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

Yue Yuan,[†] Tong-Jian Shen,[†] Priyamvada Gupta,[†] Nancy T. Ho,[†] Virgil Simplaceanu,[†] Tsuey Chyi S. Tam,[†] Michael Hofreiter,[‡] Alan Cooper,[§] Kevin L. Campbell,[⊥] and Chien Ho*[,†]

[†]Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213, United States

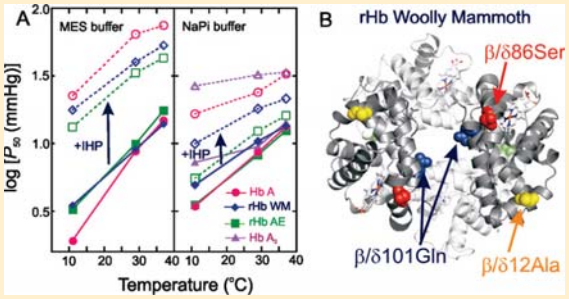
[‡]Department of Biology, University of York, York, YO10 5YW, United Kingdom

[§]Australian Centre for Ancient DNA, University of Adelaide, Adelaide, SA 5005, Australia

[⊥]Department of Biological Sciences, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada

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_2 (a minor component of human blood). We have obtained oxygen equilibrium curves and calculated O_2 affinities, Bohr effects, and the apparent heat of oxygenation (ΔH) 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 (ΔH) 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, ^{1}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_2 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 α-subunits and two β-subunits.[1] Within the bloodstream, this respiratory protein switches cyclically between high and low O_2-affinity states to control the delicate balance between the uptake of O_2 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_2 affinity decreases sharply with increasing temperature and increases with decreasing temperature.[1] Although this trait is often considered "adaptive" for enhancing O_2 offloading at warm (exercising) muscles, it may pose considerable challenges for O_2 delivery to cool extremities and peripheral tissues of heterothermic mammals and hence is often countered.[2] However, the molecular mechanisms by which the thermal sensitivity of the O_2 affinity of Hb, which is dictated by the overall enthalpy of oxygenation (ΔH), has been lowered through evolution within the various cold-adapted mammalian lineages are poorly understood.[3−5] 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 subtemperate 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.[6] 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 ΔH of the mammoth protein in the presence of naturally occurring allosteric effector molecules.[6] 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 α-globin chain (K5N) and at three positions (T12A, A86S, and E101Q) in the β-type globin chain, respectively (Table 1).[6] Of these latter residues, β/δ12Ala of the mammoth protein is near the 2,3-bisphosphoglycerate (BPG) binding cleft, β/δ86Ser is in the heme pocket, and β/δ101Gln 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.[1] Indeed, naturally occurring human mutations at β101 have illustrated that changes

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Table 1. Amino Acid Residue Differences among the α- and β-Type Globin Chains of Humans Hb A and A_2,[9] Asian Elephant (rHb AE), and Woolly Mammoth (rHb WM)[a]

α-chain

```
                10        20        30        40        50        60        70
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Hb A    VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHGKKVADALTNAVAHVDD
Hb A2   ..............................................................................
rHb AE  ...DK.......T.S...D..SD.V........F...................G.........GE...Q..G.L..
rHb WM  ...DN.......T.S...D..SD.V........F...................G.........GE...Q..G.L..

                80        90        100       110       120       130       140
        ....|....|....|....|....|....|....|....|....|....|....|....|....|.
Hb A    MPNALSALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTPAVHASLDKFLASVSTVLTSKYR
Hb A2   ..............................................................
rHb AE  L.S.............................SS.Q.T....E.........SN..........
rHb WM  L.S.............................SS.Q.T....E.........SN..........
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β-chain of Hb A, δ-chain of Hb A_2, and β/δ-chain of rHb AE and rHb WM

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                10        20        30        40        50        60        70
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Hb A    VHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDAVMGNPKVKAHGKKVLGAFSDGL
Hb A2   ........T..N.........A.........................S.........................
rHb AE  .N..AA..TQ..N.......K.L.....S.........R....H......A...LH.A..L...E...TS.GE..
rHb WM  .N..AA..TQ.AN.......K.L.....S.........R....H......A...LH.A..L...E...TS.GE..

                80        90        100       110       120       130       140
        ....|....|....|....|....|....|....|....|....|....|....|....|....|.
Hb A    AHLDNLKGTFATLSELHCDKLHVDPENFRLLGNVLVCVLAHHFGKEFTPPVQAAYQKVVAGVANALAHKYH
Hb A2   ..........SQ.............................RN.......QM...................
rHb AE  K..........D.........................I...R.........D.....E..............
rHb WM  K.........SD.............Q..........I...R.........D.....E..............
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[a]Differences from the Hb A sequence are colored in blue. Amino acid residue in rHb WM which differs with that in rHb AE is colored in red.

at this residue position alter both the intrinsic and allosteric properties of the protein, though the mechanism by which this residue exerts these effects is still unclear.[7]

The Hb of elephantids is unusual in that the β-type subunit is encoded by a chimeric β/δ-fusion gene (*HBB/D*) that arose via an unequal crossing over event long before the radiation of this group.[6,8] Consequently, the N- and C- terminal halves of the hybrid elephantid polypeptide are orthologous to the "β" and "δ" globin chains of other mammals, respectively. Thus, it would be interesting to probe the structural significance of this evolutionary change, as the physiochemical properties of human Hb A and Hb A_2 (a minor $\alpha_2\delta_2$ tetramer component of human blood)—which differ at only 10 residue positions (Table 1)[9]—show important differences in regards to antisickling properties and thermal stability.[10]

The primary objective of this study is to evaluate and compare the biochemical factors that alter the temperature dependence of O_2 binding to Asian elephant and mammoth Hb, with special reference to human Hbs A and A_2. Additionally, we aim to provide new insights into the structural mechanisms that affect Hb function, thereby aiding the design of a new generation of medically relevant Hb-based oxygen carriers (blood substitutes) for use, e.g., during certain hypothermia-dependent cardiac and brain surgery applications.[11] To this end, we obtained the ^{1}H NMR spectra of the four Hbs and measured their oxygen affinity, sensitivity to various allosteric effectors, and cooperativity at three different temperatures (37, 29, and 11 °C) and over a range of pH (pH 8.5 to 5.5).

MATERIALS AND METHODS

Materials. Hb A and Hb A_2 were isolated and purified from human normal blood samples obtained from the local blood bank using the procedures routine in our laboratory.[12,13] Chemicals and restriction enzymes were purchased from major suppliers, such as Fisher, Sigma, Bio-Rad, Boehringer Mannheim, New England BioLabs, Pharmacia, Promega, and United States Biochemicals, Inc., and were used without further purification.

Construction of Expression Plasmids. The plasmid (pHE27E) that expresses recombinant Hb AE was constructed by replacing the human α- and β-globin genes in Hb expression plasmid pHE2[12] with Asian elephant α-like and β/δ-like cDNAs. The plasmid (pHE27M) for expressing rHb WM was constructed by introducing the mammoth-specific residue mutations (αK5N, β/δT12A, β/δA86S, and β/δE101Q) into pHE27E using site-directed mutagenesis. The desired mutations were confirmed by DNA sequencing. For additional details, see ref 6.

Growth and Purification of rHbs. Plasmids for rHb AE (pHE27E) and rHb WM (pHE27M) were transformed into *E. coli* strain JM109, and the cells were grown in a 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 the expression of the rHbs. After the addition of hemin (25 mg/L), growth was continued for at least another 4 h. Harvesting was accomplished through centrifugation. Cell paste was stored at −80 °C until it was needed. The purification of rHbs followed

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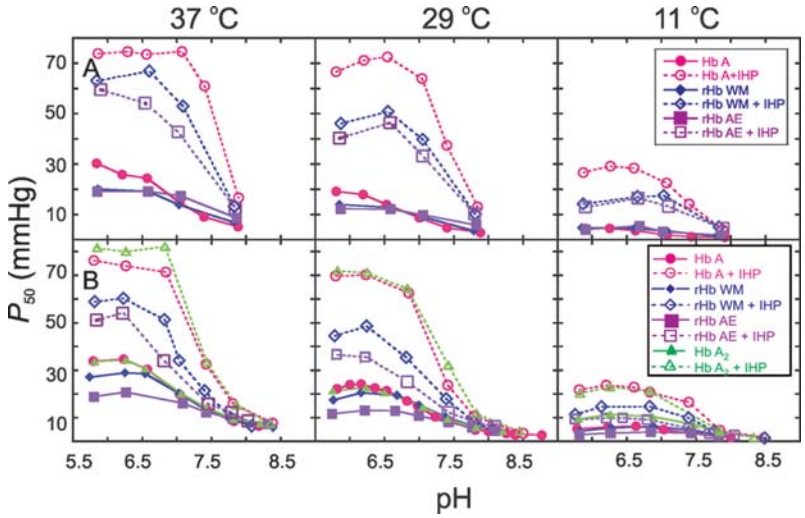

Figure 1. pH dependence of the oxygen-binding properties (P_{50}, in mmHg) of Hb A (○, red), Hb A_2 (△, green), rHb WM (◇, blue), and rHb AE (□, magenta) in (A) 0.1 M MES buffer and in (B) 0.1 M sodium phosphate buffer in the absence (filled) and presence (open) of IHP and at 37, 29, and 11 °C.

the previous procedure.[12,14] The purity of the rHbs was then checked with an electrospray ionization mass spectrometer and Edman degradation as described previously.[12,14] 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 μM (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_2 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 ±5%, and the n_{50} values have an accuracy of ±10%.

^{1}H NMR Spectroscopy. To detect changes in the tertiary or quaternary structure of the two elephantid Hbs, ^{1}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_2O) and were assessed at 11, 29, and 37 °C. A jump-and-return pulse sequence was used to suppress the water signal. ^{1}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_2 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_2 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_2 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_2 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_2 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_2-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_2 affinities of Hb A and Hb A_2 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_2 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}

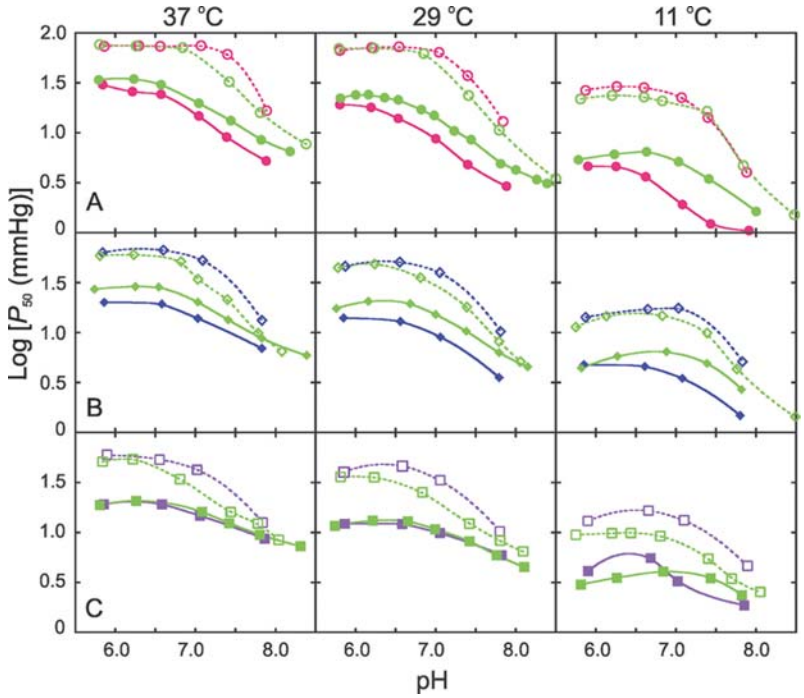

Figure 2. Comparison of pH dependence of the oxygen-binding properties [log P_{50} (mmHg)] for (A) Hb A (○, red), (B) rHb WM (◇, blue), and (C) rHb AE (□, magenta) measured in 0.1 M MES buffer and in the absence (filled) and presence (open) of IHP and at 11, 29, and 37 °C, respectively. Measurements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

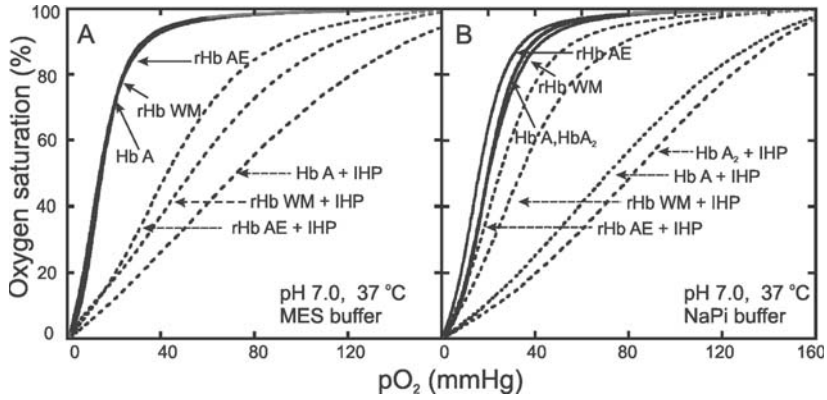

Figure 3. Percent of O_2 saturation of Hb A, Hb A2, rHb Asian elephant (AE), and rHb woolly mammoth (WM) as a function of O_2 partial pressure at pH 7.0 and 37 °C in (A) 0.1 M MES buffer and in (B) 0.1 M sodium phosphate (NaPi) buffer in the absence (solid lines) and presence (dashed lines) of IHP.

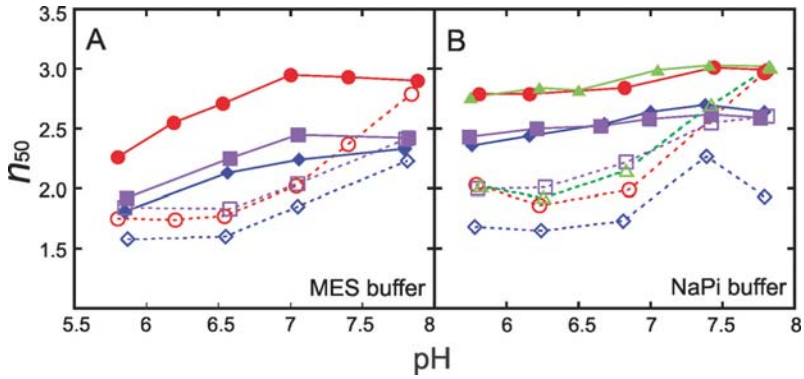

Figure 4. Hill coefficient (n_{50}) of Hb A (○, red), Hb A_2 (△, green), rHb WM (◇, blue), and rHb AE (□, magenta) as a function of pH at 29 °C in (A) 0.1 M MES buffer and in (B) 0.1 M sodium phosphate (NaPi) buffer in the absence (filled) and presence (open) of IHP.

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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 μM (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 μM 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 ($\Delta\log P_{50}/\Delta$pH between pH 6.8 and 8.0) for these Hbs (Table 2). In the "stripped" condition, the O_2-binding affinity of Hb A shows a stronger Bohr effect ($\Delta\log P_{50}/\Delta$pH = −0.53), while rHb WM and rHb AE are characterized by lower Bohr coefficients ($\Delta\log P_{50}/\Delta$pH = −0.38 and −0.28, respectively). However, upon the addition of IHP, Bohr coefficients of rHb WM and rHb AE reach their maximum ($\Delta\log P_{50}/\Delta$pH = −0.81), which is comparable to that of Hb A ($\Delta\log P_{50}/\Delta$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_2 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.

Table 2. Alkaline Bohr Effect ($\Delta\log P_{50}/\Delta$pH) of Hemoglobins from Human (Hb A and Hb A_2), Woolly Mammoth (rHb WM), and Asian Elephant (rHb AE) in the Presence and Absence of Inositol Hexaphosphate (IHP)[a]

condition		Hb A	Hb A_2	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

[a]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 μM.

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.[1,15] 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 ∼3 mmHg at 11 °C to ∼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 ΔT 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, ΔH, 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):

$$\Delta H\ (\text{kcal mol}^{-1}\ \text{O}_2) = -4.575(T_1T_2/T_2 - T_1)\Delta\log P_{50}/1000 \quad (1)$$

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

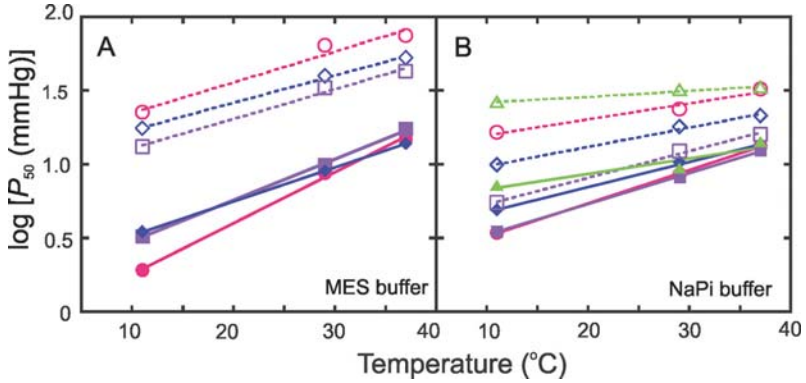

Figure 5. Comparison of temperature dependence of the oxygen-binding properties [log P_{50} (mmHg)] in the absence (filled) and presence (open) of IHP for Hb A (○, red), Hb A_2 (△, green), rHb WM (◇, blue), and rHb AE (□, magenta) measured at 11, 29, and 37 °C: (A) at pH 7.0 in 0.1 M MES buffer; (B) at pH 7.4 in 0.1 M sodium phosphate (NaPi) buffer.

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Table 3. Apparent Enthalpy of Oxygenation (ΔH; kJ mol^{-1} O_2) Values of Human (Hb A and Hb A_2), Woolly Mammoth (rHb WM), and Asian Elephant (rHb AE) Hemoglobin as a Function of pH[a]

		ΔH (kJ mol^{-1})			
buffer	pH	Hb A	Hb A_2	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
	8.03	−25.4		−28.4	−40.9
"stripped"[6]				−35	−35
"stripped" + 0.1 M Cl^-[6]		−41.0		−27	−31.5
0.1 M Cl^- + 2.5 M BPG[6]				−19.3	−28.1

[a]Mean ΔH 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 ΔH values are ±15%. Mean ΔH 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[6] are presented for comparison.

−41.0 kJ mol^{-1} at pH 5.8 to −18.7 kJ mol^{-1} at pH 7.4. At pH 7.0 in the "stripped" condition, the ΔH values of rHb WM and rHb AE are −25.9 and −30.6 kJ mol^{-1}, respectively, much lower (in absolute terms) than that of human Hb A (−46.1 kJ mol^{-1}); i.e., rHb WM and rHb AE show a smaller temperature effect than Hb A. In phosphate buffer, the ΔH values of rHb WM are less negative than those of rHb AE, while the ΔH values of Hb A and Hb A_2 are very similar over the range of experimental conditions. When IHP is present, the ΔH 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 ΔH 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 ΔH value.

^{1}H NMR Spectra of Hemoglobins. ^{1}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 α103His and α122His 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 Nε_2H group of α103His,[16−19] and the resonance at 12.9 ppm has been assigned to the side chain Nε_2H group of α122His.[18] In the CO form of Hbs (Figure 7A), the peak at 12.2 ppm (α103His) is not present in the spectra of rHb AE or rHb WM but appears in the spectra for Hb A and A_2. Additionally, the peak at 12.9 ppm (α122His) in Hb A is shifted downfield in the spectra of rHb AE and rHb WM (which have α- and β/δ-chains) and Hb A_2 (which has α- and δ-chains). In the spectrum of deoxy-Hb A (Figure 7C), the resonance at 14 ppm was assigned to the H-bond between α42Tyr and β99Asp and has been used as a T-state marker at the $\alpha_1\beta_2$ interface.[20] In the presence of IHP, the T-marker starts to appear in the spectra of the CO form of Hb A and Hb A_2 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_2 is present at the same spectral position as that of deoxy-Hb A.[13] 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 β37Trp 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).

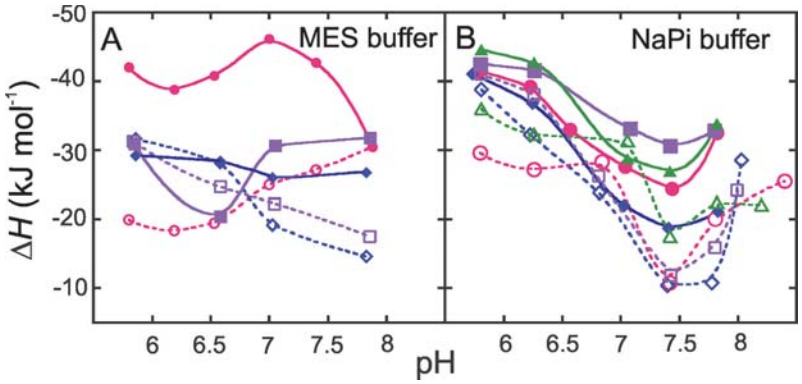

Figure 6. Apparent enthalpy of oxygenation (ΔH; kJ mol^{-1} O_2) values of Hb A (○, red), Hb A_2 (△, green), rHb WM (◇, blue), and rHb AE (□, magenta), as a function of pH. Mean ΔH values of Hbs were calculated from P_{50} values measured at 11, 29, and 37 °C (A) in 0.1 M MES buffer and (B) in 0.1 M sodium phosphate (NaPi) buffer and in the absence (filled) and presence (open) of IHP. Confidence limits of ΔH values are ±15%.

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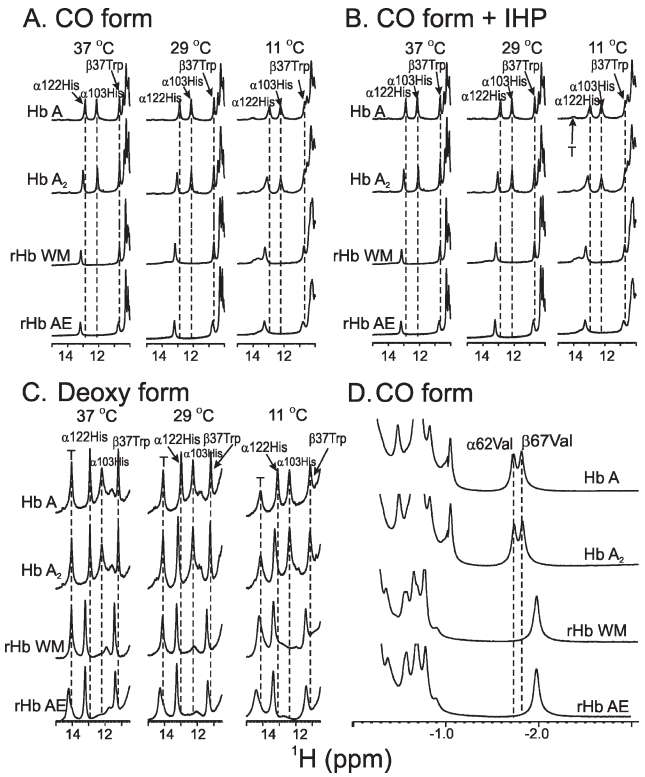

Figure 7. ^{1}H NMR spectra (600 MHz) of rHbs in 95% H_2O, 5% D_2O, and 0.1 M sodium phosphate buffer. Exchangeable proton resonances at pH 7.0 and 11, 29, and 37 °C, in the CO form in the absence (A) and presence (B) of IHP and in the deoxy form (C) are presented. (D) Ring-current-shifted resonances of the CO form of Hbs and rHbs at pH 7.0 and 29 °C.

Ring-current-shifted resonances at −1.5 to −2.0 ppm (Figure 7D) provide information about the tertiary structure around the heme pocket.[16] The resonances at −1.75 and −1.82 ppm have been assigned to the γ_2-CH_3 group of E11Val of the α- and β-chains of HbCO A, respectively.[21,22] For rHb WM and rHb AE, the E11Val methyl resonances of both the α- and β/δ-chains are shifted upfield to −2.01 ppm, while the resonances of Hb A_2 are not affected.

DISCUSSION

Response to IHP. Mammals can be divided into two groups based on how the O_2-binding affinities of their Hbs respond to allosteric effectors. The O_2-binding affinities of Hbs from humans, pigs, dogs, and most primates are decreased in the presence of organic phosphate, whereas those from cats and ruminants are not sensitive to organic phosphate but exhibit significant effects in the presence of chloride ions.[23−25] Our study has examined the properties of rHb WM and rHb AE with respect to their interactions with phosphate and IHP. Our results have confirmed that rHb WM and rHb AE belong to the group of mammalian Hbs that exhibit an intrinsically high oxygen affinity, which can be modulated by phosphate anions (i.e., phosphate and IHP). Comparisons between the P_{50} values obtained in MES and sodium phosphate buffers show that the O_2-binding affinities of Hb A, rHb WM, and rHb AE significantly decrease in phosphate buffer and/or in the presence of IHP (Figure 2). However, the IHP effect is stronger in MES buffer than in phosphate buffer, suggesting that the allosteric effects of phosphate and IHP are not synergistic with respect to the O_2 binding. Conversely, our results indicate that these two anions compete for the same binding sites. Thus, the changes of P_{50} values of these Hbs upon the addition of IHP reach their maximum in MES buffer and cannot be increased further in phosphate buffer.

On the basis of our current studies, rHb WM exhibits a stronger response to allosteric effectors (H^+, phosphate, and IHP), than rHb AE, despite possessing only three amino acid differences in their β/δ-chain sequences.[6] The functional properties of β/δ101Gln in rHb WM and β/δ101Glu in rHb AE are of particular interest because this residue is located in the $\alpha_1(\beta/\delta)_2$ contact region. In liganded Hb A, β101Glu is in close contact with α94 residue, while in the deoxy-Hb A, it has contacts with both β94Asp and α96Val residues.[1,26] Thus, this residue is involved in the stabilization of both unliganded and liganded tetramers of Hbs. Various mutations of Hb A at β101Glu have been studied, but the role of this residue is still not clear.[7] All mutant Hbs at this position have an elevated O_2 affinity relative to Hb A, except for the mutant Hb Rush (β101Glu→Gln), which is the only β101 mutant having a higher sensitivity to chloride ion than Hb A. Consequently, despite possessing a high intrinsic O_2 affinity (as in the other β101 mutants), the O_2 affinity of Hb Rush is decreased to a slightly lower value than that of Hb A in the presence of 0.1 M chloride.[7] As suggested by Campbell et al.,[6] the β/δ101Glu→Gln substitution in rHb WM might play the same role as in Hb A, which could allow the positive charge of β/δ104Arg to form an additional Cl^- binding site within the $\alpha_1(\beta/\delta)_2$ interface of the deoxy-state molecule. This proposed additional chloride-binding site in rHb WM might also be responsible for the increased allosteric effect caused by the addition of phosphate and IHP, as shown by the drastic decrease in O_2 affinity of rHb WM in the presence of these allosteric effectors.

Bohr Effect. Oxygen-binding experiments show that both inorganic phosphate and IHP affect the functional behavior of rHb WM and rHb AE, not limited only to the absolute value of the oxygen affinity, but also affecting the amplitude of the Bohr effect ($\Delta\log P_{50}/\Delta$pH). As shown in Table 2, in the "stripped" condition (i.e., MES buffer and in the absence of IHP), the $\Delta\log P_{50}/\Delta$pH values of rHb WM and rHb AE are lower (in absolute terms) than that of Hb A between pH 6.8 and 8.0. Bohr effects of the two elephantid rHbs are slightly influenced by the presence of inorganic phosphate but are markedly affected by the presence of IHP. For Hb A, it is known that there are a number of amino acid residues that contribute to the observed Bohr effects, including the N-terminal residues[27,28] and a large number of surface His residues.[29−32] In 0.1 M HEPES buffer with 0.1 M chloride, β146His contributes the most to the alkaline Bohr effect (63% at pH 7.4) among those surface histidyl residues, while β143His contributes the most to the acid Bohr effect (71% at pH 5.1).[29−32] These two histidyl residues are also present in rHb WM and rHb AE and presumably also contribute to the Bohr effect of these proteins. It is noted that the number of histidyl residues in rHb WM and rHb AE is the same as in Hb A, but the locations of these residues are different (Table 1). β2His and β116His in Hb A, which exert moderate negative and moderate positive contributions, repectively, to the Bohr effect,[31,32] have been replaced by β/δ2Asn and β/δ116Arg in rHb WM and rHb AE. Thus, the contributions from these two histidyl residues to the Bohr effect are absent in rHb WM and rHb AE. Consequently,

the low Bohr effects observed for these two Hbs (Table 2) may arise from substitutions at $\beta/\delta44$(Ser→His) and $\beta/\delta56$(Gly→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 O_2 affinity directly by weakening the hydrogen bonds between Hb and O_2.[1,33,34] 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.[3] Thus, a decrease in the temperature sensitivity of oxygen binding could be a functional strategy which allows unloading 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.[5,35,36] The apparent heat of oxygenation, ΔH, is used to evaluate the temperature effect on the O_2 affinities of Hbs. On the basis of the ΔH 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 ΔH values even in the absence of allosteric effectors.[5] The second group of Hbs can be further divided based on whether the large negative ΔH 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 ΔH is not very sensitive to the addition of 2,3-BPG, such as Hb A.[5] In the "stripped" condition, rHb WM and rHb AE have a less negative ΔH of oxygenation when compared with Hb A (Table 3 and Figure 6A). In phosphate buffer, the ΔH 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 ΔH values are observed in MES buffer, rHb WM has much less negative ΔH values in phosphate buffer (Figure 6B), which can be attributed to the fact that the O_2 affinity of rHb WM has a stronger response to phosphate, a weak allosteric effector. When IHP is present, the ΔH values of Hbs are greatly affected as the result of a change in the P_{50} values. Differences between the ΔH 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 ΔH 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,[5] a potential structural explanation has been proposed, suggesting that the amino acid residues at $\beta8$, $\beta76$, and $\beta77$ positions form an "additional" chloride-binding site (relative to Hb A), which is responsible for lowering ΔH of these proteins.[37,38] Additionally, the mutation of $\beta76$Ala→Lys might be responsible for the synergistic effect of 2,3-BPG and chloride on the O_2 affinity of these Hbs.[38] In the Hbs of arctic animals, such as reindeer and ox, the amino acid residue at $\beta76$ is Lys, and the ΔH values of these Hbs are less negative than that of horse Hb, which has Ala at $\beta76$. Both rHb WM and rHb AE have $\beta/\delta76$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 ΔH of oxygenation relative to rHb AE, thus forming part of the physiological adaptation of woolly mammoth.[6] The less negative ΔH values of rHb WM observed in the present work confirm the lower temperature effect of rHb WM compared to that of rHb AE.[6]

On the basis of our measurements, the ΔH values of rHb WM are in the same range as those of Hbs from other arctic animals obtained under similar experimental conditions.[5] For example, in 0.1 M HEPES buffer with 0.1 M NaCl in the presence of 2,3-BPG, the ΔH of reindeer Hb is −14.0 kJ mol^{-1},[5] whereas the values calculated for rHb WM are −18.7 kJ mol^{-1} in 0.1 M sodium phosphate buffer (current result) and −19.3 kJ mol^{-1} in the presence of Cl^- and BPG.[6] These low ΔH values of rHb WM are consistent with life in the arctic environment since the O_2 affinity of its Hb is less affected by low temperature, and therefore, it would be easier to unload O_2 to the cool peripheral tissues.[6] The ΔH value of rHb AE is −30.6 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 kJ mol^{-1}), a value only slightly higher than that of rHb WM (−10.4 kJ mol^{-1}). This suggests that a lower ΔH value observed from rHb AE might be attributed to the stronger allosteric effect of IHP and that the reduction in the ΔH of rHb AE is less pronounced in the presence of 2,3-BPG.[6]

In the stripped condition, the ΔH value for Hb A (−46.1 kJ mol^{-1} at pH 7.0) is more negative than that of rHb WM (−25.9 kJ mol^{-1}), and it is only slightly increased by 2,3-BPG in the presence of chloride ions.[5,39] In our study, when Hbs are saturated with IHP, the ΔH values for Hb A are significantly increased to the same range as that of rHb WM and rHb AE, suggesting that the O_2 affinity and the ΔH 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 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.[40,41]

Structural Information from ^{1}H NMR Studies. ^{1}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}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 Nε_2H group of α103His, which is hydrogen-bonded to β131Gln.[16−19] The resonance at 12.9 ppm has been assigned to the side chain Nε_2H group of α122His,[18] which forms a water-mediated H-bond with the side chain of β35Tyr. Both H-bonds are located at the $\alpha_1\beta_1$-subunit interface of Hb A. Our previous study of the mutant rHb (β131Gln→Glu) shows that the peak at 12.2 ppm in the ^{1}H NMR spectrum disappears, suggesting that the H-bond between α103His and β131Gln does not exist due to the replacement at the β131 position.[42] In rHb WM and rHb AE, the amino acid residue of α103His is the same as that in Hb A, but β131Gln existing in Hb A is changed to β/δ131Glu (Table 1). Thus, the

disappearance of the resonance at 12.2 ppm in the ^{1}H NMR spectra of rHb WM and rHb AE is likely to be caused by this mutation, as reported for the mutant rHb (β131Gln→Glu).[42] Our previous studies for rHb (β131Gln→Glu) also show a slight downfield shift of the α122His resonance to 13.1 ppm, indicating that the perturbations caused by the replacement at β131 are not just confined to the mutation site but also affect the environment of α122His.[42] This also could be true for the case of rHb WM and rHb AE. Thus, the changes in ^{1}H NMR spectra on the interface histidyl resonances of α103His and α122His could be attributed to the replacement of β131Gln→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 β37Trp 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_2. From the previous functional studies of the mutant rHb (β131Gln→Glu), it was shown that this mutation has only a small impact on the oxygen affinity of the Hb molecule.[42] Thus, although a significant change is observed in the ^{1}H NMR spectra, the replacement of β131Gln→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 γ_2-CH_3 group of the α62Val and β67Val residues of HbCO A, respectively.[15,20,21] 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_2, 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_2. Comparing the amino acid sequences of these Hbs, several replacements in rHb WM and rHb AE can be found near α62Val and β67Val, including α63Ala→Gly, α64Asp→Glu, β65Lys→Glu, and β69Gly→Thr. Previous studies have indicated that the mutations in the heme pocket of Hb A can change the O_2 affinity by affecting the tertiary structure of the protein.[34,43,44] 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_2-binding affinity of these Hbs.

Structural Information from Hb A_2. Hb A_2 exhibits a remarkable structural similarity to Hb A. The α-chains are the same in these two Hbs, and there are only 10 amino acid substitutions in the δ-chain of Hb A_2 compared to the β-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 δ-chain replacement (Figure 7D). In the ^{1}H NMR spectra of Hb A_2, 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 δ-chain replacement in Hb A_2 conserves the $\alpha_1\delta_2$ interface, but only slightly perturbs the $\alpha_1\delta_1$ interface.[13] The perturbation on the intradimer interface is shown more clearly in the ^{1}H NMR spectra of rHb WM and rHb AE due to the combination effect of the β/δ-chain replacement and the mutation of β/δ131Gln→Glu.

Perutz and Raidt reported that Hb A_2 was more resistant to thermal denaturation than Hb A.[45] They suggested that two amino acid residues in the helices G (δ116Arg) and H (δ126Met) of Hb A_2 might be responsible for its higher thermal stability. It is speculated that δ116Arg could make an extra hydrogen bond at the $\alpha_1\delta_1$ interface and that δ126Met (H4) could make intrasubunit nonpolar contacts with δ11Val of helix A.[45] However, recent X-ray crystal studies of Hb A_2 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_2.[10,46] Notably, rHb WM and rHb AE also possess Arg at position 116 of their β-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_2-binding affinity of Hb A_2 may result from the slighter larger $\alpha_1\delta_2$ interface of Hb A_2 and an additional hydrogen bond at the $\alpha_1\delta_2$ (or $\alpha_2\delta_1$) interface between α94Asp and δ37Trp in Hb A_2.[10] 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_2 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 β/δ 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_2 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.[40,41] 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.

AUTHOR INFORMATION

Corresponding Author

*Phone: 412-268-3395. Fax: 412-268-7083. E-mail: chienho@andrew.cmu.edu.

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■ ABBREVIATIONS

Hb A, human normal adult hemoglobin, the $\alpha_2\beta_2$ tetramer; Hb A_2, 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.

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