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Role of $\beta/\delta 101$ Gln in Regulating the Effect of Temperature and Allosteric Effectors on Oxygen Affinity in Woolly Mammoth Hemoglobin[†]

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Abstract

The oxygen affinity of woolly mammoth hemoglobin (rHb WM) is less affected by temperature change than that of Asian elephant hemoglobin (rHb AE) or human adult hemoglobin (Hb A). We report here a biochemical-biophysical study of Hb A, rHb AE, rHb WM and three rHb WM mutants with amino acid substitutions at $\beta/\delta 101$ ($\beta/\delta 101$ Gln→Glu, Lys, or Asp) plus a double and a triple mutant, designed to clarify the role of the $\beta/\delta 101$ residue. The $\beta/\delta 101$ Gln residue is important for responding to allosteric effectors, such as phosphate, inositol hexaphosphate (IHP), and chloride. The rHb WM mutants studied generally have higher affinity for oxygen under various conditions of pH, temperature, and salt concentration, and in the presence or absence of organic phosphate, than do rHb WM, rHb AE and Hb A. Titrations for the O₂ affinity of these mutant rHbs as a function of chloride concentration indicate a lower heterotropic effect of this anion due to the replacement of $\beta/\delta 101$ Gln in rHb WM. The alkaline Bohr effect of rHb WM and its mutants is reduced by 20–50% compared to that of Hb A and is independent of changes in temperature, in contrast to what has been observed in the hemoglobins of most mammalian species, including human. The results of our study on the temperature dependence of the O₂ affinity of rHb WM and its mutant rHbs illustrate the important role of $\beta/\delta 101$ Gln in regulating the functional properties of these hemoglobins.

Hemoglobin (Hb) is responsible for transporting oxygen (O₂) in the blood. The O₂ affinity of hemoglobin is affected by allosteric effectors (e.g. H⁺, chloride, and organic phosphate) and temperature (1). Oxygenation of Hb is an exothermic process. The oxygen affinity of Hb decreases with increasing temperature. At higher temperatures, Hb binds oxygen less tightly and gives up its oxygen more readily. Consequently, more oxygen can be delivered to tissues during vigorous exercise. However, this phenomenon is problematic for patients undergoing therapeutic hypothermia (e.g., cardiac arrest, traumatic brain injury, etc.). Under hypothermic conditions, the Hb molecule in blood has higher oxygen affinity and is less efficient in delivering oxygen to the tissues. This property may also pose considerable challenges for heterothermic mammals because the blood in their extremities could experience large and rapid temperature changes in the Arctic environment. Lower effect of temperature on the O₂ affinity of Hb has been observed previously from some arctic

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SUPPORTING INFORMATION AVAILABLE

Figures 1S and 2S show the pH dependence of the oxygen-binding properties (*P*₅₀) and Hill coefficient (*n*₅₀) of the rHbs under various experimental conditions. Figure 3S shows exchangeable proton resonances of ¹H-NMR spectra of the rHbs in the CO form at 11, 29, and 37 °C. Tables 1S and 2S show the temperature dependence of the O₂ affinity and the Bohr effect of the rHbs under various experimental conditions. This material is available free of charge via the internet at <http://pubs.acs.org>

animals, such as Eskimo dog, reindeer, and musk ox (2–8), but the key amino acid residues for this property have not been confirmed. Recently, recombinant Hbs of woolly mammoth (rHb WM) and Asian elephant (rHb AE) have been successfully expressed in *Escherichia coli* and isolated for biochemical-biophysical studies (9, 10). The O₂ affinity of rHb WM is much less dependent on temperature than that of human normal adult Hb (Hb A) or rHb AE (9, 10). This property could have an adaptive role for woolly mammoths in having a unique hemoglobin to deliver O₂ to their extremities. Results from these studies can provide a framework for further research on the evolutionary origins and the structural underpinnings of an adaptable physiological trait for an essential protein in an extinct species.

We would like to identify the key amino acid residue(s) that render the O₂ affinity of woolly mammoth Hb less dependent on temperature. In both woolly mammoth and Asian elephant, Hb is composed of two α -type globin chains, like those in Hb A, and two fused β/δ -type globin chains (9). The primary amino acid sequence of woolly mammoth Hb differs from that of Asian elephant Hb at only one position in the α -globin chain (K5N) and three positions in the β/δ -globin chain (T12A, A86S, and E101Q), [(Table 1), (9)]. Several human Hb mutants with replacements at the β 101 position (β 101Glu→Gly, Lys, Gln, Asp, or Ala) have been identified (11–16). The oxygen binding and cooperativity properties of these mutants have demonstrated clearly the importance of this residue in regulating hemoglobin function (15). The β 101 residue is located between the critical β 99Asp and β 102Asn, which form inter-subunit H-bonds in the $\alpha_1\beta_2$ interface of Hb A (17). Thus, any changes in size and/or charge at the β 101 position could adversely affect the stability of the quaternary structure (18). Our previous studies on rHb WM have further suggested that β/δ 101Gln could alter the electrostatic interactions at the sliding interface of the Hb molecule, and creates additional H⁺-linked Cl[−] binding sites in mammoth Hb (9, 10). Recently, the crystal structures of woolly mammoth Hb in the deoxy, carbonmonoxy and aquomet forms have been elucidated. The overall quaternary structure of rHb WM is quite similar to that of Hb A (19). Hence, the replacement of β/δ 101Gln in rHb WM would be expected to significantly alter the functional properties of this protein, as has been observed with the human mutants (11–15). In our present study, the rHb WM mutants with β/δ 101 substitutions (β/δ 101Gln→Glu, Lys, or Asp) have been expressed and the effect of temperature on the O₂ affinity of these mutants has been determined. Our results demonstrate clearly that the β/δ 101Gln of rHb WM participates in regulating the effect of temperature on the O₂ binding. The molecular basis of the O₂ delivery of Hb by the cold-adapted mammalian lineages, such as woolly mammoth Hb, could provide new insights into designing a new generation of hemoglobin-based oxygen carriers (HBOCs) with the oxygen affinity independent of temperature, i.e., especially suitable for treating patients undergoing therapeutic hypothermia.

MATERIALS AND METHODS

Material

Hb A was isolated and purified from human normal blood samples obtained from the local blood bank as previously described (20, 21). Chemicals and restriction enzymes were purchased from major suppliers (Fisher, Sigma, Bio-Rad, Boehringer Mannheim, New England BioLabs, and United States Biochemicals, Inc.) and were used without further purification. The QuikChange Site-Directed Mutagenesis kit was purchased from Stratagene.

Construction of Expression Plasmids

The plasmids (pHE27E and pHE27M) for expressing rHb AE and rHb WM are available in our laboratory (9, 10). They were used as the templates with appropriate primers in the

polymerase chain reactions to generate the mutants, according to instructions from the QuikChange Site-Directed Mutagenesis kit. The desired mutations were confirmed by DNA sequencing.

Expression and Purification of rHbs

Protein expression and purification were carried out according to the protocol established in our laboratory (10, 20, 22). Briefly, plasmids were transformed into *E. coli* strain JM109 and fresh colonies were grown in minimal medium in a 20-L fermentor (B. Braun Biotech International, Model Biostat C) at 32 °C until the optical density reached ~10 at 600 nm. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added at a concentration of 24 mg/L to induce rHb expression. Hemin was added at 25 mg/L and growth was continued for at least another four hours. Cells were harvested by centrifugation and stored at –80 °C until needed. Recombinant proteins were purified according to the procedures previously described (20, 22). The molecular masses and N-terminal processing of the rHbs were determined with mass spectrometry and Edman degradation, respectively (20, 22). All the rHbs used in this study had the correct molecular weights and contained less than 5% methionine at the amino termini.

Oxygen-Binding Properties of rHbs

Oxygen-equilibrium curves for the rHbs were measured in 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES) or 0.1 M sodium phosphate (NaPi) buffers at 11, 20, 29, and 37 °C with a Hemox Analyzer (TCS, Medical Products Division, Southampton, PA). Experiments were conducted in the presence and absence of 0.5 mM inositol hexaphosphate (IHP), in the pH range 5.5 to 8.5. For these measurements, the rHbs were kept at 100–120 μM (in terms of heme) to minimize tetramer-dimer dissociation (10). Each sample was checked before and after measurements for met-hemoglobin (met-Hb) in a spectrophotometer. Data from samples with greater than 5% met-Hb were discarded and the experiments were repeated with new and freshly prepared samples. The partial pressure of O₂ at 50% Hb saturation (P_{50}), a measure of O₂ affinity, and the Hill coefficient (n_{50}), a measure of the cooperativity of the oxygenation process, were calculated for each oxygen-equilibrium curve. The P_{50} values (in millimeter Hg) have an accuracy of ±5% and the n_{50} values have an accuracy of ±10%.

Enthalpy of Oxygenation of rHbs

ΔH is calculated from the van't Hoff equation (Eq. [1]) according to the P_{50} values of the rHbs obtained from two different temperatures (T_1 and T_2 , in °K).

$$\Delta H(\text{kcal mol}^{-1} \text{O}_2) = -4.575 (T_1 T_2 / T_2 - T_1) \Delta \log P_{50} / 1000 \quad [1]$$

This ΔH value includes the heat of oxygen solvation (–3 kcal mol^{–1}). The ΔH values (kJ mol^{–1}, where 1 cal = 4.184 J) presented in the results have been corrected for this heat of solvation. P_{50} values have been determined at 11, 20, 29, and 37 °C. Therefore, the standard deviations of ΔH are derived from six sets of $\Delta \log P_{50}$ values.

¹H-NMR Spectroscopy

Proton NMR spectroscopy was utilized to detect tertiary or quaternary structural changes of these rHb mutants. ¹H-NMR spectra were obtained on Bruker Avance DRX-300 (results not shown) and DRX-600 NMR spectrometers. Hb samples (5% solutions, 3.1 mM in terms of heme) in 0.1 M sodium phosphate (NaPi) buffer at pH 7.0 in 95% water and 5% deuterium oxide (D₂O) were assessed at 11, 29, and 37 °C. A jump-and-return pulse sequence was

used to suppress the water signal. ^1H chemical shifts were indirectly referenced to the methyl proton resonance of the sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) through the use of the internal reference of the water signal at 4.76 ppm downfield of DSS at 29 °C.

RESULTS

Oxygen-Binding and Cooperativity Properties of rHbs

The primary amino acid sequence of Hb WM differs from that of Hb AE at only one position in the α -type globin chain (K5N) and at three positions in the β -type globin chain (T12A, A86S, and E101Q) [(Table 1), (9)]. In this study, we have designed: (i) three single mutants with the $\beta/\delta 101\text{Gln}$ residue of rHb WM replaced with Glu, Lys, or Asp; (ii) a double mutant, rHb WM ($\alpha 5\text{Asn} \rightarrow \text{Lys}$, $\beta/\delta 101\text{Gln} \rightarrow \text{Glu}$), which has the same residues as rHb AE at the $\alpha 5$ and $\beta/\delta 101$ positions; and (iii) a triple mutant, rHb WM ($\alpha 5\text{Asn} \rightarrow \text{Lys}$, $\beta/\delta 12\text{Ala} \rightarrow \text{Thr}$, and $\beta/\delta 86\text{Ser} \rightarrow \text{Ala}$), so that the primary sequence of this mutant is the same as that of rHb AE except at the $\beta/\delta 101$ position. This triple mutant can be considered as a single mutant of rHb AE in which the $\beta/\delta 101\text{Glu}$ is substituted with Gln (Table 1).

Mutations at the $\beta/\delta 101$ position significantly change the O_2 affinity of rHb WM (Table 2 and Supporting Information Figure 1S). Under stripped conditions (MES buffer), rHb WM ($\beta/\delta 101\text{D}$) has the lowest P_{50} value (1.8 mm Hg) among the five mutants studied, suggesting that the replacement of Gln with Asp at the $\beta/\delta 101$ position increases the O_2 affinity of rHb WM (5.0 mm Hg) the most. Interestingly, replacing the $\beta/\delta 101\text{Gln}$ with a positively charged side chain [rHb WM ($\beta/\delta 101\text{K}$)] also increases the O_2 affinity of rHb WM. The P_{50} value of rHb WM ($\beta/\delta 101\text{K}$) decreases to 2.4 mm Hg, higher than that of rHb WM ($\beta/\delta 101\text{D}$). When the $\beta/\delta 101\text{Gln}$ is replaced by Glu, as appears in the Hb A or rHb AE sequence, the rHb WM ($\beta/\delta 101\text{E}$) and the rHb WM (αN5K , $\beta/\delta 101\text{E}$) mutants have P_{50} values (7.4 and 7.6 mm Hg, respectively) similar to that of rHb AE (7.7 mm Hg) and slightly higher than that of rHb WM. The triple mutant of rHb WM differs from the primary sequence of rHb AE at only one position and can be considered as a rHb AE ($\beta/\delta 101\text{Glu} \rightarrow \text{Gln}$) mutant. This single amino acid replacement drastically increases the O_2 affinity (3.6 mm Hg) of rHb AE. The order of O_2 affinities for these five mutants and the wild-type Hbs under stripped conditions (MES buffer) is rHb WM ($\beta/\delta 101\text{E}$) $\approx$ rHb WM (αN5K , $\beta/\delta 101\text{E}$) $\approx$ rHb AE $<$ rHb WM $\approx$ Hb A $<$ rHb WM (αN5K , $\beta/\delta 12\text{T}$, $\beta/\delta 86\text{A}$) $<$ rHb WM ($\beta/\delta 101\text{K}$) $<$ rHb WM ($\beta/\delta 101\text{D}$) (Table 2).

The presence of allosteric effectors diminishes the O_2 affinities of all Hbs in this study and the effects are more pronounced for rHb WM and Hb A. Under stripped conditions, rHb WM ($\beta/\delta 101\text{D}$) is 1.8-fold higher in O_2 affinity than rHb WM, and this value increases to 3- to 5.3-fold in the presence of effectors (Table 2). Hence, the differences in the P_{50} values between the mutants and rHb WM become more perceptible in the presence of effectors. rHb WM has a lower O_2 affinity than rHb AE in the presence of all allosteric effectors tested. rHb AE and the two mutants with Glu at the $\beta/\delta 101$ position [rHb WM ($\beta/\delta 101\text{E}$) and rHb WM (αN5K , $\beta/\delta 101\text{E}$)] have similar O_2 affinities under various experimental conditions. IHP has a stronger effect on the O_2 affinity of the rHbs than phosphate or chloride ions, showing a larger increase in P_{50} values (Table 2).

As reported previously (10), the Hill coefficients for rHb WM and rHb AE are lower than those of Hb A under all experimental conditions, suggesting a difference in the cooperativity of the oxygenation process for these Hbs (for details, see Supporting Information Figure 2S). The n_{50} values for the mutants rHb WM ($\beta/\delta 101\text{E}$), rHb WM (αN5K , $\beta/\delta 101\text{E}$), and rHb WM ($\beta/\delta 101\text{K}$), are maintained around the expected value of 1.8–2.5 in the range of pH 5.8–8.4 in 0.1 M MES or NaPi buffer, while the n_{50} values for rHb WM ($\beta/\delta 101\text{D}$) and

rHb WM (α N5K, β /8A12T, β /8S86A) are found to be lower (1.0–2.0) under the same experimental conditions.

Chloride Dependence of Oxygen Affinities

It is well known that chloride ions decrease the O₂ affinity of Hb A (23–26). As shown in Table 2, in MES buffer at pH 7.4 and 29 °C, Hb A has more than a two-fold increase in P_{50} value in the presence of 0.5 M NaCl (lowered O₂ affinity). Comparatively, a significant increase (2.1-fold) can be detected for the P_{50} value of rHb WM, while those for rHb AE and the five rHb WM mutants show only moderate 1.1- to 1.5-fold increases under the same conditions. That the O₂ affinities of rHb AE and the rHb WM mutants are less sensitive to the presence of chloride ions can be demonstrated by plotting the P_{50} values of the rHbs as a function of chloride ion concentrations [Cl⁻]. The results can be grouped into three categories (Figure 1). The first group includes Hb A and rHb WM, which exhibit the strongest responses to chloride. When the [Cl⁻] is increased from 0.01 to 1.0 M, the P_{50} values of Hb A and rHb WM increase more than two-fold. The second group consists of rHb WM (β /8Q101K) and the triple mutant rHb WM (α N5K, β /8A12T, β /8S86A), which exhibit moderate 1.4 to 1.5-fold increases in the P_{50} values, indicating a weaker response to [Cl⁻], compared to that of rHb WM. rHb AE and the other three mutants, rHb WM (β /8Q101E), rHb WM (α N5K, β /8Q101E), and rHb WM (β /8Q101D) are in the third group, which show at the most only a 1.2-fold increase in the P_{50} values, suggesting that Cl⁻ has minimal impact on their O₂ affinities (Figure 1).

As shown in Table 2, the presence of IHP significantly increases the P_{50} value (lowers the O₂ affinity) of all the rHbs. However, the modulation of the O₂ affinity by IHP with phosphate or chloride is not synergistic, and the IHP effect is decreased in the presence of phosphate or chloride. The antagonistic effect between IHP and chloride can also be demonstrated by plotting the changes in the P_{50} values as a function of [Cl⁻] (Figure 1). A negative correlation between [Cl⁻] and P_{50} value is observed in the presence of IHP, i.e., the P_{50} value decreases when the chloride concentration increases. For instance, in the presence of 0.5 mM IHP, the P_{50} value of rHb WM in MES buffer decreases from 20.4 to 10.6 mm Hg when the [Cl⁻] increases from 0.01 to 0.5 M. When [Cl⁻] reaches 0.5 M, the apparent P_{50} values of the rHbs measured in the presence and absence of IHP are very similar. Only rHb WM (β /8Q101D) exhibits an increased P_{50} value upon the addition of IHP. For the triple mutant rHb WM (α N5K, β /8A12T, β /8S86A), the antagonistic effect between IHP and chloride is so strong that when [Cl⁻] reaches 0.5 M and higher, the P_{50} value observed in the presence of IHP is even lower than that in the absence of IHP (Figure 1).

Bohr Effect

It has been observed that the alkaline Bohr effect of rHb WM is slightly lower than that of Hb A under stripped conditions (MES buffer), meaning that the P_{50} value of rHb WM increases less when the pH decreases from 8.0 to 6.5 (10). Allosteric effectors, such as IHP, have comparable effects on the alkaline Bohr effect of rHb WM and Hb A. In phosphate buffer and/or in the presence of IHP, the alkaline Bohr effects of rHb WM and Hb A are increased, while rHb AE has a lower alkaline Bohr effect than those of rHb WM and Hb A even in the presence of the allosteric effectors (Table 3). Among the rHb WM mutants, rHb WM (β /8Q101E) and rHb WM (α N5K, β /8Q101E) behave very similarly to rHb AE, exhibiting a 40% reduction of the alkaline Bohr effect compared to that of rHb WM (Table 3). The alkaline Bohr effects of mutants rHb WM (β /8Q101K) and rHb WM (β /8Q101D) are not significantly different from that of rHb WM, although the P_{50} values of these two mutants are decreased compared to that of rHb WM. Addition of 0.5 mM IHP increases the alkaline Bohr effect to nearly the same extent in these two mutants as in rHb WM, demonstrating normal binding of IHP to the mutants and a normal additional alkaline Bohr

effect due to anion binding. The triple mutant, rHb WM (α N5K, β / δ A12T, β / δ S86A), has the lowest alkaline Bohr effect among all the mutant rHbs studied under various experimental conditions. The alkaline Bohr effect of this triple mutant is reduced by 50% compared to that of rHb WM even in the presence of the allosteric effectors. It is also noticed that in the absence of IHP, the Bohr effect curves of rHb WM and its mutants are shifted toward the left. Thus, the pH_{max} (the pH at which maximum proton release occurs) is about 0.6 pH unit lower in rHb WM compared to that in Hb A. In the presence of IHP, the pH_{max} is increased by 0.6 pH unit in rHb WM compared to 0.4 pH unit in Hb A (results not shown).

It is known that the alkaline Bohr effect of Hb A is affected by temperature as well as by allosteric effectors. Hb A exhibits a stronger Bohr effect at lower temperature (27). We have determined the temperature dependence of the alkaline Bohr effects of rHb WM and its mutants, and compared them to that of Hb A. In MES buffer, the alkaline Bohr effect ($\Delta P_{50}/\Delta \text{pH}$) of rHb WM and the mutants is always lower than that of Hb A between 11 and 37 °C (results not shown). The temperature dependencies [$(\Delta P_{50}/\Delta \text{pH})/\Delta T$] of rHb WM and Hb A are approximately the same, while that of the rHb WM mutants is lower. In the presence of IHP or chloride, the alkaline Bohr effect of rHb WM becomes less dependent on temperature, as does that of the mutants (Supporting Information Table 1S).

Effect of Temperature on Oxygen Affinity

The P_{50} values were measured for Hb A and the rHbs at 11, 20, 29, and 37 °C under various experimental conditions. The $\Delta \log P_{50}/\Delta T$ values were calculated and used to demonstrate the effect of temperature on oxygen binding in the presence and absence of allosteric effectors. A smaller $\Delta \log P_{50}/\Delta T$ value signifies a lesser change of the O_2 affinity upon temperature changes. This value differs for each Hb molecule under the various experimental conditions, as shown in Figure 2 and Supporting Information Table 2S. For instance, Hb A has the largest $\Delta \log P_{50}/\Delta T$ value (0.034) under stripped conditions (MES buffer), which becomes lower in phosphate buffer (0.022) or in the presence of chloride (0.029), and decreases further upon adding IHP (0.018). For rHb WM (Figure 2B), the O_2 affinities decrease as a function of temperature in the presence of either phosphate or chloride, but the $\Delta \log P_{50}/\Delta T$ values remain the same as that under stripped conditions. In the presence of IHP, the $\Delta \log P_{50}/\Delta T$ value has a slight decrease and it drops further in the presence of phosphate or chloride, suggesting that the O_2 affinity of rHb WM is more constant as a function of temperature in the presence of the two allosteric effectors. The $\Delta \log P_{50}/\Delta T$ values of rHb AE and the two mutants with Glu at the β /101 position, i.e., rHb WM (β /101E) and rHb WM (α N5K, β /101E), exhibit the same trend under different experimental conditions. They are less affected by the presence of phosphate and IHP, while Cl^- causes a slight decrease in the slopes of the $\Delta \log P_{50}$ versus ΔT plot. The presence of allosteric effector does not decrease the $\Delta \log P_{50}/\Delta T$ value of the other three rHb WM mutants. For instance, the $\Delta \log P_{50}/\Delta T$ value of rHb WM (β /101D) is even higher in the presence of IHP than under stripped conditions. For the $\Delta \log P_{50}/\Delta T$ values of these rHbs under various conditions, see Supporting Information Table 2S.

Aside from using the $\Delta \log P_{50}/\Delta T$ values to gauge the effect of temperature on O_2 affinity, the enthalpy of oxygenation ΔH ($\text{kJ mol}^{-1} \text{O}_2$) has been calculated on the basis of these values. Consistent with the changes in the $\Delta \log P_{50}/\Delta T$ values, the ΔH values of the mutants vary with the type of the amino acid residue at the β /101 position, and give more insight into the effect of temperature. Since the oxygenation of Hb is exothermic, a negative enthalpy, ΔH value, is observed (Figure 3). In analyzing these results, one must note that a smaller ΔH (absolute value) suggests a lower effect of temperature on the O_2 binding. In previous studies, it has been observed that the influence of temperature on rHb WM and rHb AE differs from that on Hb A (10). The ΔH value of $-26.7 \text{ kJ mol}^{-1}$ for rHb WM is 40%

lower than that for Hb A ($-43.9 \text{ kJ mol}^{-1}$) (Figure 3). This observation, made in MES buffer, indicates that the O_2 affinity of rHb WM is more constant as a function of temperature compared to that of Hb A.

In our present study, the replacement of $\beta/\delta 101\text{Gln}$ modulates the effect of temperature on the O_2 affinity of the mutants. Under stripped conditions (MES buffer), the ΔH values of rHb WM ($\beta/\delta\text{Q101D}$) and rHb WM (αN5K , $\beta/\delta\text{A12T}$, $\beta/\delta\text{S86A}$) are -16.1 and -7.4 kJ mol^{-1} , respectively, significantly lower than that of rHb WM (Figure 3). rHb AE, rHb WM ($\beta/\delta\text{Q101E}$) and rHb WM (αN5K , $\beta/\delta\text{Q101E}$) have ΔH values similar to that of rHb WM, while the ΔH value of the rHb WM ($\beta/\delta\text{Q101K}$) mutant is $-22.0 \text{ kJ mol}^{-1}$, slightly lower than that of rHb WM.

The differences in ΔH values between rHb WM and its mutants change significantly under various experimental conditions, with rHb WM having a stronger response to the allosteric effectors. The ΔH value of rHb WM ($-26.7 \text{ kJ mol}^{-1}$ in MES buffer) decreases to -21.8 and $-18.9 \text{ kJ mol}^{-1}$ in phosphate buffer and in MES buffer with IHP, respectively. Compared to phosphate and IHP, chloride has less effect on the ΔH values of rHb WM and its mutants, although the P_{50} values of rHb WM exhibit a strong chloride dependence (Table 2). In the presence of 0.1 M NaCl , the ΔH value of rHb WM changes slightly to $-26.2 \text{ kJ mol}^{-1}$, which is very similar to that of rHb AE, rHb WM ($\beta/\delta\text{Q101E}$), rHb WM (αN5K , $\beta/\delta\text{Q101E}$) and rHb WM ($\beta/\delta\text{Q101K}$). Under this condition, rHb WM ($\beta/\delta\text{Q101D}$) and rHb WM (αN5K , $\beta/\delta\text{A12T}$, $\beta/\delta\text{S86A}$) still exhibit a lower temperature dependence (ΔH values of -13.2 and $-14.6 \text{ kJ mol}^{-1}$) than that of rHb WM. In the presence of chloride, Hb A shows a significant decrease of the ΔH value from $-44.4 \text{ kJ mol}^{-1}$ to $-35.7 \text{ kJ mol}^{-1}$, but it is still numerically higher than that of rHb AE, rHb WM and its mutants.

The most dramatic decrease of the ΔH value of rHb WM is observed in the presence of two allosteric effectors. The ΔH value of rHb WM is $-12.9 \text{ kJ mol}^{-1}$ in phosphate buffer with IHP, and -7.3 kJ mol^{-1} in MES buffer in the presence of IHP and chloride, much lower than those of Hb A and rHb AE under the same experimental conditions (Figure 3). Like rHb AE, rHb ($\beta/\delta\text{Q101E}$) and rHb WM (αN5K , $\beta/\delta\text{Q101E}$) exhibit a less dramatic change in their ΔH values. The ΔH values of these three rHbs also decrease, but to a lesser extent in the presence of allosteric effectors. The triple mutant, rHb WM (α5K , $\beta/\delta\text{A12T}$, $\beta/\delta\text{S86A}$), having the same $\beta/\delta 101\text{Gln}$ residue as rHb WM, always exhibits a lower ΔH value than the other mutant rHbs, especially in the presence of chloride and IHP. Conversely, the ΔH values of rHb WM ($\beta/\delta\text{Q101K}$) and rHb WM ($\beta/\delta\text{Q101D}$) are lower than that of rHb WM in MES buffer, and exhibit a slight increase in the presence of IHP or phosphate, but become higher than that of rHb WM in the presence of IHP and chloride, signifying the importance of the $\beta/\delta 101\text{Gln}$ residue to the effect of temperature on the O_2 affinity (Figure 3).

^1H -NMR Spectra of Hemoglobins

In the ^1H -NMR spectra of Hb A in the CO form at 29°C (Figure 4A), the resonances at 12.2 ppm and 12.9 ppm have been assigned to the exchangeable side chain $\text{N}\epsilon_2\text{H}$ group of $\alpha 103\text{His}$ and $\alpha 122\text{His}$, respectively (28–31). The peak at 12.2 ppm for the $\alpha 103\text{His}$ residue is absent from the spectra of rHb AE, rHb WM (10) and its mutants. In this region, only the resonance for the residue $\alpha 122\text{His}$ can be observed at 13.2 ppm (Figure 4A). More significant differences are detected in the spectra obtained from the rHb WM mutants in the deoxy form. In the region between 11 and 15 ppm , two important resonances are related to the H-bond network in the $\alpha_1\beta_2$ interface of deoxy Hb A. The peak at 11.4 ppm is assigned to the H-bond between $\beta 37\text{Trp}$ and $\alpha 94\text{Asp}$ (32). This peak disappears in the spectrum of rHb WM ($\beta/\delta\text{Q101D}$), and shifts 0.2 ppm upfield in the spectra of the other four rHb WM mutants (Figure 4B). The peak at 14 ppm is assigned to the H-bond between $\alpha 42\text{Tyr}$ and $\beta 99\text{Asp}$ and is used as a T-state marker of Hb A (33). Compared to that of Hb A, this T-state

marker shifts 0.2 ppm upfield in the spectrum of the triple mutant, but 0.1–0.3 ppm downfield in the spectra of rHb WM, rHb AE, and the remaining four mutants (Figure 4B).

Upon the addition of IHP, no significant change is detected in spectra from the CO form of Hb A and rHb AE at 29 °C. However, in the spectra of rHb WM and its mutants, the resonance at 13.2 ppm becomes broader, suggesting that chemical exchange occurs at the $\alpha 103$ His residue. To confirm this change, ^1H -NMR spectra were also obtained for the Hbs at 11 and 37 °C (Figure 4C and Supporting Information Figure 3S). An additional peak starts to appear at 29 and 37 °C in the range of 13.2–14.0 ppm in the CO form of these rHbs, and becomes more obvious at lower temperature (11 °C) and in the presence of IHP. Under the same experimental conditions, the T-state marker is observed at 14 ppm from the CO form of Hb A, while it is not seen in the spectra of rHb AE (Figure 4A and C).

Ring-current-shifted resonances at –1.5 ppm to –2.0 ppm (Figure 4D) provide information about the tertiary structure around the heme pockets (28). For rHb WM and rHb AE, the E11Val methyl resonances of both α - and β/δ -chains are shifted upfield to –2.01 ppm (10). The rHb WM mutants show no significant differences in this region.

DISCUSSION

Oxygen Affinity of rHb WM $\beta/\delta 101$ Gln mutants

The $\alpha_1\beta_2$ ($\alpha_2\beta_1$) subunit interface in Hb A is very important in the allosteric transition between deoxy- and oxy-Hb (1). Several critical amino acid residues in this region form H-bonds, such as those between $\beta 37$ Trp and $\beta 102$ Asn in the CO-form of Hb A, and those between $\beta 99$ Asp and $\alpha 42$ Tyr in the deoxy-form of Hb A. These H-bonds are essential in maintaining a functional Hb (1, 34). $\beta 101$ Glu is in the central cavity of Hb A and in close proximity to $\beta 99$ Asp and $\beta 102$ Asn. Previous studies of Hb A mutants with minimum chloride under stripped conditions indicated that substitution of $\beta 101$ Glu with Gly, Lys, Asp and Gln amplified oxygen affinity (or destabilized the deoxy tetramer) (15). However, the role of $\beta/\delta 101$ in oxygen binding is not fully understood. Hb A and Hb AE have Glu, while Hb WM has a Gln residue at the $\beta/\delta 101$ position. This substitution in the primary sequence of the Hb molecule could be an evolutionary necessity for the woolly mammoth to adapt to the Arctic environment, as rHb WM has a lower effect of temperature on the O_2 affinity. Recently, the structural models for rHb WM in the CO and deoxy forms have been determined from X-ray crystallographic studies (19). The overall structures of rHb WM and Hb A are very similar. Analogous to the $\beta 101$ Glu of Hb A, the $\beta/\delta 101$ Gln of rHb WM is located in the $\alpha_1(\beta/\delta)_2$ [or $\alpha_2(\beta/\delta)_1$] interface. The region around $\beta/\delta 101$ Gln also overlaps extremely well with the region around the $\beta 101$ Glu in Hb A (19). Thus, the rHb WM and rHb AE mutants in this study can illustrate the role of this amino acid residue in regulating Hb function.

The central cavity of the Hb A tetramer has more cationic residues ($\alpha 99$ Lys, $\alpha 103$ His, $\beta 2$ His, $\beta 82$ Lys, $\beta 104$ Arg, $\beta 143$ His, and the amino termini of $\alpha 1$ Val and $\beta 1$ Val) than anionic residues ($\alpha 94$ Asp, $\alpha 126$ Asp, and $\beta 101$ Glu) (18). Therefore, this region has excess positive charges balanced by a few well-placed negatively charged side-chains. The electrostatic potential in this area of rHb WM is similar to that of Hb A (19). Thus, if the repulsion between α Lys99 and $\beta/\delta 104$ Arg (and their symmetry mates) in this region is alleviated by counter ions (such as $\beta 101$ Glu), the deoxy form of Hb can be stabilized with a consequent decrease in O_2 affinity. Conversely, the addition of positive charge(s) or the removal of negative charge(s) in this region can destabilize the deoxy form and increase the O_2 affinity of the tetramer. Ultimately, upon O_2 binding and converting Hb into the oxy form, the negative charges on O_2 have a neutralization effect and stabilize the Hb molecule. Compared to rHb AE with Glu at the $\beta/\delta 101$ position, the Gln in rHb WM increases the

repulsion between $\alpha 99\text{Lys}$ and $\beta/\delta 104\text{Arg}$. Thus, rHb WM exhibits a higher O_2 affinity than rHb AE, as observed under stripped conditions (MES buffer) (Table 2). The same behavior was observed in the human mutant Hb Rush ($\beta 101\text{Glu} \rightarrow \text{Gln}$) reported by Shih et al. (15). The change in net positive charge in the central cavity is also responsible for the higher O_2 affinity of human mutant Hb British Columbia ($\beta 101\text{Glu} \rightarrow \text{Lys}$) and the mutant rHb WM ($\beta/\delta 101\text{Gln} \rightarrow \text{Lys}$). In these two mutants, introducing the positively charged Lys destabilizes the central cavity and increases the O_2 affinity of the Hb molecule [(15), (Table 2)].

According to the discussion above, rHb WM with a $\beta/\delta 101\text{Gln}$ to Asp replacement could have a favorable electrostatic potential to stabilize the center cavity. However, the rHb WM ($\beta/\delta 101\text{Gln} \rightarrow \text{Asp}$) has the highest oxygen affinity among the mutants studied, suggesting that besides the positive charges at the $\beta/\delta 101$ position, other factor(s) can adversely affect the stability of the quaternary structure of Hb WM. The same observation has also been reported by Shih et al. (15). The Hb Potomac ($\beta 101\text{Asp}$) has a higher oxygen affinity than those of Hb A, Hb British Columbia ($\beta 101\text{Lys}$) and Hb Rush ($\beta 101\text{Gln}$). The NMR spectrum of rHb WM ($\beta/\delta 101\text{Gln} \rightarrow \text{Asp}$) provides a direct evidence for the loss of an intersubunit H-bond, which could result in destabilizing the T-structure of this mutant (Figure 4B). Intersubunit H-bonds are critical for Hb function by affecting the stabilization of the ligated or unligated structure of the Hb molecule (35). X-ray crystal structures of rHb WM in the CO and deoxy forms (19) suggest that mammoth Hb and Hb A share the same key residues in forming hydrogen bonds that are characteristic of the T and R states as determined previously (35). Accordingly, the side-chain NH proton of $\beta/\delta 37\text{Trp}$ in the rHb WM forms an intrasubunit H-bond with $\beta/\delta 102\text{Asn}$ in the CO form and an intersubunit H-bond with $\alpha 94\text{Asp}$ in the deoxy form. These two H-bonds can be assigned to 10.7 and 11.2 ppm in the NMR spectra for the CO and deoxy forms of rHb WM, respectively. However, the peak at 11.2 ppm disappears from the spectrum of the deoxy form of rHb WM ($\beta 101\text{Gln} \rightarrow \text{Asp}$) (Figure 4B), while in the CO form, the peak at 10.7 ppm is still visible (Figure 4A). This suggests that in the deoxy form, the H-bond network in the $\alpha_1(\beta/\delta)_2$ interface is disturbed by replacing $\beta/\delta 101\text{Gln}$ with Asp. The missing H-bond may destabilize the deoxy form of this mutant and be responsible for the increased oxygen affinity and decreased cooperativity.

Besides $\beta/\delta 101\text{Gln}$, Hb WM differs from Hb AE in three other positions. Our study shows that the double mutant rHb WM ($\alpha 5\text{Asn} \rightarrow \text{Lys}$, $\beta/\delta 101\text{Gln} \rightarrow \text{Glu}$) exhibits a functional property different from that of rHb WM (with $\beta/\delta 101\text{Gln}$), but very similar to that of rHb AE (with $\beta/\delta 101\text{Glu}$) and the single mutant rHb WM ($\beta/\delta 101\text{Gln} \rightarrow \text{Glu}$), suggesting that the $\beta/\delta 101$ residue plays a more important role in the O_2 binding of rHb WM than does $\alpha 5\text{Asn}$. Further observations from the triple mutant of rHb WM demonstrate that replacements at $\beta/\delta 12$ ($\text{Thr} \rightarrow \text{Ala}$) and $\beta/\delta 86$ ($\text{Ala} \rightarrow \text{Ser}$) could potentially cooperate with the change at the $\beta/\delta 101$ position. The triple mutant, having a Gln at the $\beta/\delta 101$ position, but the rest of the sequence identical to that of rHb AE, exhibits a higher chloride dependence and a lower effect of temperature than rHb AE. However, it also exhibits a much higher O_2 affinity and very poor cooperativity compared to rHb WM. Meanwhile, the broad signals of $\alpha 103\text{His}$ observed from the ^1H -NMR spectra of rHb WM and the triple mutant suggest that the $\alpha_1(\beta/\delta)_1$ interface is disturbed, which is responsible for the lower cooperativity (shown as lower n_{50} values) of rHb WM. Thus, the replacements at the $\beta/\delta 12$ and $\beta/\delta 86$ positions are needed to stabilize the $\alpha_1(\beta/\delta)_1$ interface of rHb WM.

Replacement of $\beta/\delta 101\text{Gln}$ Changes the Chloride Dependence of rHb WM

It is well known that chloride ions play an allosteric role in regulating the O_2 affinity of hemoglobin (36, 37). However, X-ray structural analyses have shown that Hb A, bovine Hb, and rHb WM do not have specific binding sites for chloride ions (18, 19, 38). Thus, chloride

exerts its effect on O₂ affinity via different amino acid residue(s) in each Hb molecule. For instance, the central cavity of the Hb A tetramer has a lot fewer anionic than cationic residues. The excessive positive charges in this region present a destabilizing factor (18). As proposed by Perutz et al., the diffusion of chloride ions into this cavity could neutralize these charges, even though chloride ions were randomly distributed in the central cavity (18, 38). The central cavity has a larger width in the T- than in the R- structure. Diffusion of chloride ions into the cavity could have a more significant effect for Hb in the deoxy state and lower the overall oxygen affinity (18). Following this argument, rHb WM and the human mutant Hb Rush with $\beta/\delta 101\text{Gln}$ behave similarly towards chloride by providing an additional binding site for chloride. rHb WM exhibits a more significant chloride effect on the O₂ affinity than rHb AE, as does Hb Rush compared to Hb A. The O₂ affinity of rHb WM also shows a much stronger chloride dependence than those of the three rHb WM mutants which have either Glu or Asp residues at the $\beta/\delta 101$ position.

Similarly, one would expect that the triple mutant, rHb WM (αN5K , $\beta/\delta\text{A12T}$, $\beta/\delta\text{S86A}$), and the mutant rHb WM ($\beta/\delta\text{Q101K}$) could have exhibited a similar or an even stronger chloride effect on O₂ affinity than rHb WM. However, their response to chloride observed in our experiments is much weaker than that of rHb WM, suggesting that the chloride effect on the O₂ affinity of these rHbs is not solely related to the charge of the residue at the $\beta/\delta 101$ position. Even though the replacement of the residue $\beta/\delta 101\text{Gln}$ with Lys increases the net positive charge in the central cavity, the bulkiness of the Lys side-chain might destabilize this region and hence increase the O₂ affinity. The potential effect obtained from the enhanced chloride binding could be cancelled out due to the destabilization. Thus, the apparent chloride effect on the O₂ affinity of the mutant rHb WM ($\beta/\delta 101\text{Lys}$) is weaker than that of rHb WM.

Replacement of $\beta/\delta 101\text{Gln}$ Changes the Temperature Dependence of the O₂ Affinity

The effect of temperature on the O₂ affinity of rHb WM and its mutants is affected by the presence of allosteric effectors. The allosteric effects of phosphate, chloride, and IHP vary under different temperatures and increase the P_{50} values of the rHbs to different extents, as shown in the current study. For instance, rHb WM ($\beta/\delta\text{Q101D}$) has a higher O₂ affinity (lower P_{50} value) and weaker response to allosteric effectors than rHb WM and the other mutants. IHP increases the P_{50} value of rHb WM ($\beta/\delta\text{Q101D}$) more significantly at higher temperature (37 °C) than at lower temperature (11 °C) (Figure 2). Thus, in the presence of IHP, enhanced $\Delta P_{50}/\Delta T$ values or (absolute) ΔH values are observed for this mutant. Compared to rHb AE and the mutants, rHb WM shows a stronger response to the allosteric effectors by exhibiting a more significant increase in the P_{50} values, especially at lower temperatures (Figure 2). The $\Delta P_{50}/\Delta T$ values or (absolute) ΔH values of rHb WM decrease in the presence of allosteric effectors. This feature could play a most important role in keeping the O₂ affinity constant when temperature decreases. The replacement of $\beta/\delta 101\text{Gln}$ by Glu decreases the allosteric effect. Thus, for rHb AE, rHb WM ($\beta/\delta\text{Q101E}$), and rHb WM (αN5K , $\beta/\delta\text{Q101E}$), the O₂ affinity is more dependent on temperature, and the $\Delta P_{50}/\Delta T$ values or (absolute) ΔH values are less affected by the presence of the allosteric effectors.

It is also noted that the effect of temperature on the O₂ affinity is less significant when only one effector is used in the measurements (Figures 2 and 3). For instance, in the presence of 0.1 M chloride, rHb WM exhibits a lower O₂ affinity (higher P_{50} value) than rHb AE (Table 2), but the effect of temperature on the O₂ affinities is similar (Figure 3). The lowest effect of temperature on the O₂ affinity and the most dramatic differences between rHb WM and the other rHbs studied have only been observed in the presence of two or more effectors, such as IHP and chloride, or IHP and phosphate, suggesting that the change in the effect of temperature on the O₂ affinities of Hbs is not the result of any single effector alone.

CONCLUSION

For woolly mammoth living in the Arctic environment, a lower temperature dependence of the O₂ affinity of its Hb is beneficial, while the O₂ affinity needs to be at the same level as that of Asian elephant Hb. In the current study, the replacements of the $\beta/\delta 101$ Gln residue in rHb WM with Glu, Lys, or Asp allow us to investigate the role of this residue in the functional properties of Hbs, and to speculate on why the $\beta/\delta 101$ Glu residue in Hb AE is changed to Gln in Hb WM. With a Glu at the $\beta/\delta 101$ position, as shown in rHb WM ($\beta/\delta Q101E$) and rHb WM ($\alpha N5K, \beta/\delta Q101E$), the mutants exhibit functional properties very similar to rHb AE and do not have a lower effect of temperature on O₂ affinity. If rHb WM has a Lys or Asp instead of a Gln at its $\beta/\delta 101$ position, the effect of temperature on O₂ affinity could not be lower than that of rHb WM, and the O₂ affinity also becomes much stronger than that of rHb AE and rHb WM. These results suggest that the $\beta/\delta 101$ Gln residue is responsible for the lower effect of temperature of rHb WM. Once the $\beta/\delta 101$ Glu residue of rHb AE is replaced by Gln as in rHb WM, as shown in the triple mutant, the mutant does gain a lower temperature dependence of the O₂ affinity than that of rHb AE and rHb WM. However, the O₂ affinity of this mutant is much higher, and the cooperativity is much lower than that of rHb WM, suggesting that the replacement of $\beta/\delta 101$ Glu also needs to combine with the other three unique residues in the woolly mammoth Hb to provide the structural basis for its function. This information provides a guide in designing a general purpose hemoglobin-based oxygen carriers (HBOCs) whose oxygen affinities are independent of temperature, i.e., especially beneficial for treating patients undergoing therapeutic hypothermia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Hb A	human normal adult hemoglobin composed of $\alpha_2\beta_2$ tetramers
rHb	recombinant Hb
rHb WM	recombinant woolly mammoth Hb composed of $\alpha_2(\beta/\delta)_2$ tetramers
rHb AE	recombinant Asian elephant Hb composed of $\alpha_2(\beta/\delta)_2$ tetramers
HbCO	carbonmonoxyhemoglobin
deoxy-Hb	deoxyhemoglobin
met-Hb	methemoglobin
NMR	nuclear magnetic resonance
DSS	2,2-dimethyl-2-silapentane-5-sulfonate
MES	2-(<i>N</i> -morpholino) ethanesulfonic acid
and IHP	inositol hexaphosphate

References

1. Dickerson, RE.; Geis, I. Hemoglobin: Structure, Function, Evolution, and Pathology. Benjamin/Cummings; Menlo Park, CA: 1983.
2. Bårdgard AJ, Brix O. Functional characterisation of Eskimo dog hemoglobin: II. The interplay of HCO_3^- and Cl^- . Comparative Biochemistry and Physiology Part A: Physiology. 1997; 117:375–381.
3. Bårdgard AJ, Strand I, Nuutinen M, Jul E, Brix O. Functional characterisation of Eskimo dog Hemoglobin: I. Interaction of Cl^- and 2,3-DPG and its importance to oxygen unloading at low temperature. Comparative Biochemistry and Physiology Part A: Physiology. 1997; 117:367–373.
4. Brix O, Bardgard A, Mathisen S, Elsherbini S, Condo SG, Giardina B. Arctic life adaptation. 2 The function of musk ox (*Ovibos moschatus*) hemoglobin. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology. 1989; 94:135–138.
5. Clementi ME, Condo SG, Castagnola M, Giardina B. Hemoglobin-function under extreme life conditions. Eur J Biochem. 1994; 223:309–317. [PubMed: 7519981]
6. Coletta M, Clementi ME, Ascenzi P, Petruzzelli R, Condo SG, Giardina B. A comparative study of the temperature dependence of the oxygen-binding properties of mammalian hemoglobins. Eur J Biochem. 1992; 204:1155–1157. [PubMed: 1551393]
7. De Rosa MC, Castagnola M, Bertonati C, Galtieri A, Giardina B. From the Arctic to fetal life: physiological importance and structural basis of an 'additional' chloride-binding site in haemoglobin. Biochem J. 2004; 380:889–896. [PubMed: 14979874]
8. Giardina B, Condo SG, el Sherbini S, Mathisen S, Tyler N, Nuutinen M, Bardgard A, Brix O. Arctic life adaptation--I. The function of reindeer hemoglobin. Comp Biochem Physiol B. 1989; 94:129–133. [PubMed: 2598627]
9. Campbell KL, Roberts JEE, Watson LN, Stetefeld J, Sloan AM, Signore AV, Howatt JW, Tame JRH, Rohland N, Shen TJ, Austin JJ, Hofreiter M, Ho C, Weber RE, Cooper A. Substitutions in woolly mammoth hemoglobin confer biochemical properties adaptive for cold tolerance. Nature Genet. 2010; 42:536–540. [PubMed: 20436470]
10. Yuan Y, Shen T-J, Gupta P, Ho NT, Simplaceanu V, Tam TCS, Hofreiter M, Cooper A, Campbell KL, Ho C. A biochemical–biophysical study of hemoglobins from woolly mammoth, Asian elephant, and humans. Biochemistry. 2011; 50:7350–7360. [PubMed: 21806075]
11. Adams JG, Winter WP, Tausk K, Heller P. Hemoglobin Rush $\beta 101$ (G3) glutamine - New unstable hemoglobin causing mild hemolytic-anemia. Blood. 1974; 43:261–269. [PubMed: 4129558]
12. Mant MJ, Salkie ML, Cope N, Appling F, Bolch K, Jayalakshmi M, Gravely M, Wilson JB, Huisman THJ. Hb-Alberta or $\alpha_2\beta_2$ 101(G3)Glu→Gly, a new high-oxygen-affinity hemoglobin variant causing erythrocytosis. Hemoglobin. 1977; 1:183–194. [PubMed: 1052180]
13. Jones RT, Brimhall B, Gray G. Hemoglobin British-Columbia $\alpha_2\beta_2$ 101(G3)Glu→Lys a new variant with high oxygen-affinity. Hemoglobin. 1977; 1:171–182. [PubMed: 1052179]
14. Charache S, Jacobson R, Brimhall B, Murphy EA, Hathaway P, Winslow R, Jones R, Rath C, Simkovich J. Hb-Potomac (101Glu→Asp) - Speculations on placental oxygen-transport in carriers of high-affinity hemoglobins. Blood. 1978; 51:331–338. [PubMed: 563749]
15. Shih DT, Jones RT, Imai K, Tyuma I. Involvement of Glu G3(101) β in the function of hemoglobin. Comparative O_2 equilibrium studies of human mutant hemoglobins. J Biol Chem. 1985; 260:5919–5924. [PubMed: 3997804]
16. Wild BJ, Green BN, Cooper EK, Lalloz MRA, Erten S, Stephens AD, Layton DM. Rapid identification of hemoglobin variants by electrospray ionization mass spectrometry. Blood Cells, Molecules, and Diseases. 2001; 27:691–704.
17. Perutz MF. Stereochemistry of cooperative effects in haemoglobin. Nature. 1970; 228:726–739. [PubMed: 5528785]
18. Perutz MF, Fermi G, Poyart C, Pagnier J, Kister J. A novel allosteric mechanism in hemoglobin - Structure of bovine deoxyhemoglobin, absence of specific chloride-binding sites and origin of the chloride-linked Bohr effect in bovine and human hemoglobin. J Mol Biol. 1993; 233:536–545. [PubMed: 8411160]

19. Noguchi H, Campbell KL, Ho C, Unzai S, Park SY, Tame JR. Structures of haemoglobin from woolly mammoth in liganded and unliganded states. *Acta Crystallogr D Biol Crystallogr*. 2012; 68:1441–1449. [PubMed: 23090393]
20. Shen TJ, Ho NT, Simplaceanu V, Zou M, Green BN, Tam MF, Ho C. Production of unmodified human adult hemoglobin in *Escherichia coli*. *Proc Natl Acad Sci U S A*. 1993; 90:8108–8112. [PubMed: 8367471]
21. Russu IM, Lin A, Ferro-Dosch S, Ho C. A proton nuclear magnetic-resonance investigation of human hemoglobin A₂ – Implications on the intermolecular contacts in Sickle hemoglobin fibers and on the Bohr effect of human normal adult hemoglobin. *Biochim Biophys Acta*. 1984; 785:123–131. [PubMed: 6704402]
22. Shen TJ, Ho NT, Zou M, Sun DP, Cottam PF, Simplaceanu V, Tam MF, Bell DA, Ho C. Production of human normal adult and fetal hemoglobins in *Escherichia coli*. *Protein Eng*. 1997; 10:1085–1097. [PubMed: 9464574]
23. Benesch RE, Benesch R, Yu CI. Oxygenation of hemoglobin in presence of 2,3-diphosphoglycerate. Effect of temperature pH ionic strength and hemoglobin concentration. *Biochemistry*. 1969; 8:2567–2571. [PubMed: 5799137]
24. Bunn HF, Guidotti G. Stabilizing interactions in hemoglobin. *J Biol Chem*. 1972; 247:2345–2350. [PubMed: 5019951]
25. Nigen AM, Manning JM. Interaction of anions with hemoglobin carbamylated on specific NH₂-terminal residues. *J Biol Chem*. 1975; 250:8248–8250. [PubMed: 1176467]
26. Haire RN, Hedlund BE. Thermodynamic aspects of linkage between binding of chloride and oxygen to human hemoglobin. *Proc Natl Acad Sci U S A*. 1977; 74:4135–4138. [PubMed: 270660]
27. Antonini E, Wyman J, Brunori M, Frontice C, Bucci E, Rossifan A. Studies on relations between molecular and functional properties of hemoglobin. V Influence of temperature on Bohr effect in human and in horse hemoglobin. *J Biol Chem*. 1965; 240:1096–1103. [PubMed: 14284708]
28. Ho, C. *Adv Protein Chem*. Academic Press; San Diego, California: 1992. Proton Nuclear-Magnetic-Resonance studies on hemoglobin – Cooperative interactions and partially ligated intermediates; p. 153–312.
29. Russu IM, Ho NT, Ho C. A proton nuclear Overhauser effect investigation of the subunit interfaces in human normal adult hemoglobin. *Biochim Biophys Acta*. 1987; 914:40–48. [PubMed: 3607061]
30. Simplaceanu V, Lukin JA, Fang TY, Zou M, Ho NT, Ho C. Chain-selective isotopic labeling for NMR studies of large multimeric proteins: Application to hemoglobin. *Biophys J*. 2000; 79:1146–1154. [PubMed: 10920044]
31. Tsai CH, Shen TJ, Ho NT, Ho C. Effects of substitutions of lysine and aspartic acid for asparagine at β 108 and of tryptophan for valine at α 96 on the structural and functional properties of human normal adult hemoglobin: Roles of $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces in the cooperative oxygenation process. *Biochemistry*. 1999; 38:8751–8761. [PubMed: 10393550]
32. Fang TY, Simplaceanu V, Tsai CH, Ho NT, Ho C. An additional H-bond in the $\alpha_1\beta_2$ interface as the structural basis for the low oxygen affinity and high cooperativity of a novel recombinant hemoglobin (β L105W). *Biochemistry*. 2000; 39:13708–13718. [PubMed: 11076510]
33. Fung LWM, Ho C. Proton nuclear magnetic-resonance study of quaternary structure of human hemoglobins in water. *Biochemistry*. 1975; 14:2526–2535. [PubMed: 1138870]
34. Perutz MF, Ladner JE, Simon SR, Ho C. Influence of globin structure on state of heme. 1 Human deoxyhemoglobin. *Biochemistry*. 1974; 13:2163–2173. [PubMed: 4826890]
35. Park SY, Yokoyama T, Shibayama N, Shiro Y, Tame JRH. 1.25 Å resolution crystal structures of human haemoglobin in the oxy, deoxy and carbonmonoxy forms. *J Mol Biol*. 2006; 360:690–701. [PubMed: 16765986]
36. Perutz MF, Imai K. Regulation of oxygen affinity of mammalian haemoglobins. *J Mol Biol*. 1980; 136:183–191. [PubMed: 7373649]
37. Fronticelli C, Bucci E, Orth C. Solvent regulation of oxygen-affinity in hemoglobin - Sensitivity of bovine hemoglobin to chloride ions. *J Biol Chem*. 1984; 259:10841–10844. [PubMed: 6469985]

38. Kavanaugh JS, Rogers PH, Case DA, Arnone A. High-resolution X-ray study of deoxyhemoglobin Rothschild-37- β Trp $\rightarrow$ Arg: a mutation that creates an intersubunit chloride-binding site. *Biochemistry*. 1992; 31:4111–4121. [PubMed: 1567857]

Figure 1.

The chloride dependence of the oxygen-binding properties [$\log P_{50}$ (mm Hg)] for Hb A, rHb AE, rHb WM and the five mutants of rHbs WM measured at 29 °C, pH 7.4 in 0.1 M MES buffer with 0.01 M, 0.1 M, 0.5 M and 1.0 M sodium chloride, in the absence (*solid lines*) and presence (*dotted lines*) of 0.5 mM IHP, respectively.

Figure 2. Comparison of temperature dependence of the oxygen-binding properties [$\log P_{50}$ (mm Hg)] in the absence (solid lines and filled symbols) and presence (dotted lines and unfilled symbols) of 0.5 mM IHP for Hb A, rHb AE, rHb WM, and the five mutants of rHb WM, measured at 11, 20, 29, and 37 °C in 0.1 M MES (●, circles) and sodium phosphate (NaPi) (◆, diamonds) buffers at pH 7.0, and in 0.1 M MES buffer with 0.1 M sodium chloride (▲, triangles) at pH 7.4.

Figure 3.

Apparent enthalpy of oxygenation ΔH ($\text{kJ mol}^{-1} \text{O}_2$) values of Hb A and the rHbs under various experimental conditions. Mean ΔH values of Hbs and the standard deviation values were calculated from P_{50} values measured at 11, 20, 29, and 37 °C.

Figure 4. ^1H -NMR spectra (600 MHz) of the rHbs in 95% H_2O , 5% D_2O , and 0.1 M sodium phosphate (NaPi) buffer. Exchangeable proton resonances at pH 7.0 in the CO (A) and deoxy form (B) at 29 °C, and the CO form of the rHbs in the presence of 5 mM IHP at 11 °C (C). Ring-current-shifted resonances of the CO form of the rHbs at pH 7.0 and 29 °C (D).

Table 1

Sequence differences among the α - and β/δ -type globin chains of Asian elephant Hb (rHb AE), woolly mammoth Hb (rHb WM), and the five mutants of rHb WM. The corresponding amino acid residues of human adult Hb (Hb A) ($\alpha_2\beta_2$ tetramer) are included for comparison.

Hemoglobin	$\alpha 5$	$\beta/\delta 12$	$\beta/\delta 86$	$\beta/\delta 101$
Hb A	Ala	Thr	Ala	Glu
rHb AE	Lys	Thr	Ala	Glu
rHb WM	Asn	Ala	Ser	Gln
rHb WM ($\beta/\delta Q101E$)	Asn	Ala	Ser	Glu
rHb WM ($\alpha N5K$, $\beta/\delta Q101E$)	Lys	Ala	Ser	Glu
rHb WM ($\beta/\delta Q101K$)	Asn	Ala	Ser	Lys
rHb WM ($\beta/\delta Q101D$)	Asn	Ala	Ser	Asp
rHb WM ($\alpha N5K$, $\beta/\delta A12T$, $\beta/\delta S86A$)	Lys	Thr	Ala	Gln

Oxygen affinities (P_{50} , in mm Hg) of the rHbs at 29 °C and pH 7.4 in 0.1 M MES (“stripped” condition) or 0.1 M sodium phosphate (NaPi) buffer, in the absence and presence of 0.5 M sodium chloride and/or 0.5 mM inositol hexaphosphate (IHP).

Table 2

Hemoglobin	P_{50} (mm Hg)					
	MES buffer			NaPi buffer		
	“Stripped”	+ Cl ⁻	+ IHP	+ Cl ⁻ + IHP	- IHP	+ IHP
Hb A	5.2	13.9	40.1	15.3	8.6	27.6
rHb AE	7.7	9.2	17.6	9.2	8.4	12.3
rHb WM	5.0	10.9	20.4	10.6	10.5	15.0
rHb WM (β8Q101E)	7.4	8.2	18.3	8.4	8.6	10.9
rHb WM (αN5K,β8Q101E)	7.6	8.6	16.1	9.4	8.5	12.0
rHb WM (β8Q101K)	2.4	3.7	11.6	5.1	3.0	5.7
rHb WM (β8Q101D)	1.8	2.5	5.1	3.5	2.0	3.6
rHb WM (αN5K, β8A12T, β8S86A)	3.6	5.2	6.2	4.1	3.9	5.0

Table 3

Alkaline Bohr effect ($\Delta\log P_{50}/\Delta\text{pH}$) of Hb A, rHb WM, rHb AE, and the five mutants of rHb WM in the presence and absence of 0.5 mM IHP. Measurements were conducted at 29 °C and over the pH range 6.5–7.8 in 0.1 M MES and 0.1 M sodium phosphate (NaPi) buffers, at a hemoglobin concentration of 100 μM .

Hemoglobin	Alkaline Bohr effect			
	MES buffer		NaPi buffer	
	“Stripped”	+ IHP	– IHP	+ IHP
Hb A	–0.51	–0.87	–0.48	–0.77
rHb AE	–0.26	–0.68	–0.32	–0.47
rHb WM	–0.46	–0.77	–0.44	–0.68
rHb WM ($\beta/\delta\text{Q101E}$)	–0.27	–0.57	–0.26	–0.48
rHb WM (αN5K , $\beta/\delta\text{Q101E}$)	–0.24	–0.66	–0.26	–0.45
rHb WM ($\beta/\delta\text{Q101K}$)	–0.36	–0.74	–0.34	–0.68
rHb WM ($\beta/\delta\text{Q101D}$)	–0.54	–0.76	–0.47	–0.60
rHb WM (αN5K , $\beta/\delta\text{A12T}$, $\beta/\delta\text{S86A}$)	–0.21	–0.37	–0.21	–0.37