysis of their ^{1}H NMR signal intensities, and the reported oxygen equilibrium curve (OEC)[17] for the protein. We found that the O_2 affinities of the α and β subunits of Hb A are almost indistinguishable, in the absence of an allosteric effector, inositol hexaphosphate (IHP), and lower by factors of ≈8.4 and ≈10, respectively, in the presence of IHP at pH 7.40 and 25 °C.

Experimental

Sample Preparation. Hb A was prepared from blood obtained from the Medical Center of the University of Tsukuba using the reported procedure.[18] Met-aquo Hb A was prepared by the addition of a 5-fold molar excess of potassium ferricyanide (Wako Pure Chem. Ind., Ltd.). The protein was separated from the residual reagents with a Sephadex G-25 (Sigma Chemical Co.) column equilibrated with 20 mM Tris (Sigma Chemical Co.), pH 8.0, and 20 mM NaC1. Then, the protein was concentrated to about 1 mM in nominal ^2H_2O (80% ^1H_2O/20% ^2H_2O) in an ultrafiltration cell (Amicon). Fully deoxygenated Hb A (deoxy Hb A) was prepared from met-aquo Hb A by the addition of a 1.5-fold molar excess of sodium dithionite (Nakalai Chem., Ltd.). Deoxy Hb A was fully and partially exposed to air to prepare fully oxygenated Hb A (oxy Hb A), and a mixture of intermediately oxygenated species in addition to deoxy and oxy Hb A's, respectively. In order to attain full equilibration as to oxygenation of the protein at a given P_{O_2} value, the protein solution was thoroughly mixed in the NMR sample tube. Slow autoxidation of the protein did not disturb the measurements.[19] In order to determine the effect of IHP on the O_2 binding properties of Hb A, a 25-fold molar excess of IHP was added to the protein. Considering that the binding constant of IHP to Hb A at pH 7.40 and 25 °C is $2.7 \times 10^7\,M^{-1}$,[17] the addition of a 25-fold molar excess of IHP should be enough to achieve complete complexation between them.

NMR Spectroscopy. NMR spectra of the protein were recorded on a Bruker Avance 400 FT NMR spectrometer

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operating at the ^{1}H frequency of 400 MHz. Typical spectra of the protein required a 100 kHz spectral width, 32×10^3 data points, a ≈6 μs 45° pulse, a recycle time of 1.0 s, and 4000 scans, and the water signal was suppressed with a 100 ms presaturation pulse. In order to ensure that the relative concentrations of deoxy and oxy Hb A's could be accurately determined through analysis of the NMR signal intensities, the pulse width and recycle time were optimized. Since the spin–lattice relaxation time (T_1) of the ValE11 $C_{\gamma'}H_3$ proton signal of oxy Hb A was determined to be 0.98 ± 0.15 s (result not shown), the magnetization for the ValE11 $C_{\gamma'}H_3$ proton is expected to assure >99% recovery after each cycle in NMR measurements using a 45° pulse and a recycle time of 1.0 s. Obviously, the magnetization for the HisF8 $N_\delta H$ proton of deoxy Hb A should be fully recovered after each cycle in the measurements because of its extremely short T_1, possibly <1 ms, due to effective paramagnetic relaxation mechanisms. Chemical shifts are given in ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate, with H_2O as an internal reference.

Analysis of OECs. The OECs were analyzed by the following equation proposed by Monod, Wyman, and Changeux,[20] in which the cooperative O_2 binding of the protein is accounted for by a P_{O_2}-dependent transition between the two different quaternary structures exhibiting low and high O_2 affinities, which are generally represented by the O_2 equilibrium constants K_T and K_R, respectively.

$$Y_i(P_{O_2}) = \{L_{0i}\ K_{Ti}\ P_{O_2}(1 + K_{Ti}\ P_{O_2})^3 + K_{Ri}\ P_{O_2}(1 + K_{Ri}\ P_{O_2})^3\} / \{L_{0i}(1 + K_{Ti}\ P_{O_2})^4 + (1 + K_{Ri}\ P_{O_2})^4\} \quad (1)$$

where $Y_i(P_{O_2})$ is the fractional saturation of subunit i (α or β) of the protein at a given P_{O_2} value and L_{0i} is the allosteric constant of the subunit. The fitting of the OEC for subunit i to the equation yielded values for L_0, K_T, and K_R of the subunit. Nonlinear least-squares fitting was carried out in order to determine these values using the Levenberg–Marquardt algorithm implemented in KaleidaGraph 3.5 package (Synergy Software, Reading, PA).

Results

In a ^{1}H NMR spectrum of deoxy Hb A (Figure 1A), the signals due to the HisF8$N_\delta H$ protons of the subunits are resolved at ≈70 ppm, and the signals at 63.4 and 75.7 ppm have been assigned to the NH protons of the α and β subunits, respectively.[21,22] On the other hand, upon oxygenation of the protein to yield oxy Hb A (Figure 1C), the ValE11 $C_{\gamma'}H_3$ proton signals of the subunits are most readily observed at −2.3 ppm,[23,24] because of their high intensities. Furthermore, upon insufficient oxygenation of the protein to yield a mixture of intermediately oxygenated species in addition to deoxy and oxy Hb A's (Figure 1B), the signals of the deoxy and oxy subunits are separately observed, because the time scale of the O_2 binding kinetics of deoxy Hb A is much slower than that of NMR, as in the case of O_2 storage hemoprotein myoglobin (Mb).[25] With such a slow exchange regime, the concentrations of the deoxy and oxy subunits are separately reflected in the intensities of their HisF8 $N_\delta H$ and ValE11 $C_{\gamma'}H_3$ signals, respectively. Since exchange rates of 0.8×10^{-4} and 1.0×10^{-5} s^{-1} have been obtained for the HisF8 $N_\delta H$ protons of the α and β subunits of deoxy Hb A at pH 7.24 and 25 °C, respectively,[26] the extremely slow exchange of the HisF8 $N_\delta H$ protons of the protein supported the validity of the quantitative analysis of the concentrations of deoxy subunits based on the intensities of their exchangeable $N_\delta H$ signals. Hence, the concentrations of the deoxy α and β subunits of the protein can be estimated through analysis of the intensities of the signals at 63.4 and 75.7 ppm, respectively, together with consideration of the solvent $^1H_2O/^2H_2O$ composition of the protein solution. On the other hand, analysis of the intensities of the ValE11 $C_{\gamma'}H_3$ signals at −2.3 ppm provides the total concentrations of oxy α and β subunits. Thus, the Φ_α and Φ_β values at a given P_{O_2} value can be calculated from the concentrations determined through analysis of these signals' intensities, and then the degree of oxygenation of the intact protein (Φ_{Hb}) can be calculated as the average of the Φ_α and Φ_β values. Finally, since the OEC of the protein represents the P_{O_2}-dependence of the Φ_{Hb} value, the P_{O_2} values used for the NMR measurements can be estimated from the OEC of the protein using the calculated Φ_{Hb} value. Consequently, the OECs of the α and β subunits of the intact protein can be obtained as plots of the Φ_α and Φ_β values against the estimated P_{O_2} values, respectively.

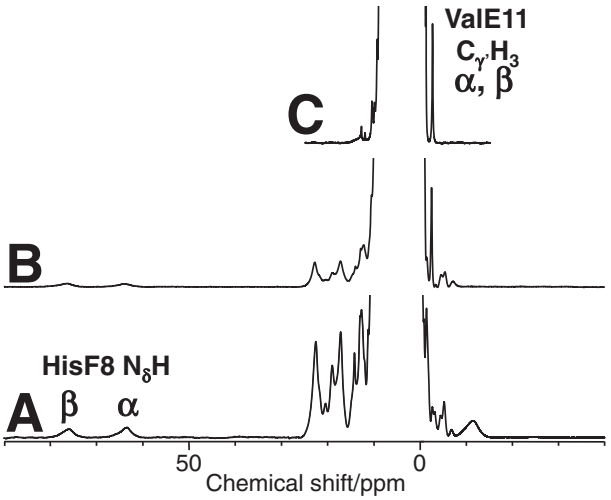

Figure 1. ^{1}H NMR spectra of deoxy Hb A (A), a mixture of intermediately oxygenated species in addition to deoxy and oxy Hb A's (B), and oxy Hb A (C) in 100 mM NaCl, 90% ^1H_2O/10% ^2H_2O at pH 7.40 and 25 °C. The assignments of selected signals[21–24] are given with the spectra.

We next analyzed the effects of IHP on the O_2 binding properties of the individual subunits within intact Hb A. The previously reported OECs of Hb A at pH 7.40 and 25 °C in the absence and presence of a 13-fold molar excess of IHP[17] are given in Figure 2. The P_{50} values, which represent the partial O_2 pressures required to achieve 50% oxygenation of the protein, were determined to be 5.3 and 48.8 mmHg for the protein in the absence and presence of IHP, respectively.[17] IHP has been shown to bind between the β subunits, and to significantly decrease the O_2 affinity of the protein.[27,28] The OEC results demonstrated that the binding of IHP to the protein increased the P_{50} value by a factor of ≈9.2.

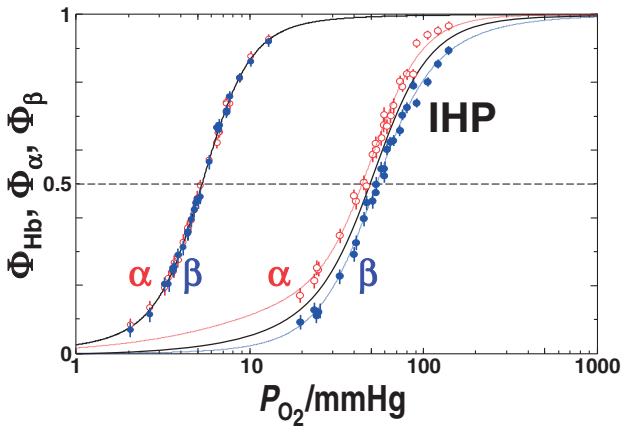

Figure 2. Oxygen equilibrium curves (OEC) for Hb A (solid curves) in the absence (left) and presence (right) of a 25-fold molar excess of inositol hexaphosphate (IHP), and 100 mM NaCl at pH 7.40 and 25 °C. OECs were taken from the literature.[17] Considering that the binding constant of IHP as to Hb A at pH 7.40 and 25 °C is $2.7 \times 10^7\,M^{-1}$,[17] the addition of a 25-fold molar excess of IHP to the protein is enough to achieve complete complexation between them. Plots of the Φ_α and Φ_β values, determined through analysis of the NMR signal intensities, against the P_{O_2} values, estimated from the Φ_{Hb} ($=(\Phi_\alpha + \Phi_\beta)/2$) value using the reported OEC[17] of Hb A, are indicated by red open circles and blue filled circles, respectively. The curves indicated by broken lines represent the fitting of the plots for the individual subunits using the equation of Monod, Wyman, and Changeux.[20]

The HisF8 $N_\delta H$ and ValE11 $C_{\gamma'}H_3$ signals of Hb A, in the absence and presence of a 25-fold molar excess of IHP, at a series of P_{O_2} values are compared in Figure 3. In the absence of IHP, the intensities of the HisF8 $N_\delta H$ proton signals of the α and β subunits decreased similarly to each other with increasing P_{O_2} (Figure 3A). The calculated Φ_α and Φ_β values are plotted against the P_{O_2} values in Figure 2, the plots yielding P_{50} values of 5.28 ± 0.11 and 5.36 ± 0.13 mmHg for the α and β subunits, respectively. These results are consistent with those of previous studies, which suggested that the O_2 affinities of the individual subunits of intact Hb A are almost indistinguishable in the absence of IHP.[29] In contrast, in the presence of IHP, the P_{50} value of the α subunits was found to be distinctly smaller than that of the β values, as reflected in the ^{1}H NMR spectra in Figure 3B, which revealed that the intensity of the HisF8 $N_\delta H$ signal of the α subunits is smaller than that of the β values at any P_{O_2} value insufficient for complete oxygenation of the protein. P_{50} values of 44.3 ± 1.9 and 53.7 ± 2.7 mmHg were obtained for the α and β subunits, respectively, from the plots of the Φ_α and Φ_β values against the P_{O_2} values (Figure 2). Hence, the ≈9.2-times increase in the P_{50} value of Hb A due to the IHP binding[17] was found to have resulted from the ≈8.4- and ≈10-times increases in the P_{50} values of the α and β subunits, respectively.

We then analyzed the OECs of the individual subunits, in the presence of IHP, using the well-known equation proposed by Monod, Wyman, and Changeux[20] to determine the K_T and K_R values. The cooperative O_2 binding of the protein is accounted for by a P_{O_2}-dependent transition between the two different quaternary structures exhibiting low and high O_2 affinities represented by K_T and K_R, respectively (Table 1). The K_T and

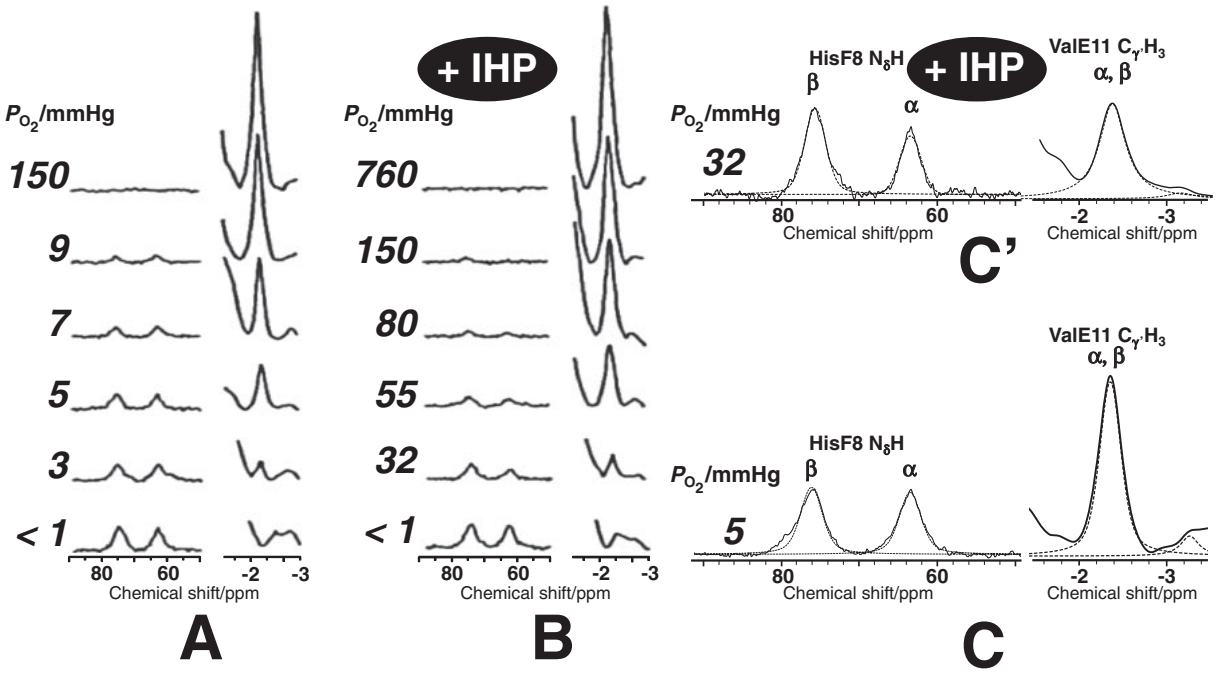

Figure 3. The HisF8 $N_\delta H$ proton signals of the deoxy subunits and the ValE11 $C_{\gamma'}H_3$ proton signals of the oxy subunits at various partial oxygen pressure values (P_{O_2}) in the absence (A) and presence (B) of a 25-fold molar excess inositol hexaphosphate (IHP) in 100 mM NaCl, 90% ^1H_2O/10% ^2H_2O at pH 7.40 and 25 °C. The numbers represent the P_{O_2} values in mmHg. Fitting was performed on both the HisF8 and ValE11 proton signals at $P_{O_2} = 5$ mmHg, in the absence of IHP, (C) and at $P_{O_2} = 32$ mmHg, in the presence of a 25-molar excess of IHP, (C′) to the Lorentzian function for analysis of the NMR signal intensities.

Table 1. P_{50} and O_2 Equilibrium Constants, K_T and K_R, of Hb A and Its Subunits at pH 7.40 and 25 °C

	P_{50}/mmHg	K_T/mmHg^{-1}	K_R/mmHg^{-1}
Hb A	5.3[a)]	≈0.0218[a),b)]	≈3.45[a),b)]
	(48.8[a)])[c)]	(≈0.00502[a),b)])[c)]	(≈0.915[a),b)])[c)]
α subunit	5.28 ± 0.11[d)]	≈0.0218[f)]	≈3.45[f)]
	$(44.3 \pm 1.9$[d)])[e)]	$(0.0084 \pm 0.00012$[g)])[e)]	$(14 \pm 12$[g)])[e)]
β subunit	5.36 ± 0.13[d)]	≈0.0218[f)]	≈3.45[f)]
	$(53.7 \pm 2.7$[d)])[e)]	$(0.0025 \pm 0.00019$[g)])[e)]	$(0.089 \pm 0.0048$[g)])[e)]

a) Taken from Ref. 17. b) Under physiological conditions, K_T and K_R values can be assumed to approximately equal to Adair constants[30,31] K_1 and K_4, respectively.[17] c) Measured in the presence of a 13-fold molar excess of inositol hexaphosphate (IHP). d) Obtained through analysis of the O_2 equilibrium curves of the individual subunits determined using the proposed technique. e) Measured in the presence of a 25-fold molar excess of IHP. f) The values of α and β subunits should be close to the corresponding ones of Hb A (see text). g) Obtained through analysis of the O_2 equilibrium curves of the individual subunits determined using the proposed technique. The allosteric constants L_o of α and β subunits of Hb A were calculated to be 2.1×10^7 and 4.7×10^2, respectively.

K_R values of the α subunit were determined to be 0.0084 ± 0.00012 and 14 ± 12 mmHg^{-1}, respectively, and those of the β subunit were 0.0025 ± 0.00019 and 0.089 ± 0.0048 mmHg^{-1}, respectively. The considerably large error in the K_R value of the α subunit was primarily due to the disagreement between the experimental and theoretical values at higher P_{O_2} values, particularly P_{O_2} > ca. 90 mmHg, as can be seen in the plots (Figure 3).

The cooperative O_2 binding of Hb A has been analyzed in detail on the basis of the intrinsic O_2 equilibrium constant K_i ($i = 1$–4) for the ith O_2 binding step, yielded on fitting of the OECs to the well-known Adair equation.[30,31] Under physiological conditions, the K_T and K_R values can be assumed to approximately equal to the K_1 and K_4 values, respectively.[17] Furthermore, in the absence of IHP, the K_T and K_R values of the individual subunits can also be considered equal to those of Hb A, because O_2 affinities of the constituent subunits were found to be almost indistinguishable from each other (Figure 3). The effects of IHP on the O_2 binding properties of the individual subunits of intact Hb A were clearly manifested in the K_T and K_R values of the subunits (Table 1). The IHP binding decreased the K_T values of the α and β subunits by factors of ≈1/3 and ≈1/9, respectively, i.e., upon the addition of IHP, the K_T value decreased from 0.0218 to 0.0084 ± 0.00012 mmHg^{-1} for the former and to 0.0025 ± 0.00019 mmHg^{-1} for the latter. As a result, the IHP binding resulted in remarkable enhancement of the degree of nonequivalence in the functional properties between the constituent subunits.

Discussion

Making use of the high sensitivity of ^{1}H NMR for distinguishing not only the individual subunits of Hb A,[29,30] but also the deoxy and oxy forms of the subunits, we have demonstrated here that the O_2 binding properties of the constituent subunits of the protein can be quantitatively characterized by the combined use of the ^{1}H NMR and conventional spectrophotometric methods. But, since the HisF8 N_δH proton signals of the deoxy protein are so broad, i.e., their line widths are >1000 Hz (Figures 1A and 1B), the eight possible intermediately oxygenated species as a result of the statistical distribution of Fe-bound O_2 among the four subunits within a Hb A molecule[3] cannot be differentiated even in its NMR spectra. Consequently, plots of the Φ_α and Φ_β values against the P_{O_2} values, determined with the present technique, could be considered as the OECs reflecting statistically averaged O_2 binding of the α and β subunits of the intact protein, respectively. In fact, the OEC of Hb A measured by the conventional spectrophotometric method also reflects such statistically averaged O_2 binding among the subunits within a protein molecule.

Characterization of the O_2 binding properties of Hb A using the present technique revealed that the P_{50} values of the α and β subunits of the protein at pH 7.40 and 25 °C are 5.28 ± 0.11 and 5.36 ± 0.13 mmHg, respectively, and that the addition of a 25-fold molar excess of IHP increases the values of the α and β subunits to 44.3 ± 1.9 and 53.7 ± 2.7 mmHg, respectively. Thus, upon the binding of IHP to Hb A, the O_2 affinity of the β subunit was found to more significantly decrease than that of the α one. This finding provides significant clues for elucidating the molecular mechanism responsible for control of the O_2 binding properties of Hb A.

The effects of IHP on the O_2 binding properties of the individual subunits of intact Hb A were characterized on the basis of the K_T and K_R values of the subunits (Table 1). The IHP binding decreased the K_T value of the β subunit more considerably than that of the α one. Since IHP binds between the β subunits of the protein,[28] the greater effect of the IHP binding on the O_2 affinity of the β subunit than that of the α subunit could be due to the direct interaction of IHP with the β subunits, although the molecular basis of this remains to be elucidated. Thus, the present study revealed that the O_2 affinity of the β subunit decreases to a much larger extent compared with that of the α one, although structural changes of Hb A induced upon the IHP binding have been shown to be within the $\alpha_1\beta_1$ (or $\alpha_2\beta_2$) dimer itself.[8] Additionally, the difference in the K_R value between the α and β subunits was much greater than that in the K_T value between them. This suggests, in terms of the Adair scheme,[31,32] that IHP remains bound to the protein until the binding of the fourth O_2 molecule.

In oxy Hb A and Mb, the binding of O_2 to the heme Fe atom has been shown to be stabilized significantly through distal hydrogen-bonding between the Fe-bound O_2 and distal His side chain.[33,34] The distal hydrogen-bonding in Hb A has been shown to be sensitive to changes in pH and temperature, and

such pH- and temperature-dependent properties of the distal hydrogen-bonding reasonably explained the changes in the O_2 affinity of the protein as a function of pH and temperature.[13] However, the greater effect of the IHP binding on the O_2 affinity of the β subunit than that of the α one could not be due to changes in the distal hydrogen-bonding, because this hydrogen-bonding has been shown to be affected only slightly by IHP binding.[13] On the other hand, protein dynamics have also been considered as an important factor for control of the functional properties of Hb A.[10–12] The dynamic features on the ms–μs time scale have been shown to be affected upon IHP binding to the protein.[12] Detailed characterization of the structural and dynamic properties of Hb A as well as the functional properties of the individual subunits of the protein is needed to elucidate the structure–function relationship in the protein.

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