

# A Biochemical–Biophysical Study of Hemoglobins from Woolly Mammoth, Asian Elephant, and Humans

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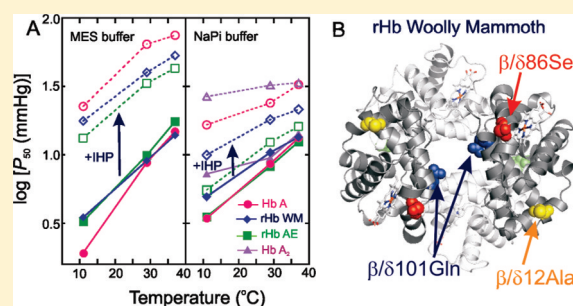
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## Supporting Information

**ABSTRACT:** This study is aimed at investigating the molecular basis of environmental adaptation of woolly mammoth hemoglobin (Hb) to the harsh thermal conditions of the Pleistocene ice ages. To this end, we have carried out a comparative biochemical–biophysical characterization of the structural and functional properties of recombinant hemoglobins (rHb) from woolly mammoth (rHb WM) and Asian elephant (rHb AE) in relation to human hemoglobins Hb A and Hb A<sub>2</sub> (a minor component of human blood). We have obtained oxygen equilibrium curves and calculated O<sub>2</sub> affinities, Bohr effects, and the apparent heat of oxygenation ( $\Delta H$ ) in the presence and absence of allosteric effectors [inorganic phosphate and inositol hexaphosphate (IHP)]. Here, we show that the four Hbs exhibit distinct structural properties and respond differently to allosteric effectors. In addition, the apparent heat of oxygenation ( $\Delta H$ ) for rHb WM is less negative than that of rHb AE, especially in phosphate buffer and the presence of IHP, suggesting that the oxygen affinity of mammoth blood was also less sensitive to temperature change. Finally, <sup>1</sup>H NMR spectroscopy data indicates that both  $\alpha_1(\beta/\delta)_1$  and  $\alpha_1(\beta/\delta)_2$  interfaces in rHb WM and rHb AE are perturbed, whereas only the  $\alpha_1\delta_1$  interface in Hb A<sub>2</sub> is perturbed compared to that in Hb A. The distinct structural and functional features of rHb WM presumably facilitated woolly mammoth survival in the Arctic environment.



Human normal adult hemoglobin (Hb A) is a heterotetramer consisting of two  $\alpha$ -subunits and two  $\beta$ -subunits.<sup>1</sup> Within the bloodstream, this respiratory protein switches cyclically between high and low O<sub>2</sub>-affinity states to control the delicate balance between the uptake of O<sub>2</sub> at the alveoli and its optimal unloading at the tissues. However, because Hb oxygenation is exothermic, this balance is disrupted by changes in temperature such that O<sub>2</sub> affinity decreases sharply with increasing temperature and increases with decreasing temperature.<sup>1</sup> Although this trait is often considered “adaptive” for enhancing O<sub>2</sub> offloading at warm (exercising) muscles, it may pose considerable challenges for O<sub>2</sub> delivery to cool extremities and peripheral tissues of heterothermic mammals and hence is often countered.<sup>2</sup> However, the molecular mechanisms by which the thermal sensitivity of the O<sub>2</sub> affinity of Hb, which is dictated by the overall enthalpy of oxygenation ( $\Delta H$ ), has been lowered through evolution within the various cold-adapted mammalian lineages are poorly understood.<sup>3–5</sup> Elephantids are a particularly good model system to investigate the effects of temperature on Hb structure–function relationships as they possess both warm- and cold-adapted members. Specifically, the group evolved in warm subtropical climates, where extant Asian and African lineages are still found, with the ancestors of the extinct woolly

mammoth (*Mammuthus primigenius*) abruptly invading high-latitude environments of Eurasia near the start of the Pleistocene ice ages some 1.2–2.0 million years ago.<sup>6</sup> Consistent with this environmental diaspora, the functional properties of woolly mammoth Hb were found to differ substantially from those of Asian elephant Hb, with the most notable change being a significant reduction in the overall  $\Delta H$  of the mammoth protein in the presence of naturally occurring allosteric effector molecules.<sup>6</sup> Interestingly, this functional shift comes about despite the fact that the primary sequences of Asian elephant Hb differ from those of the mammoth at only one position in the  $\alpha$ -globin chain (KSN) and at three positions (T12A, A86S, and E101Q) in the  $\beta$ -type globin chain, respectively (Table 1).<sup>6</sup> Of these latter residues,  $\beta/\delta 12\text{Ala}$  of the mammoth protein is near the 2,3-bisphosphoglycerate (BPG) binding cleft,  $\beta/\delta 86\text{Ser}$  is in the heme pocket, and  $\beta/\delta 101\text{Gln}$  is located between 99Asp and 102Asn of the same chain in the intersubunit  $\alpha_1(\beta/\delta)_2$  interface, which are all critical to the function of Hb.<sup>1</sup> Indeed, naturally occurring human mutations at  $\beta 101$  have illustrated that changes

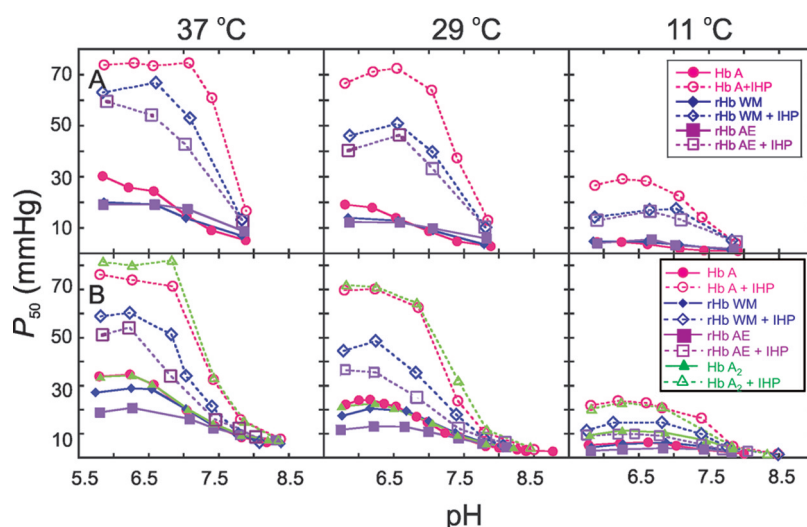
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**Figure 1.** pH dependence of the oxygen-binding properties ( $P_{50}$ , in mmHg) of Hb A (○, red), Hb A<sub>2</sub> (△, green), rHb WM (◇, blue), and rHb AE (□, magenta) in (A) 0.1 M MES buffer and in (B) 0.1 M sodium phosphate buffer in the absence (filled) and presence (open) of IHP and at 37, 29, and 11 °C.

the previous procedure.<sup>12,14</sup> The purity of the rHbs was then checked with an electrospray ionization mass spectrometer and Edman degradation as described previously.<sup>12,14</sup> All rHbs used in this study had the correct molecular weights and contained less than 5% methionine at the amino termini.

**Oxygen-Binding Properties of Hbs.** Oxygen-equilibrium curves for these four Hbs were measured in both 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) and 0.1 M sodium phosphate buffers at 11, 29, and 37 °C with a Hemox Analyzer. Experiments were conducted in the presence and absence of three-time molar concentrations of inositol hexaphosphate (IHP), in the pH range 5.5 to 8.5. The sample concentration of Hbs used 100–120  $\mu$ M (in terms of heme) was selected to avoid tetramer–dimer dissociation. Each sample was checked before and after each measurement for methemoglobin (met-Hb) in a spectrophotometer. Any sample with greater than 5% met-Hb was discarded. The partial pressure of O<sub>2</sub> at 50% Hb saturation ( $P_{50}$ ), a measure of oxygen 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 (mmHg) have an accuracy of  $\pm 5\%$ , and the  $n_{50}$  values have an accuracy of  $\pm 10\%$ .

**<sup>1</sup>H NMR Spectroscopy.** To detect changes in the tertiary or quaternary structure of the two elephantid Hbs, <sup>1</sup>H NMR spectra were obtained on Bruker Avance DRX-300 (results not shown) and DRX-600 NMR spectrometers. Samples consisted of aqueous solutions of Hb at a concentration of 5% (3.1 mM in terms of heme) in 0.1 M sodium phosphate buffer at pH 7.0 in 95% water and 5% deuterium oxide (D<sub>2</sub>O) and were assessed at 11, 29, and 37 °C. A jump-and-return pulse sequence was used to suppress the water signal. <sup>1</sup>H chemical shifts were indirectly referenced to the methyl proton resonance of the sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) through use of the internal reference of the water signal at 4.76 ppm downfield of DSS at 29 °C.

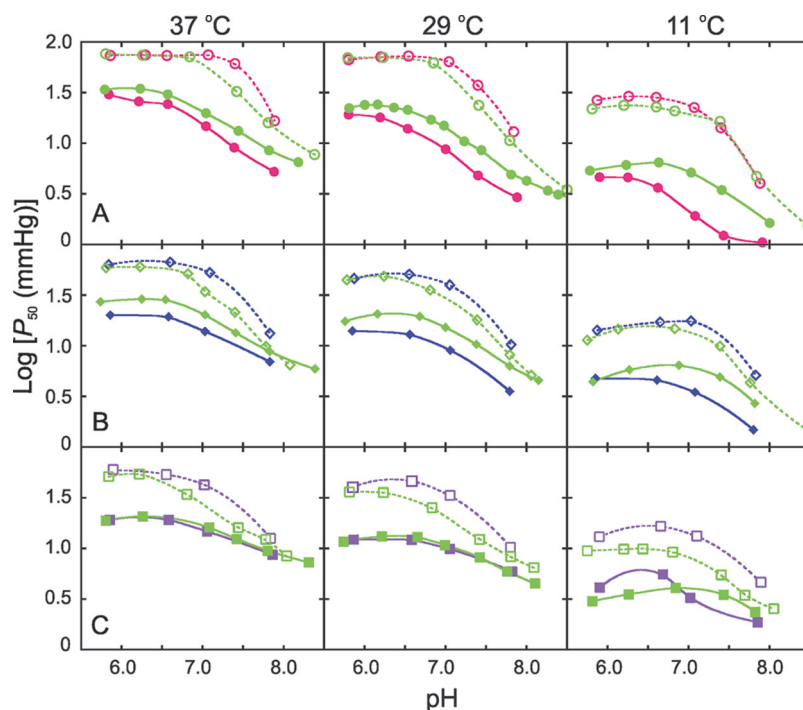
## RESULTS

**Oxygen-Binding Properties in MES and Phosphate Buffers.** The functional properties of the four Hbs were studied by measuring their oxygen-binding affinities as a

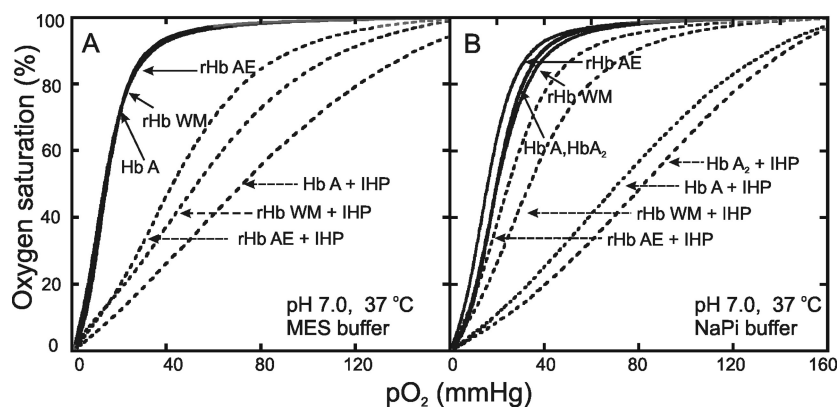
function of buffer, pH, and temperature in the presence or absence of IHP (Figure 1). Each of the Hbs studied exhibits distinct functional properties, with  $P_{50}$  values varying widely between Hbs. Notably, rHb WM has a lower O<sub>2</sub> affinity (higher  $P_{50}$  value) than that of rHb AE under the various experimental conditions (Figure 1). The  $P_{50}$  values of Hb A and Hb A<sub>2</sub> are very similar and always higher than those of rHb WM and rHb AE. The  $P_{50}$  values are also affected remarkably by buffer condition. For instance, in MES buffer and in the absence of IHP (“stripped” condition), the differences of the  $P_{50}$  values between rHb WM and rHb AE are very small (Figure 1A) but become significant in phosphate buffer (Figure 1B) due to their different response to phosphate ion. Similarly, the addition of IHP to Hbs significantly decreases the O<sub>2</sub> affinities in both MES buffer and phosphate buffer, but the relative IHP effect is much stronger in MES buffer. Thus, in the presence of IHP, the O<sub>2</sub> affinities of these Hbs are always lower in MES buffer than in phosphate buffer (Figure 2). The effects of the buffer and IHP on the O<sub>2</sub> affinities are also illustrated by the dramatic shift of the oxygen-binding curves. For example, at pH 7 and 37 °C, in the absence of IHP, the O<sub>2</sub>-binding curves for these Hbs are very close in MES buffer (Figure 3A) but become scattered in phosphate buffer (Figure 3B). Upon the addition of IHP, all of the binding curves are shifted to the right in both buffers. Finally, it is noted that the addition of IHP affects the O<sub>2</sub> affinities of Hb A and Hb A<sub>2</sub> the most, followed by rHb WM, whereas rHb AE is affected the least by IHP (Figure 3).

The cooperativity of the oxygenation process for all Hbs can be assessed by the Hill coefficient,  $n_{50}$ . The  $n_{50}$  values for Hbs under various experimental conditions are taken as the slope of the Hill plots at 50% saturation and are summarized in the Supporting Information Table 1S. The  $n_{50}$  values change significantly in the presence of IHP but are only slightly affected by buffer, temperature, and pH. For example, as shown in Figure 4, the  $n_{50}$  values for both Hb A and Hb A<sub>2</sub> are maintained around the expected value of 2.8–3.0 in the range of pH 5.8–8.4 in 0.1 M phosphate buffer at 29 °C, while  $n_{50}$  values for rHb WM and rHb AE are found to have lower values (2.0–2.5) under the same experimental conditions. Upon the addition of IHP, a broader spectrum of differences in the  $n_{50}$

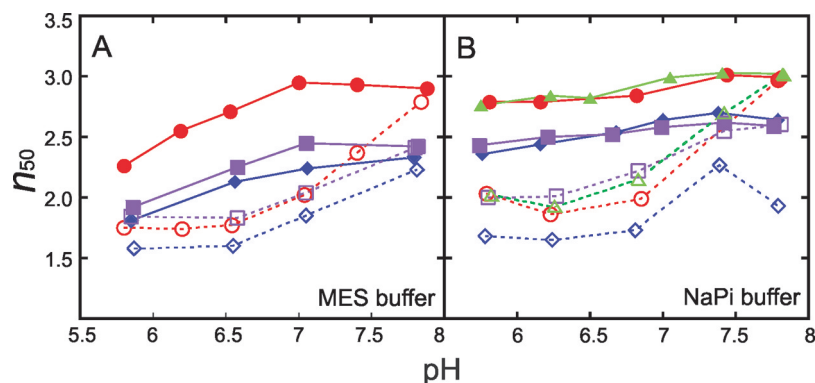




**Figure 2.** Comparison of pH dependence of the oxygen-binding properties [ $\log P_{50}$  (mmHg)] for (A) Hb A ( $\circ$ , red), (B) rHb WM ( $\diamond$ , blue), and (C) rHb AE ( $\square$ , magenta) measured in 0.1 M MES buffer and in the absence (filled) and presence (open) of IHP and at 11, 29, and 37 °C, respectively. Measurements conducted for the three Hbs in 0.1 M sodium phosphate buffer (green) are also presented.



**Figure 3.** Percent of  $O_2$  saturation of Hb A, Hb A<sub>2</sub>, rHb Asian elephant (AE), and rHb woolly mammoth (WM) as a function of  $O_2$  partial pressure at pH 7.0 and 37 °C in (A) 0.1 M MES buffer and in (B) 0.1 M sodium phosphate (NaPi) buffer in the absence (solid lines) and presence (dashed lines) of IHP.



**Figure 4.** Hill coefficient ( $n_{50}$ ) of Hb A ( $\circ$ , red), Hb A<sub>2</sub> ( $\triangle$ , green), rHb WM ( $\diamond$ , blue), and rHb AE ( $\square$ , magenta) as a function of pH at 29 °C in (A) 0.1 M MES buffer and in (B) 0.1 M sodium phosphate (NaPi) buffer in the absence (filled) and presence (open) of IHP.

values is observed with  $n_{50}$  being generally reduced at lower pH values and close to  $n_{50}$  values measured in the absence of IHP at higher pHs. Of note, the  $n_{50}$  values of rHb WM are generally lower than those of the other Hbs under all experimental conditions (Figure 4).

The usual Hb concentration used for our  $O_2$ -binding measurements is 100–120  $\mu\text{M}$  (in terms of heme). In order to investigate if rHb WM and rHb AE exhibit unusual tetramer–dimer dissociation, we have also carried out a concentration-dependent study of the  $O_2$ -binding measurements of these two Hbs together with Hb A over a Hb concentration range of 25–100  $\mu\text{M}$  in 0.1 M sodium phosphate buffer at pH 7.4 and 37 °C. No observable differences in both  $P_{50}$  and  $n_{50}$  values among these concentrations were found, indicating that there is no significant tetramer–dimer dissociation over the range of experimental conditions studied (see Supporting Information Table 2S).

**Bohr Effect.** The pH dependence of the oxygen affinity of Hbs is measured over a range of pH 8.5 to 5.5, and a significant difference is found in the amplitude of the alkaline Bohr effect ( $\Delta\log P_{50}/\Delta\text{pH}$  between pH 6.8 and 8.0) for these Hbs (Table 2). In the “stripped” condition, the  $O_2$ -binding affinity

**Table 2. Alkaline Bohr Effect ( $\Delta\log P_{50}/\Delta\text{pH}$ ) of Hemoglobins from Human (Hb A and Hb A<sub>2</sub>), Woolly Mammoth (rHb WM), and Asian Elephant (rHb AE) in the Presence and Absence of Inositol Hexaphosphate (IHP)<sup>a</sup>**

condition		Hb A	Hb A <sub>2</sub>	rHb WM	rHb AE
MES buffer	–IHP	–0.53		–0.38	–0.28
	+IHP	–0.79		–0.81	–0.81
NaPi buffer	–IHP	–0.45	–0.43	–0.46	–0.32
	+IHP	–0.67	–0.71	–0.72	–0.47

<sup>a</sup>Measurements were conducted at 37 °C and over the pH range 7.0–7.8 in 0.1 M MES and sodium phosphate (NaPi) buffers at a hemoglobin concentration of 100  $\mu\text{M}$ .

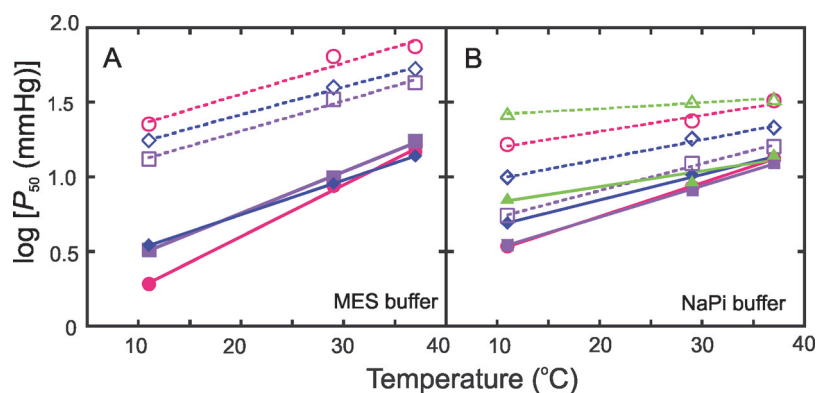
of Hb A shows a stronger Bohr effect ( $\Delta\log P_{50}/\Delta\text{pH} = -0.53$ ), while rHb WM and rHb AE are characterized by lower Bohr coefficients ( $\Delta\log P_{50}/\Delta\text{pH} = -0.38$  and  $-0.28$ , respectively). However, upon the addition of IHP, Bohr coefficients of rHb WM and rHb AE reach their maximum ( $\Delta\log P_{50}/\Delta\text{pH} = -0.81$ ), which is comparable to that of Hb A ( $\Delta\log P_{50}/\Delta\text{pH} = -0.79$ ). The Bohr effect of rHb WM is

slightly increased ( $-0.46$ ) in phosphate buffer and is further increased to about  $-0.70$  upon the addition of IHP, very close to that of Hb A and A<sub>2</sub> under the same experimental conditions (Table 2). However, the Bohr effect of rHb AE remains low ( $-0.32$ ) in phosphate buffer and only increase slightly ( $-0.47$ ) in the presence of IHP, a value still considerably lower than that of the other Hbs.

**Effect of Temperature on Oxygen Affinity.** The oxygen-binding affinity of Hbs is affected not only by allosteric effectors and pH but also by temperature.<sup>1,15</sup> The temperature dependence of the oxygen affinity of Hbs has been measured at 37, 29, and 11 °C, in the pH range of 5.5–8.5 in MES and phosphate buffers, respectively, and the results are summarized in Figure 5 and Supporting Information Figure 1S. As expected,  $P_{50}$  values of Hbs are lower at 11 °C than at 37 °C, indicating that  $O_2$  binds tighter to Hbs at lower temperatures (Figure 5). In the absence of IHP, the  $P_{50}$  values of these Hbs are in the same range, increasing from  $\sim 3$  mmHg at 11 °C to  $\sim 16$  mmHg at 37 °C. However, the  $P_{50}$  values of rHb WM exhibit a smaller increase for the same experimental temperature change. As illustrated in Figure 5A, rHb WM has a lower  $\Delta\log P_{50}/\Delta T$  value (0.023) in MES buffer, compared to Hb A (0.034) and rHb AE (0.028), where  $\Delta T$  represents the difference between two temperatures (in °C) and  $\Delta\log P_{50}$  is the difference in  $P_{50}$  (mmHg) at the two temperatures. In phosphate buffer, the  $\Delta\log P_{50}/\Delta T$  values for these Hbs are all reduced, through that of rHb WM (0.019) remains consistently lower than that of Hb A (0.023) and rHb AE (0.024). Upon the addition of IHP, all of the  $P_{50}$  values at each experimental temperature increase, but the absolute values vary for each Hb because of their different response to IHP. In order to illustrate the temperature effect more clearly, the apparent heat of oxygenation,  $\Delta H$ , is calculated from the van't Hoff eq 1 on the basis of  $\Delta\log P_{50}$  at the two different temperatures ( $T_1$  and  $T_2$ , in K):

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

$\Delta H$  includes the heat of oxygen solvation ( $-3 \text{ kcal mol}^{-1}$ ). The results of the exothermic oxygenation enthalpy ( $\Delta H$ , kJ  $\text{mol}^{-1}$ , where  $1 \text{ cal} = 4.184 \text{ J}$ ) presented in Table 3 have been corrected for this value. It is noted that the  $\Delta H$  values for the four Hbs are all pH dependent (Figure 6). For example, in phosphate buffer, the  $\Delta H$  value for rHb WM changes from



**Figure 5.** Comparison of temperature dependence of the oxygen-binding properties [ $\log P_{50}$  (mmHg)] in the absence (filled) and presence (open) of IHP for Hb A (O, red), Hb A<sub>2</sub> (Δ, green), rHb WM (◇, blue), and rHb AE (□, magenta) measured at 11, 29, and 37 °C: (A) at pH 7.0 in 0.1 M MES buffer; (B) at pH 7.4 in 0.1 M sodium phosphate (NaPi) buffer.

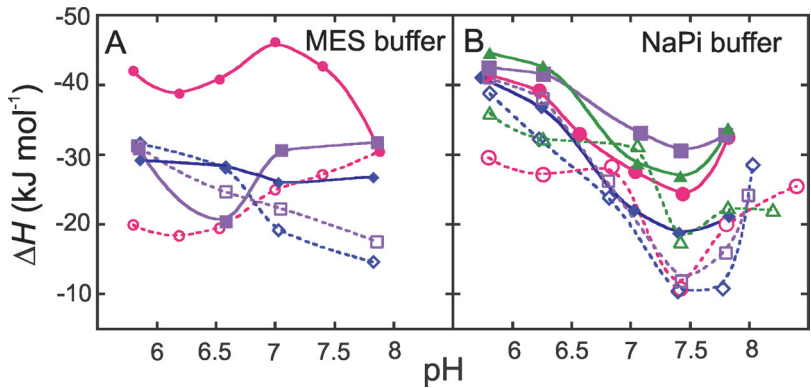
**Table 3. Apparent Enthalpy of Oxygenation ( $\Delta H$ ;  $\text{kJ mol}^{-1} \text{O}_2$ ) Values of Human (Hb A and Hb A<sub>2</sub>), Woolly Mammoth (rHb WM), and Asian Elephant (rHb AE) Hemoglobin as a Function of pH<sup>a</sup>**

buffer	pH	$\Delta H$ ( $\text{kJ mol}^{-1}$ )			
		Hb A	Hb A <sub>2</sub>	rHb WM	rHb AE
MES	5.80	−42.0		−29.1	−30.9
	6.19	−38.7			
	6.53	−40.8		−28.3	−20.4
	7.00	−46.1		−25.9	−30.6
	7.40	−42.7			
	7.88	−30.4		−26.7	−31.7
MES + IHP	5.80	−19.9		−31.5	−31.1
	6.19	−18.3			
	6.53	−19.4		−28.1	−24.6
	7.00	−24.9		−19.1	−22.2
	7.40	−27.1			
	7.88	−30.4		−14.6	−17.5
NaPi	5.79	−41.4	−44.5	−41.0	−42.5
	6.23	−39.2	−42.7	−36.7	−41.6
	6.57	−33.0			
	7.04	−27.5	−28.7	−21.9	−33.1
	7.44	−24.3	−26.8	−18.7	−30.6
	7.82	−32.4	−33.7	−21.0	−32.8
NaPi + IHP	5.80	−29.5	−35.9	−38.8	−40.9
	6.26	−27.1	−32.2	−32.2	−38.2
	6.84	−28.3		−23.7	−26.0
	7.06		−31.3		−11.8
	7.42	−10.7	−17.4	−10.4	−15.9
	7.81	−20.0	−22.4	−10.7	−24.0
"stripped" <sup>6</sup>	8.03	−25.4		−28.4	−40.9
				−35	−35
"stripped" + 0.1 M Cl <sup>−</sup> <sup>6</sup>		−41.0		−27	−31.5
0.1 M Cl <sup>−</sup> + 2.5 M BPG <sup>6</sup>				−19.3	−28.1

<sup>a</sup>Mean  $\Delta H$  values of Hbs were calculated from  $P_{50}$  values measured in the absence and presence of inositol hexaphosphate (IHP) in 0.1 M MES and sodium phosphate (NaPi) buffers at 11, 29, and 37 °C. Confidence limits of  $\Delta H$  values are  $\pm 15\%$ . Mean  $\Delta H$  values previously determined for woolly mammoth and Asian elephant rHbs in 0.1 M HEPES buffer at pH 7.0 and 7.4 and over the temperature ranges 10 and 25 °C and 25 and 37 °C<sup>6</sup> are presented for comparison.

−41.0  $\text{kJ mol}^{-1}$  at pH 5.8 to −18.7  $\text{kJ mol}^{-1}$  at pH 7.4. At pH 7.0 in the "stripped" condition, the  $\Delta H$  values of rHb WM and rHb AE are −25.9 and −30.6  $\text{kJ mol}^{-1}$ , respectively, much lower (in absolute terms) than that of human Hb A (−46.1  $\text{kJ mol}^{-1}$ ); i.e., rHb WM and rHb AE show a smaller temperature effect than Hb A. In phosphate buffer, the  $\Delta H$  values of rHb WM are less negative than those of rHb AE, while the  $\Delta H$  values of Hb A and Hb A<sub>2</sub> are very similar over the range of experimental conditions. When IHP is present, the  $\Delta H$  values of the four Hbs are all significantly decreased (in absolute terms), with that of rHb WM remaining slightly lower than that of rHb AE. For all four Hbs, the lowest  $\Delta H$  values (in absolute terms) are observed when both phosphate and IHP are present as functional modulators, suggesting an additive effect of phosphate and IHP to the  $\Delta H$  value.

**<sup>1</sup>H NMR Spectra of Hemoglobins.** <sup>1</sup>H NMR spectra were recorded for the Hbs in the deoxy and the CO forms in the absence and presence of IHP at 11, 29, and 37 °C. The exchangeable proton resonances of  $\alpha 103\text{His}$  and  $\alpha 122\text{His}$  of the four Hbs studied in the CO and deoxy forms are shown in Figure 7. In Hb A, the resonance at 12.2 ppm has been assigned to the side chain  $\text{Ne}_2\text{H}$  group of  $\alpha 103\text{His}$ ,<sup>16–19</sup> and the resonance at 12.9 ppm has been assigned to the side chain  $\text{Ne}_2\text{H}$  group of  $\alpha 122\text{His}$ .<sup>18</sup> In the CO form of Hbs (Figure 7A), the peak at 12.2 ppm ( $\alpha 103\text{His}$ ) is not present in the spectra of rHb AE or rHb WM but appears in the spectra for Hb A and A<sub>2</sub>. Additionally, the peak at 12.9 ppm ( $\alpha 122\text{His}$ ) in Hb A is shifted downfield in the spectra of rHb AE and rHb WM (which have  $\alpha$ - and  $\beta/\delta$ -chains) and Hb A<sub>2</sub> (which has  $\alpha$ - and  $\delta$ -chains). In the spectrum of deoxy-Hb A (Figure 7C), the resonance at 14 ppm was assigned to the H-bond between  $\alpha 42\text{Tyr}$  and  $\beta 99\text{Asp}$  and has been used as a T-state marker at the  $\alpha_1\beta_2$  interface.<sup>20</sup> In the presence of IHP, the T-marker starts to appear in the spectra of the CO form of Hb A and Hb A<sub>2</sub> at a lower temperature (11 °C) but is not seen in the spectra of the CO form of rHb AE and rHb WM (Figure 7B). Significant differences are observed in the deoxy state of the Hbs (Figure 7C). As reported previously, the T-marker in deoxy-Hb A<sub>2</sub> is present at the same spectral position as that of deoxy-Hb A.<sup>13</sup> These T-state markers shift 0.1–0.3 ppm downfield in the spectra of rHb WM and rHb AE, and the peak at 11.1 ppm assigned to  $\beta 37\text{Trp}$  also shifts 0.2 ppm downfield at all temperatures, indicating a perturbation of the  $\alpha_1(\beta/\delta)_2$  interface in rHb WM and rHb AE (Figure 7C).



**Figure 6.** Apparent enthalpy of oxygenation ( $\Delta H$ ;  $\text{kJ mol}^{-1} \text{O}_2$ ) values of Hb A (○, red), Hb A<sub>2</sub> (△, green), rHb WM (◇, blue), and rHb AE (□, magenta), as a function of pH. Mean  $\Delta H$  values of Hbs were calculated from  $P_{50}$  values measured at 11, 29, and 37 °C (A) in 0.1 M MES buffer and (B) in 0.1 M sodium phosphate (NaPi) buffer and in the absence (filled) and presence (open) of IHP. Confidence limits of  $\Delta H$  values are  $\pm 15\%$ .





the low Bohr effects observed for these two Hbs (Table 2) may arise from substitutions at  $\beta/\delta 44(\text{Ser} \rightarrow \text{His})$  and  $\beta/\delta 56(\text{Gly} \rightarrow \text{His})$ , which presumably contribute negatively to the Bohr effect of these two proteins. Although rHb AE has the identical histidyl residues as rHb WM, it exhibits a weaker Bohr effect in phosphate buffer, even in the presence of IHP, suggesting that the contributions from the histidyl residues might not account for all of the Bohr effect of these two rHbs. The lower Bohr effect of rHb AE is consistent with its weaker response to phosphate and IHP relative to rHb WM (Table 2).

**Temperature Effects.** The oxygenation of Hb is exothermic, with increasing the temperature lowering the  $\text{O}_2$  affinity directly by weakening the hydrogen bonds between Hb and  $\text{O}_2$ .<sup>1,33,34</sup> Arctic ruminants (e.g., musk ox and reindeer) are routinely subjected to extremely low environmental temperatures. Hence, to reduce the heat loss under these conditions, these animals exploit countercurrent heat exchangers in the extremities that allow them to maintain markedly lower tissue temperatures at these sites.<sup>3</sup> Thus, a decrease in the temperature sensitivity of oxygen binding could be a functional strategy which allows unloading  $\text{O}_2$  to the cool peripheral tissues. This phenomenon has been observed in the previous studies for the Hbs of a number of subarctic and arctic mammals, including Eskimo dog, musk ox, and reindeer.<sup>5,35,36</sup> The apparent heat of oxygenation,  $\Delta H$ , is used to evaluate the temperature effect on the  $\text{O}_2$  affinities of Hbs. On the basis of the  $\Delta H$  values, the Hbs of various species can be broadly divided into two groups. The Hbs of the first group, including reindeer Hb and musk ox Hb, have intrinsically low  $\Delta H$  values even in the absence of allosteric effectors.<sup>5</sup> The second group of Hbs can be further divided based on whether the large negative  $\Delta H$  of these Hbs becomes less negative in the presence of 2,3-bisphosphoglyceric acid (2,3-BPG), such as pig Hb and fetal human Hb (Hb F), or whether the  $\Delta H$  is not very sensitive to the addition of 2,3-BPG, such as Hb A.<sup>5</sup> In the “stripped” condition, rHb WM and rHb AE have a less negative  $\Delta H$  of oxygenation when compared with Hb A (Table 3 and Figure 6A). In phosphate buffer, the  $\Delta H$  values of rHb WM and rHb AE decrease slightly and remain in the same range as in MES buffer, while that of Hb A dramatically decrease (in absolute terms) compared to that in MES buffer (Table 3 and Figure 6B). Further comparisons between rHb WM and rHb AE show that although similar  $\Delta H$  values are observed in MES buffer, rHb WM has much less negative  $\Delta H$  values in phosphate buffer (Figure 6B), which can be attributed to the fact that the  $\text{O}_2$  affinity of rHb WM has a stronger response to phosphate, a weak allosteric effector. When IHP is present, the  $\Delta H$  values of Hbs are greatly affected as the result of a change in the  $P_{50}$  values. Differences between the  $\Delta H$  values of the various Hbs remain but become smaller due to the strong allosteric effect of IHP (Figure 6). The less negative values of the  $\Delta H$  values observed upon the presence of phosphate and/or IHP suggest that the apparent temperature effect is dependent on the ability of each of the Hbs to respond to the allosteric effectors. On the basis of the previous studies on the Hbs of various arctic animals,<sup>5</sup> a potential structural explanation has been proposed, suggesting that the amino acid residues at  $\beta 8$ ,  $\beta 76$ , and  $\beta 77$  positions form an “additional” chloride-binding site (relative to Hb A), which is responsible for lowering  $\Delta H$  of these proteins.<sup>37,38</sup> Additionally, the mutation of  $\beta 76\text{Ala} \rightarrow \text{Lys}$  might be responsible for the synergistic effect of 2,3-BPG and chloride on the  $\text{O}_2$  affinity of these Hbs.<sup>38</sup> In the Hbs of arctic animals, such as reindeer and ox, the amino acid residue at  $\beta 76$

is Lys, and the  $\Delta H$  values of these Hbs are less negative than that of horse Hb, which has Ala at  $\beta 76$ . Both rHb WM and rHb AE have  $\beta/\delta 76\text{Lys}$ , the same as in reindeer Hb. Our previous study of rHb WM suggests the E101Q substitution in rHb WM creates an additional binding site for allosteric effectors, which contributes to the less negative  $\Delta H$  of oxygenation relative to rHb AE, thus forming part of the physiological adaptation of woolly mammoth.<sup>6</sup> The less negative  $\Delta H$  values of rHb WM observed in the present work confirm the lower temperature effect of rHb WM compared to that of rHb AE.<sup>6</sup>

On the basis of our measurements, the  $\Delta H$  values of rHb WM are in the same range as those of Hbs from other arctic animals obtained under similar experimental conditions.<sup>5</sup> For example, in 0.1 M HEPES buffer with 0.1 M NaCl in the presence of 2,3-BPG, the  $\Delta H$  of reindeer Hb is  $-14.0 \text{ kJ mol}^{-1}$ ,<sup>5</sup> whereas the values calculated for rHb WM are  $-18.7 \text{ kJ mol}^{-1}$  in 0.1 M sodium phosphate buffer (current result) and  $-19.3 \text{ kJ mol}^{-1}$  in the presence of  $\text{Cl}^-$  and BPG.<sup>6</sup> These low  $\Delta H$  values of rHb WM are consistent with life in the arctic environment since the  $\text{O}_2$  affinity of its Hb is less affected by low temperature, and therefore, it would be easier to unload  $\text{O}_2$  to the cool peripheral tissues.<sup>6</sup> The  $\Delta H$  value of rHb AE is  $-30.6 \text{ kJ mol}^{-1}$  in phosphate buffer, which is more negative than that of rHb WM. However, it is reduced by the addition of IHP ( $-15.9 \text{ kJ mol}^{-1}$ ), a value only slightly higher than that of rHb WM ( $-10.4 \text{ kJ mol}^{-1}$ ). This suggests that a lower  $\Delta H$  value observed from rHb AE might be attributed to the stronger allosteric effect of IHP and that the reduction in the  $\Delta H$  of rHb AE is less pronounced in the presence of 2,3-BPG.<sup>6</sup>

In the stripped condition, the  $\Delta H$  value for Hb A ( $-46.1 \text{ kJ mol}^{-1}$  at pH 7.0) is more negative than that of rHb WM ( $-25.9 \text{ kJ mol}^{-1}$ ), and it is only slightly increased by 2,3-BPG in the presence of chloride ions.<sup>5,39</sup> In our study, when Hbs are saturated with IHP, the  $\Delta H$  values for Hb A are significantly increased to the same range as that of rHb WM and rHb AE, suggesting that the  $\text{O}_2$  affinity and the  $\Delta H$  value of Hbs are dependent on the property and the concentration of the allosteric effectors. It could also be the case for Hbs in the blood cells. For example, Asian and African elephant blood have slightly different functional properties, with the  $\text{O}_2$  affinity of Asian elephant blood being lower than that of African elephant due to an increased 2,3-BPG concentration in the red cells.<sup>40,41</sup>

**Structural Information from  $^1\text{H}$  NMR Studies.**  $^1\text{H}$  NMR spectra of Hbs in 0.1 M sodium phosphate buffer at pH 7.0 were assessed at 11, 29, and 37 °C. One significant change shown in the  $^1\text{H}$  NMR spectra is that the peak at 12.2 ppm disappears in the CO and deoxy forms of rHb WM and rHb AE (Figure 7). In Hb A, this resonance has been assigned to the side chain  $\text{Ne}_2\text{H}$  group of  $\alpha 103\text{His}$ , which is hydrogen-bonded to  $\beta 131\text{Gln}$ .<sup>16–19</sup> The resonance at 12.9 ppm has been assigned to the side chain  $\text{Ne}_2\text{H}$  group of  $\alpha 122\text{His}$ ,<sup>18</sup> which forms a water-mediated H-bond with the side chain of  $\beta 35\text{Tyr}$ . Both H-bonds are located at the  $\alpha_1\beta_1$ -subunit interface of Hb A. Our previous study of the mutant rHb ( $\beta 131\text{Gln} \rightarrow \text{Glu}$ ) shows that the peak at 12.2 ppm in the  $^1\text{H}$  NMR spectrum disappears, suggesting that the H-bond between  $\alpha 103\text{His}$  and  $\beta 131\text{Gln}$  does not exist due to the replacement at the  $\beta 131$  position.<sup>42</sup> In rHb WM and rHb AE, the amino acid residue of  $\alpha 103\text{His}$  is the same as that in Hb A, but  $\beta 131\text{Gln}$  existing in Hb A is changed to  $\beta/\delta 131\text{Glu}$  (Table 1). Thus, the



disappearance of the resonance at 12.2 ppm in the  $^1\text{H}$  NMR spectra of rHb WM and rHb AE is likely to be caused by this mutation, as reported for the mutant rHb ( $\beta 131\text{Gln}\rightarrow\text{Glu}$ ).<sup>42</sup> Our previous studies for rHb ( $\beta 131\text{Gln}\rightarrow\text{Glu}$ ) also show a slight downfield shift of the  $\alpha 122\text{His}$  resonance to 13.1 ppm, indicating that the perturbations caused by the replacement at  $\beta 131$  are not just confined to the mutation site but also affect the environment of  $\alpha 122\text{His}$ .<sup>42</sup> This also could be true for the case of rHb WM and rHb AE. Thus, the changes in  $^1\text{H}$  NMR spectra on the interface histidyl resonances of  $\alpha 103\text{His}$  and  $\alpha 122\text{His}$  could be attributed to the replacement of  $\beta 131\text{Gln}\rightarrow\text{Glu}$  in rHb WM and rHb AE. In the deoxy state of rHb WM and rHb AE (Figure 7C), the T-state markers located at 14.2 ppm and the peak of  $\beta 37\text{Trp}$  at 11.1 ppm are shifted downfield, indicating a perturbation of the  $\alpha_1(\beta/\delta)_2$  interface as well (Figure 7). Thus, in rHb AE and rHb WM, both  $\alpha_1(\beta/\delta)_1$  and  $\alpha_1(\beta/\delta)_2$  interfaces are perturbed in both the deoxy and CO forms in the absence and presence of IHP as compared to those of Hb A and Hb A<sub>2</sub>. From the previous functional studies of the mutant rHb ( $\beta 131\text{Gln}\rightarrow\text{Glu}$ ), it was shown that this mutation has only a small impact on the oxygen affinity of the Hb molecule.<sup>42</sup> Thus, although a significant change is observed in the  $^1\text{H}$  NMR spectra, the replacement of  $\beta 131\text{Gln}\rightarrow\text{Glu}$  might not have an important effect on the function of rHb WM and rHb AE.

On the basis of the previous studies of Hb A, the non-exchangeable ring current-shifted proton resonances at  $-1.75$  and  $-1.82$  ppm have been assigned to the  $\gamma_2\text{-CH}_3$  group of the  $\alpha 62\text{Val}$  and  $\beta 67\text{Val}$  residues of HbCO A, respectively.<sup>15,20,21</sup> These resonances provide information about the geometry/environment of the heme pocket. The ring-current-shifted resonances for these two valyl residues are resolved in the CO form of Hb A and Hb A<sub>2</sub>, but both shift upfield to  $-2.0$  ppm in the CO form of rHb WM and rHb AE (Figure 7D). These results suggest that the distal heme pockets of rHb WM and rHb AE are altered as compared to those of Hb A and Hb A<sub>2</sub>. Comparing the amino acid sequences of these Hbs, several replacements in rHb WM and rHb AE can be found near  $\alpha 62\text{Val}$  and  $\beta 67\text{Val}$ , including  $\alpha 63\text{Ala}\rightarrow\text{Gly}$ ,  $\alpha 64\text{Asp}\rightarrow\text{Glu}$ ,  $\beta 65\text{Lys}\rightarrow\text{Glu}$ , and  $\beta 69\text{Gly}\rightarrow\text{Thr}$ . Previous studies have indicated that the mutations in the heme pocket of Hb A can change the O<sub>2</sub> affinity by affecting the tertiary structure of the protein.<sup>34,43,44</sup> Our NMR studies reported here provide direct evidence showing that the geometry/environment of the heme pockets of rHb WM and rHb AE is different with respect to those of human Hbs and that these changes may be related to the higher O<sub>2</sub>-binding affinity of these Hbs.

**Structural Information from Hb A<sub>2</sub>.** Hb A<sub>2</sub> exhibits a remarkable structural similarity to Hb A. The  $\alpha$ -chains are the same in these two Hbs, and there are only 10 amino acid substitutions in the  $\delta$ -chain of Hb A<sub>2</sub> compared to the  $\beta$ -chain of Hb A. On the basis of the ring-current-shifted resonances of E11Val, no significant change in the distal heme pocket was detected due to the  $\delta$ -chain replacement (Figure 7D). In the  $^1\text{H}$  NMR spectra of Hb A<sub>2</sub>, the resonance at 13.1 ppm shows a slight downfield shift, similar to that of rHb WM and rHb AE, while the resonance at 12.2 ppm is unchanged, suggesting that the  $\delta$ -chain replacement in Hb A<sub>2</sub> conserves the  $\alpha_1\delta_2$  interface, but only slightly perturbs the  $\alpha_1\delta_1$  interface.<sup>13</sup> The perturbation on the intradimer interface is shown more clearly in the  $^1\text{H}$  NMR spectra of rHb WM and rHb AE due to the combination

effect of the  $\beta/\delta$ -chain replacement and the mutation of  $\beta/\delta 131\text{Gln}\rightarrow\text{Glu}$ .

Perutz and Raidt reported that Hb A<sub>2</sub> was more resistant to thermal denaturation than Hb A.<sup>45</sup> They suggested that two amino acid residues in the helices G ( $\delta 116\text{Arg}$ ) and H ( $\delta 126\text{Met}$ ) of Hb A<sub>2</sub> might be responsible for its higher thermal stability. It is speculated that  $\delta 116\text{Arg}$  could make an extra hydrogen bond at the  $\alpha_1\delta_1$  interface and that  $\delta 126\text{Met}$  (H4) could make intrasubunit nonpolar contacts with  $\delta 11\text{Val}$  of helix A.<sup>45</sup> However, recent X-ray crystal studies of Hb A<sub>2</sub> did not support these additional interactions, but nonpolar contacts formed between helices A and H were found, which presumably contribute to the higher thermal stability of Hb A<sub>2</sub>.<sup>10,46</sup> Notably, rHb WM and rHb AE also possess Arg at position 116 of their  $\beta$ -type chains (Table 1), which may similarly elevate the thermal stability of these two Hbs. The X-ray structural studies also suggest that the higher O<sub>2</sub>-binding affinity of Hb A<sub>2</sub> may result from the slighter larger  $\alpha_1\delta_2$  interface of Hb A<sub>2</sub> and an additional hydrogen bond at the  $\alpha_1\delta_2$  (or  $\alpha_2\delta_1$ ) interface between  $\alpha 94\text{Asp}$  and  $\delta 37\text{Trp}$  in Hb A<sub>2</sub>.<sup>10</sup> This could also be true for rHb WM and rHb AE.

## CONCLUSION

It is important to understand how the oxygen transport of Hbs is controlled by the combined action of temperature and ligands. Our studies on rHb WM and rHb AE have confirmed that these two Hbs respond differently to changes in experimental conditions. In general, the O<sub>2</sub> affinity of rHb WM has a larger response to allosteric effectors and is less sensitive to temperature change than rHb AE. These features are related to its structure and appear to arise from at least two of the three mutations found on the  $\beta/\delta$  chain of this extinct species. It should be noted that our biochemical–biophysical study is limited to only the Hb molecule. In red cells, the factors that affect the oxygen affinity are more complex. Thus, the negative influences, such as a decrease in oxygen unloading due to a lower temperature, may be compensated by the action of one or more other factors. For example, it has been shown that blood of African and Asian elephants possess slightly different functional properties, with the O<sub>2</sub> affinity of Asian elephant blood being lower than that of African elephant blood due to an increased 2,3-BPG concentration in the red cells.<sup>40,41</sup> It is possible that such subtle difference also existed in woolly mammoth blood. The distinct structural features of rHb WM provide a part of the basis for woolly mammoth survival in the arctic environment. Further investigations are needed for applying these structural features to the design of a new generation of medically relevant Hb-based oxygen carriers.

## ASSOCIATED CONTENT

### Supporting Information

Table 1S showing the Hill coefficient ( $n_{50}$ ); Table 2S and Figure 1S showing the concentration and temperature dependence of the oxygen-binding properties ( $P_{50}$ ) of Hbs under various experimental conditions, respectively. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## ABBREVIATIONS

Hb A, human normal adult hemoglobin, the  $\alpha_2\beta_2$  tetramer; Hb A<sub>2</sub>, a minor component of human normal adult hemoglobin, the  $\alpha_2\delta_2$  tetramer; rHb, recombinant Hb; rHb WM, recombinant woolly mammoth Hb; rHb AE, recombinant Asian elephant Hb; HbCO, carbonmonoxyhemoglobin; deoxy-Hb, deoxyhemoglobin; met-Hb, methemoglobin; NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; MES, 2-(N-morpholino)ethanesulfonic acid; 2,3-BPG, 2,3-bisphosphoglycerate; IHP, inositol hexaphosphate.

## REFERENCES

- (1) Dickerson, R. E., and Geis, I. (1983) *Hemoglobin: Structure, Function, Evolution, and Pathology*, Benjamin/Cummings, Menlo Park, CA.
- (2) Weber, R. E., and Campbell, K. L. (2011) Temperature dependence of haemoglobin-oxygen affinity in heterothermic vertebrates: mechanisms and biological significance. *Acta Physiol.* 202, 549–562.
- (3) Irving, L., and Krog, J. (1955) Temperature of skin in the arctic as a regulator of heat. *J. Appl. Physiol.* 7, 355–364.
- (4) Clementi, M. E., Condo, S. G., Castagnola, M., and Giardina, B. (1994) Hemoglobin-function under extreme life conditions. *Eur. J. Biochem.* 223, 309–317.
- (5) De Rosa, M. C., Castagnola, M., Bertonati, C., Galtieri, A., and Giardina, B. (2004) From the Arctic to fetal life: physiological importance and structural basis of an 'additional' chloride-binding site in haemoglobin. *Biochem. J.* 380, 889–896.
- (6) Campbell, K. L., Roberts, J. E. E., Watson, L. N., Stetefeld, J., Sloan, A. M., Signore, A. V., Howatt, J. W., Tame, J. R. H., Rohland, N., Shen, T. J., Austin, J. J., Hofreiter, M., Ho, C., Weber, R. E., and Cooper, A. (2010) Substitutions in woolly mammoth hemoglobin confer biochemical properties adaptive for cold tolerance. *Nature Genet.* 42, 536–540.
- (7) Shih, D. T., Jones, R. T., Imai, K., and Tyuma, I. (1985) Involvement of Glu G3(101) $\beta$  in the function of hemoglobin. Comparative O<sub>2</sub> equilibrium studies of human mutant hemoglobins. *J. Biol. Chem.* 260, 5919–5924.
- (8) Opazo, J. C., Sloan, A. M., Campbell, K. L., and Storz, J. F. (2009) Origin and ascendency of a chimeric fusion gene: the beta/delta-globin gene of paenungulate mammals. *Mol. Biol. Evol.* 26, 1469–1478.
- (9) Bunn, H. F., and Forget, B. G. (1986) *Hemoglobin: Molecular, Genetic and Clinical Aspects*, W.B. Saunders, Philadelphia, PA.
- (10) Sen, U., Dasgupta, J. L., Choudhury, D., Datta, P., Chakrabarti, A., Chakrabarty, S. B., Chakrabarty, A., and Dattagupta, J. K. (2004) Crystal structures of HbA<sub>2</sub> and HbE and modeling of hemoglobin  $\delta_4$ : Interpretation of the thermal stability and the antisickling effect of HbA<sub>2</sub> and identification of the ferrocyanide binding site in Hb. *Biochemistry* 43, 12477–12488.
- (11) Dexter, F., Kern, F. H., Hindman, B. J., and Greeley, W. J. (1997) The brain uses mostly dissolved oxygen during profoundly hypothermic cardiopulmonary bypass. *Ann. Thorac. Surg.* 63, 1725–1729.
- (12) Shen, T. J., Ho, N. T., Simplaceanu, V., Zou, M., Green, B. N., Tam, M. F., and Ho, C. (1993) Production of unmodified human adult hemoglobin in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* 90, 8108–8112.
- (13) Russu, I. M., Lin, A., Ferro-Dosch, S., and Ho, C. (1984) A proton nuclear magnetic-resonance investigation of human hemoglobin A<sub>2</sub> – Implications on the intermolecular contacts in Sick cell hemoglobin fibers and on the Bohr effect of human normal adult hemoglobin. *Biochim. Biophys. Acta* 785, 123–131.
- (14) Shen, T. J., Ho, N. T., Zou, M., Sun, D. P., Cottam, P. F., Simplaceanu, V., Tam, M. F., Bell, D. A., and Ho, C. (1997) Production of human normal adult and fetal hemoglobins in *Escherichia coli*. *Protein Eng.* 10, 1085–1097.
- (15) Doyle, M. L., Gill, S. J., Decristofaro, R., Castagnola, M., and Dicerca, E. (1989) Temperature-dependence and pH-dependence of the oxygen-binding reaction of human-fetal hemoglobin. *Biochem. J.* 260, 617–619.
- (16) Ho, C. (1992) Proton Nuclear-Magnetic-Resonance studies on hemoglobin – Cooperative interactions and partially ligated intermediates. *Adv. Protein Chem.*, 153–312.
- (17) Russu, I. M., Ho, N. T., and Ho, C. (1987) A proton nuclear Overhauser effect investigation of the subunit interfaces in human normal adult hemoglobin. *Biochim. Biophys. Acta* 914, 40–48.
- (18) Simplaceanu, V., Lukin, J. A., Fang, T. Y., Zou, M., Ho, N. T., and Ho, C. (2000) Chain-selective isotopic labeling for NMR studies of large multimeric proteins: Application to hemoglobin. *Biophys. J.* 79, 1146–1154.
- (19) Tsai, C. H., Shen, T. J., Ho, N. T., and Ho, C. (1999) Effects of substitutions of lysine and aspartic acid for asparagine at  $\beta$ 108 and of tryptophan for valine at  $\alpha$ 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* 38, 8751–8761.
- (20) Fung, L. W. M., and Ho, C. (1975) Proton nuclear magnetic-resonance study of quaternary structure of human hemoglobins in water. *Biochemistry* 14, 2526–2535.
- (21) Dalvit, C., and Ho, C. (1985) Proton nuclear Overhauser effect investigation of the heme pockets in ligated hemoglobin - Conformational differences between oxy and carbonmonoxy forms. *Biochemistry* 24, 3398–3407.
- (22) Lindstrom, T. R., Lehmann, H., Charache, S., Noren, I. B. E., and Ho, C. (1972) Nuclear magnetic-resonance studies of hemoglobins. 7. Tertiary structure around ligand binding-site in carbonmonoxyhemoglobin. *Biochemistry* 11, 1677–1681.
- (23) Bunn, H. F. (1971) Differences in interaction of 2,3-diphosphoglycerate with certain mammalian hemoglobins. *Science* 172, 1049–1050.
- (24) Bunn, H. F. (1980) Regulation of hemoglobin-function in mammals. *Am. Zool.* 20, 199–211.
- (25) Perutz, M. F., and Imai, K. (1980) Regulation of oxygen affinity of mammalian haemoglobins. *J. Mol. Biol.* 136, 183–191.
- (26) Perutz, M. F. (1970) Stereochemistry of cooperative effects in haemoglobin. *Nature* 228, 726–733.
- (27) Kilmartin, J. V., and Rossi-Bernardi, L. (1969) Inhibition of CO<sub>2</sub> combination and reduction of the Bohr effect in haemoglobin chemically modified at its  $\alpha$ -amino groups. *Nature* 222, 1243–1246.
- (28) Kilmartin, J. V., and Rossi-Bernardi, L. (1973) Interaction of hemoglobin with hydrogen ions, carbon dioxide, and organic phosphates. *Physiol. Rev.* 53, 836–890.
- (29) Busch, M. R., Mace, J. E., Ho, N. T., and Ho, C. (1991) Roles of the  $\beta$ 146 histidyl residue in the molecular-basis of the Bohr effect of hemoglobin - a proton nuclear magnetic resonance study. *Biochemistry* 30, 1865–1877.
- (30) Sun, D. Z.P., Zou, M., Ho, N. T., and Ho, C. (1997) Contribution of surface histidyl residues in the  $\alpha$ -chain to the Bohr effect of human normal adult hemoglobin: Roles of global electrostatic effects. *Biochemistry* 36, 6663–6673.

- (31) Fang, T. Y., Zou, M., Simplaceanu, V., Ho, N. T., and Ho, C. (1999) Assessment of roles of surface histidyl residues in the molecular basis of the Bohr effect and of  $\beta$ 143 histidine in the binding of 2,3-bisphosphoglycerate in human normal adult hemoglobin. *Biochemistry* 38, 13423–13432.
- (32) Lukin, J. A., and Ho, C. (2004) The structure-function relationship of hemoglobin in solution at atomic resolution. *Chem. Rev.* 104, 1219–1230.
- (33) Weber, R. E., Campbell, K. L., Fago, A., Malte, H., and Jensen, F. B. (2010) ATP-induced temperature independence of hemoglobin- $O_2$  affinity in heterothermic billfish. *J. Exp. Biol.* 213, 1579–1585.
- (34) Yuan, Y., Simplaceanu, V., Ho, N. T., and Ho, C. (2010) An investigation of the distal histidyl hydrogen bonds in oxyhemoglobin: Effects of temperature, pH, and inositol hexaphosphate. *Biochemistry* 49, 10606–10615.
- (35) Bårdgard, A. J., and Brix, O. (1997) Functional characterisation of Eskimo dog hemoglobin: II. The interplay of  $HCO_3^-$  and  $Cl^-$ . *Comp. Biochem. Physiol., Part A: Physiol.* 117, 375–381.
- (36) Bårdgard, A. J., Strand, I., Nuutinen, M., Jul, E., and Brix, O. (1997) Functional characterisation of Eskimo dog Hemoglobin: I. Interaction of  $Cl^-$  and 2,3-DPG and its importance to oxygen unloading at low temperature. *Comp. Biochem. Physiol., Part A: Physiol.* 117, 367–373.
- (37) Fronticelli, C. (1990) A possible new mechanism of oxygen affinity modulation in mammalian hemoglobins. *Biophys. Chem.* 37, 141–146.
- (38) Fronticelli, C., Sanna, M. T., Perez-Alvarado, G. C., Karavitis, M., Lu, A.-L., and Brinigar, W. S. (1995) Allosteric modulation by tertiary structure in mammalian hemoglobins - Introduction of the functional characteristics of bovine hemoglobin into human hemoglobin by five amino acid substitutions. *J. Biol. Chem.* 270, 30588–30592.
- (39) Giardina, B., Scatena, R., Clementi, M. E., Cerroni, L., Nuutinen, M., Brix, O., Sletten, S. N., Castagnola, M., and Condo, S. G. (1993) Physiological relevance of the overall  $\Delta H$  of oxygen binding to fetal human hemoglobin. *J. Mol. Biol.* 229, 512–516.
- (40) Brown, I. R. F., and White, P. T. (1980) Elephant Blood Hematology and Chemistry. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* 65, 1–12.
- (41) Dhindsa, D. S., Sedgwick, C. J., and Metcalfe, J. (1972) Comparative studies of the respiratory functions of mammalian blood. 8. Asian elephant (*Elephas maximus*) and African elephant (*Loxodonta africana africana*). *Respir. Physiol.* 14, 332–342.
- (42) Chang, C. K., Simplaceanu, V., and Ho, C. (2002) Effects of amino acid substitutions at  $\beta$ 131 on the structure and properties of hemoglobin: Evidence for communication between  $\alpha_1\beta_1$ - and  $\alpha_1\beta_2$ -subunit interfaces. *Biochemistry* 41, 5644–5655.
- (43) Mailliet, D. H., Simplaceanu, V., Shen, T. J., Ho, N. T., Olson, J. S., and Ho, C. (2008) Interfacial and distal-heme pocket mutations exhibit additive effects on the structure and function of hemoglobin. *Biochemistry* 47, 10551–10563.
- (44) Wiltrout, M.E., Giovannelli, J. L., Simplaceanu, V., Lukin, J. A., Ho, N. T., and Ho, C. (2005) A biophysical investigation of recombinant hemoglobins with aromatic B10 mutations in the distal heme pockets. *Biochemistry* 44, 7207–7217.
- (45) Perutz, M. F., and Raidt, H. (1975) Stereochemical Basis of Heat-Stability in Bacterial Ferredoxins and in Hemoglobin- $A_2$ . *Nature* 255, 256–259.
- (46) Dasgupta, J., Sen, U., Choudhury, D., Datta, P., Chakrabarti, A., Chakrabarty, S. B., Chakrabarty, A., and Dattagupta, J. K. (2003) Crystallization and preliminary X-ray structural studies of hemoglobin  $A_2$  and hemoglobin E, isolated from the blood samples of  $\beta$ -thalassemic patients. *Biochem. Biophys. Res. Commun.* 303, 619–623.