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Bohr effect and temperature sensitivity of hemoglobins from highland and lowland deer mice

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Abstract

An important means of physiological adaptation to environmental hypoxia is an increased oxygen (O_2) affinity of the hemoglobin (Hb) that can help secure high O_2 saturation of arterial blood. However, the trade-off associated with a high Hb-O_2 affinity is that it can compromise O_2 unloading in the systemic capillaries. High-altitude deer mice (*Peromyscus maniculatus*) have evolved an increased Hb-O_2 affinity relative to lowland conspecifics, but it is not known whether they have also evolved compensatory mechanisms to facilitate O_2 unloading to respiring tissues. Here we investigate the effects of pH (Bohr effect) and temperature on the O_2-affinity of high- and low-altitude deer mouse Hb variants, as these properties can potentially facilitate O_2 unloading to metabolizing tissues.

Our experiments revealed that Bohr factors for the high- and low-altitude Hb variants are very similar in spite of the differences in O_2-affinity. The Bohr factors of deer mouse Hbs are also comparable to those of other mammalian Hbs. In contrast, the high- and low-altitude variants of deer mouse Hb exhibited similarly low temperature sensitivities that were independent of red blood cell anionic cofactors, suggesting an appreciable endothermic allosteric transition upon oxygenation. In conclusion, high-altitude deer mice have evolved an adaptive increase in Hb-O_2 affinity, but this is not associated with compensatory changes in sensitivity to changes in pH or temperature. Instead, it appears that the elevated Hb-O_2 affinity in high-altitude deer mice is compensated by an associated increase in the tissue diffusion capacity of O_2 (via increased muscle capillarization), which promotes O_2 unloading.

Keywords

high-altitude; hypoxia; globin; adaptation; enthalpy of oxygenation; Bohr effect

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1. Introduction

Adjustment of blood oxygen (O_2)[1] affinity in response to ambient O_2 availability occurs by two means: (1) by genetically-based modifications of the structure of the hemoglobin (Hb, the O_2 carrier of the blood) that affect the intrinsic heme-O_2 affinity or the sensitivity to allosteric cofactors, or (2) by changes in the red blood cell concentration of allosteric cofactors, organic phosphates in particular (Weber and Fago 2004; Weber 2007; Mairbaurl and Weber 2012).

Vertebrates living at high altitudes provide excellent examples of adaptive changes in Hb structure and function (Weber 2007; Storz et al. 2010b). To cope with the reduced ambient PO_2 at high altitudes, the Hb-O_2 affinity of highland natives is often increased (Weber 2007, as this will enhance blood-O_2 loading in pulmonary capillaries, thereby securing high saturation of arterial blood. However, there is a trade-off, as a high affinity Hb may compromise O_2 unloading to the tissues in the systemic capillaries. To facilitate O_2 unloading to metabolizing tissues, mammalian Hbs are typically sensitive to the organic phosphate 2,3-diphosphoglycerate (DPG), chloride, protons and CO_2, which all bind to specific sites and stabilize the low-affinity T (tense) state relative to the high-affinity R (relaxed) state of the Hb (Weber and Fago 2004). The well-known effect of a pH change on Hb-O_2 affinity, the Bohr effect, facilitates unloading of O_2 in systemic capillaries in response to an increase in blood lactic acid and CO_2, the end-products of energy metabolism, to locally match O_2 supply with O_2 consumption. The magnitude of the Bohr effect varies greatly among the Hbs of different species, but it is unclear whether changes in the Bohr effect have evolved as an adaptive response to changes in environmental O_2 availability (Jensen 2004; Giardina et al. 2004; Weber 2007).

Another factor that can effectively decrease the O_2 affinity and promote O_2 delivery is an increase in temperature, due to the exothermic nature of Hb oxygenation. While a substantial temperature effect would be beneficial in favoring O_2 unloading to warm exercising muscles, it would also impair O_2 delivery to cold limbs and extremities in mammals living in cold environments. The possession of Hbs with reduced temperature sensitivity could compensate for this impairment, and may therefore have adaptive significance in regional heterotherms that live in polar or alpine environments (Brix et al. 1990; Weber and Campbell 2011). Consistent with this idea, a number of arctic or sub-arctic mammals have been shown to possess Hbs with a low temperature sensitivity (Weber and Campbell 2011). Whether the same is true in alpine mammals remains to be investigated.

To better understand how O_2 transport is regulated in mammalian species adapted to high altitude hypoxia, we here investigate whether the Hbs of highland and lowland populations of the deer mouse (*Peromyscus maniculatus*) differ in their sensitivity to pH and temperature. The deer mouse is particularly well suited for such study because this species has the broadest altitudinal distribution of any North American mammal. Moreover, high-altitude deer mice from the Rocky Mountains have evolved an increased Hb-O_2 affinity

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[1]Abbreviations used: O_2, oxygen; Hb, hemoglobin; P_{50}, oxygen partial pressure at half-saturation; PO_2, oxygen partial pressure; n_{50}, Hill cooperativity coefficient; DPG, 2,3-diphosphoglycerate; H, enthalpy of oxygenation; IEF, isoelectrofocusing; Φ, Bohr factor.

relative to lowland conspecifics from the Great Plains (Storz et al. 2009; Storz et al. 2010a). The increased Hb-O_2 affinity in high-altitude mice is attributable to the additive and nonadditive effects of numerous amino acid replacements in duplicated genes that encode the α- and β-type subunits of the Hb tetramer (Storz et al. 2009; Storz et al. 2010a; Natarajan et al. 2013; Natarajan et al. 2015a). The increased Hb-O_2 affinity in high-altitude mice is expected to enhance pulmonary O_2 loading under hypoxia. Here we investigate whether this modification of Hb function is associated with compensatory mechanisms to promote O_2 unloading in the systemic circulation.

2. Materials and methods

2.1 Sample collection and analysis

We analyzed a set of Hb variants that were representative of high- and low-altitude deer mouse populations (Storz et al. 2009; Storz et al. 2010a; Natarajan et al. 2013; Natarajan et al. 2015a). As described previously (Storz et al. 2010a), deer mice were live-trapped at high-altitude (4347 m, Mount Evans, Clear Creek County, Colorado, 39°15'24"N, 106°10'54"W) and low-altitude locations (1158 m, prairie grassland habitat, Bonny Reservoir, Yuma County, Colorado, 39°37'30"N, 102°10'27"W). After killing each animal by cervical dislocation, blood samples were drawn by cardiac puncture and were snap frozen in liquid nitrogen. Animal handling was in accordance with the guidelines approved by the University of Nebraska Institutional Animal Care and Use Committee (IACUC no. 07-07-030D) and the National Institutes of Health (NIH publication no. 78-23). Purified hemolysates were prepared and examined for Hb multiplicity by isoelectric focusing (IEF) on polyacrylamide gels in the pH range 3–9 (PhastSystem, GE Healthcare) as described previously (Storz et al. 2010a). Individual samples from each elevation that showed identical patterns of Hb multiplicity were pooled. In the present study we examined purified hemolysates representing the largest class of Hb variants from high-altitude and the largest class from the low-altitude population. , corresponding to the "sample B" and "sample I" described in a previous study (Storz et al. 2010a), respectively. Each of these two samples consisted of pooled hemolysates from eight (N=8) individual deer mouse specimen showing identical IEF patterns.

2.2. O_2 equilibria and calculations

Pooled hemolysates from high- and low-altitude deer mice were stripped of organic phosphates by using a mixed-bed resin MB-1 AG501-X8 (BioRad), concentrated by ultrafiltration (to [heme]>3 mM), dialyzed in CO-equilibrated 10 mM Hepes buffer, pH 7.6, and stored in aliquots at −80 °C. Hb concentration (heme basis) was measured using previously reported extinction coefficients (Antonini and Brunori 1971). O_2 equilibrium curves of 4 μl samples (0.3 mM heme) were measured using a modified diffusion chamber method previously described (Weber 1981; Weber 1992). In this method, samples are stepwise equilibrated to gas mixtures of atmospheric air or O_2 and pure (>99.998 %) N_2 made by two serially coupled gas mixing pumps (Wösthoff, Bochum, Germany), while absorbance at 436 nm (where oxy and deoxy Hb differ in absorbance) is continuously measured in order to obtain the fractional saturation at each equilibration step (4–6 steps for each curve). Experiments were made in 0.1 M Hepes buffers in the absence (stripped) and

presence of anionic cofactors KCl (0.09 ± 0.01 M) and/or DPG (2-fold molar excess over tetrameric Hb) added at physiological levels and approximating conditions existing within red blood cells (Imai 1982; Mairbaurl and Weber 2012). Curves were measured at different pH values to estimate the Bohr effect and at 25° and 37 °C to estimate the enthalpy of oxygenation. Prior to the measurements of O_2-equilibrium curves, CO was removed from each sample by repeated equilibrations with pure O_2 and nitrogen. In the hemolysate solutions used in O_2 equilibrium experiments, Cl^- concentration was measured using a model 926S Mark II chloride analyzer (Sherwood Scientific Ldt, Cambridge, UK) and pH was measured using a InLab micro pH-electrode equipped with a SevenCompact pH/Ion Meter S220 (Mettler Toledo, Greifensee, Switzerland) after bringing samples at the same temperature of the experiments using a HLC BioTech block thermostat Model TK23 (Bovenden, Germany).

For each O_2 equilibrium experiment, the parameters P_{50} (the O_2 partial pressure at half-saturation) and n_{50} (Hill cooperativity coefficient) were calculated by fitting the Hill's sigmoidal equation $[SO_2 = PO_2^{n50} / (P_{50}^{n50} + PO_2^{n50})]$ to the saturation data, where SO_2 is the fractional saturation. The nonlinear regression fitting was based on 4–6 saturation steps and yielded means $\pm$ sem values for P_{50} and n_{50} parameters under each condition used. The Bohr factor (Φ, expressing the number of protons bound per O_2) was quantified by the slope of linear plots of $\log P_{50}$ as a function of pH ($\Phi = \Delta \log P_{50}/\Delta$ pH). The overall enthalpy of oxygenation (ΔH, J mol O_2^{-1}, expressing the heat liberated upon oxygenation) at pH 7.2 under varying anionic conditions was calculated by using the van't Hoff equation $\Delta H = 2.303R (\Delta \log P_{50}/\Delta 1/T)$, where R is the gas constant (8.314 J·K^{-1}·mol O_2^{-1}) and T the absolute temperature in Kelvin. Values of $\log P_{50}$ at pH 7.20 were interpolated from the linear $\log P_{50}$ vs pH plots.

3. Results

The common patterns of Hb multiplicity in deer mouse populations living at high and low altitudes are shown in the IEF gel images (Fig. 1). The high-altitude deer mice that we examined primarily expressed one major and one minor Hb component, whereas low altitude mice expressed one major and two minor Hb components (Fig. 1). This difference in Hb multiplicity in the hemolysates reflects altitudinal variation in the frequencies of alternative α- and β-chain amino acid variants (Storz and Kelly 2008; Storz et al. 2009; Storz et al. 2010a; Storz et al. 2012; Natarajan et al. 2015a). In particular, the set of Hb variants comprising high- and low-altitude samples exhibit fixed differences at four sites in the β-chain subunit, reflecting the fact that the four-site β-globin haplotype '62Gly/72Gly/ 128Ala/135Ala' is nearly fixed in the high-altitude, Rocky Mountain population, and the alternative four-site haplotype '62Ala/72Ser/128Ser/135Ser' is nearly fixed in the low-altitude, Great Plains population (Storz et al. 2009; Storz et al. 2010a; Storz et al. 2012).

O_2 equilibrium curves of the purified hemolysates from highland and lowland deer mice were measured at different pH values and two different temperatures to estimate possible altitude-related differences in the Bohr effect and in the enthalpy of oxygenation. These experiments were performed in the absence and presence of anionic cofactors to determine

whether their allosteric effects were influenced by changes in pH or temperature. An overview of all O_2 equilibrium data is presented in Table 1.

An increase in P_{50} with decreasing pH indicated the presence of Bohr effects of roughly similar magnitudes in high- and low-altitude samples, as shown by similar slopes of the $\log P_{50}$ vs pH linear plots (Fig. 2). However, the measured P_{50} values in the presence of anionic cofactors were consistently lower in high-altitude samples, indicating an increased O_2 affinity under physiological conditions (Table 1, Fig. 2C,D). Overall, we observed similar shifts in $\log P_{50}$ with pH under the buffer and temperature conditions here investigated in Hbs from highland and lowland deer mice, indicating that the allosteric binding of anionic cofactors (DPG or Cl^-) was largely independent of both temperature and pH (Fig. 2C,D). Consistently, the Bohr factors (Φ, indicating the magnitude of the Bohr effect and the slope of the plots in Fig. 2) were roughly similar in Hbs from highland and lowland deer mice (Table 2). Slight differences in Bohr factors were observed in the presence of Cl^- possibly related to small variations in Cl^- concentration in the samples.

An increase in temperature from 25° to 37°C produced a small right-shift of the O_2 binding curve (Fig. 3, Table 1), with an average $\log P_{50}$ shift of 0.02 per °C. The correlated changes in P_{50} and H were similar for high- and low-altitude samples under all anionic conditions investigated (Table 1), although addition of DPG alone resulted in a less exothermic oxygenation (i.e., a less negative overall H), particularly in high-altitude samples (Table 1). Interestingly, addition of Cl^- had no noticeable effect on H, even though it exerted a substantial effect on P_{50} (Table 1).

4. Discussion

A major finding of this study is that deer mice living at high- and low-altitudes have Hbs with similar Bohr effects. In this study we examined purified hemolysates with IEF profiles that were representative of the high- and low-altitude deer mouse populations (Storz et al. 2010a). The fact that Hbs of high-altitude deer mice have an unaltered Bohr effect implies that the increase in O_2 delivery in response to an increase in energy metabolism in tissues does not involve particular molecular adaptations of the Hb molecule. Instead, other physiological adjustments of the cardio-circulatory system may facilitate O_2 unloading in the systemic circulation. For example, high-altitude deer mice have evolved significantly higher muscle capillary densities relative to lowland mice (Lui et al. 2015; Scott et al. 2015), a change which should increase the O_2 diffusion capacity, thereby enhancing aerobic capacity under hypoxia (Wagner 1996; Scott and Milsom 2006). Moreover, deer mice exhibit plasticity in a number of hematological traits such as hematocrit, Hb concentration, and the intraerythrocytic concentration of DPG that can potentially enhance blood-O_2 carrying capacity in response to hypoxia (Tufts et al. 2013; Lui et al. 2015). Thus, it appears that genetic adaptation to high-altitude in deer mice involves an evolved increase in Hb-O_2 affinity, which maximizes pulmonary O_2 loading, in conjunction with an evolved increase in tissue diffusion capacity of O_2 (via increased muscle capillarization) that should promote O_2 unloading. The results of this study are in overall good agreement with those of a previous investigation on the same samples (Storz et al. 2010a) but performed under slightly different experimental conditions (e.g. in the presence of an enzymatic met-reducing system and at a

lower heme concentration) and confirm the consistently higher O_2-affinity of the high-altitude deer mouse Hbs (Table 1).

Overall, the magnitudes of the Bohr effect found in this study (Table 2) are close to those typical of mammalian Hbs measured under in vitro standard conditions (e.g. ~−0.5 in human HbA) (Shih et al. 1993; Giardina et al. 2004; Revsbech et al. 2013), although other studies have found that the Bohr effect scales with body size and is larger in Hbs from small animals with higher mass-specific metabolic rates (Riggs 1960). The Hbs from high- and low-altitude deer mice have similar Bohr effects at each of the two temperatures and in the presence and absence of anionic allosteric cofactors (Table 2). This suggests that there is no difference in the number of Bohr groups between the Hb samples, corresponding to the O_2-linked dissociation of ~1.5–2.5 protons per tetrameric Hb. That DPG does not increase appreciably the magnitude of the Bohr effect implies that negatively-charged DPG does not alter the affinity for protons of positively-charged groups in the DPG binding central cavity of the T-state Hb molecule. Similarly to the deer mouse, Hbs from closely related pairs of high- and low-altitude waterfowl from the Andes exhibited differences in O_2-affinity (higher affinities in the high-altitude taxa) but no associated differences in the Bohr factor (Natarajan et al. 2015b).

The temperature effect of deer mouse Hbs, quantified as the enthalpy of oxygenation H (on average −10.5 kJ mol O_2^{-1}, Table 1), was significantly lower than that of human HbA (−50.7 kJ mol O_2^{-1}) (Weber et al. 2014) and similar to that of bovine Hb, (Coletta et al. 1992; Weber et al. 2014), a known example of a temperature-insensitive Hb. H values of deer mouse Hbs were also similar to those of ground squirrels (Revsbech et al., 2013) and moles (Signore et al. 2012). Moreover, the enthalpy of oxygenation was similar in high-altitude and low-altitude deer mouse Hb and was largely unaffected by the presence of anionic cofactors. This observation suggests that – as in bovine Hb (Weber et al. 2014) – the T-R shift of deer mouse Hb upon oxygenation is considerably endothermic in nature, whereas the endothermic dissociation of ionic cofactors upon oxygenation plays a minor role. This endothermic transition in quaternary structure correlates with a tighter $\alpha 1\beta 2$ sliding subunit interface identified in crystallographic studies of deer mouse recombinant Hb (Inoguchi et al. 2013), with additional interactions between αArg92 and βGln39 and between αArg92 and βAsp43, which are both lacking in human HbA. However, the molecular mechanisms underlying why DPG markedly affects O_2 affinity but not the enthalpy of oxygenation remains to be clarified.

In conclusion, adaptive modifications of the oxygenation properties of deer mouse Hbs do not affect the magnitude of the Bohr effect, indicating that O_2 unloading at high-altitude is instead facilitated by an increased muscle capillary density, and possibly also by plastic changes in hematocrit, Hb concentration, and intraerythrocytic DPG concentration that increase blood-O_2 carrying capacity (Tufts et al. 2013; Lui et al. 2015). Furthermore, the low temperature effect on Hb-O_2 affinity, which is comparable to that of temperature-insensitive ruminant Hbs, suggests that O_2 delivery to extremities that are below the body core temperature may be facilitated by a lower heat of oxygenation of the Hb, as reported in polar mammals (Weber and Campbell 2011).

Acknowledgments

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Figure 1. Expression of different Hb isoforms in high- and low-altitude deer mice
Isoelectrofocusing (pH 3–9) of the pooled hemolysates from high-altitude (left) and low-altitude deer mice (right). Samples in lanes 2 and 4 are dilutions of samples in lanes 1 and 3, respectively. Cathode is at the bottom.
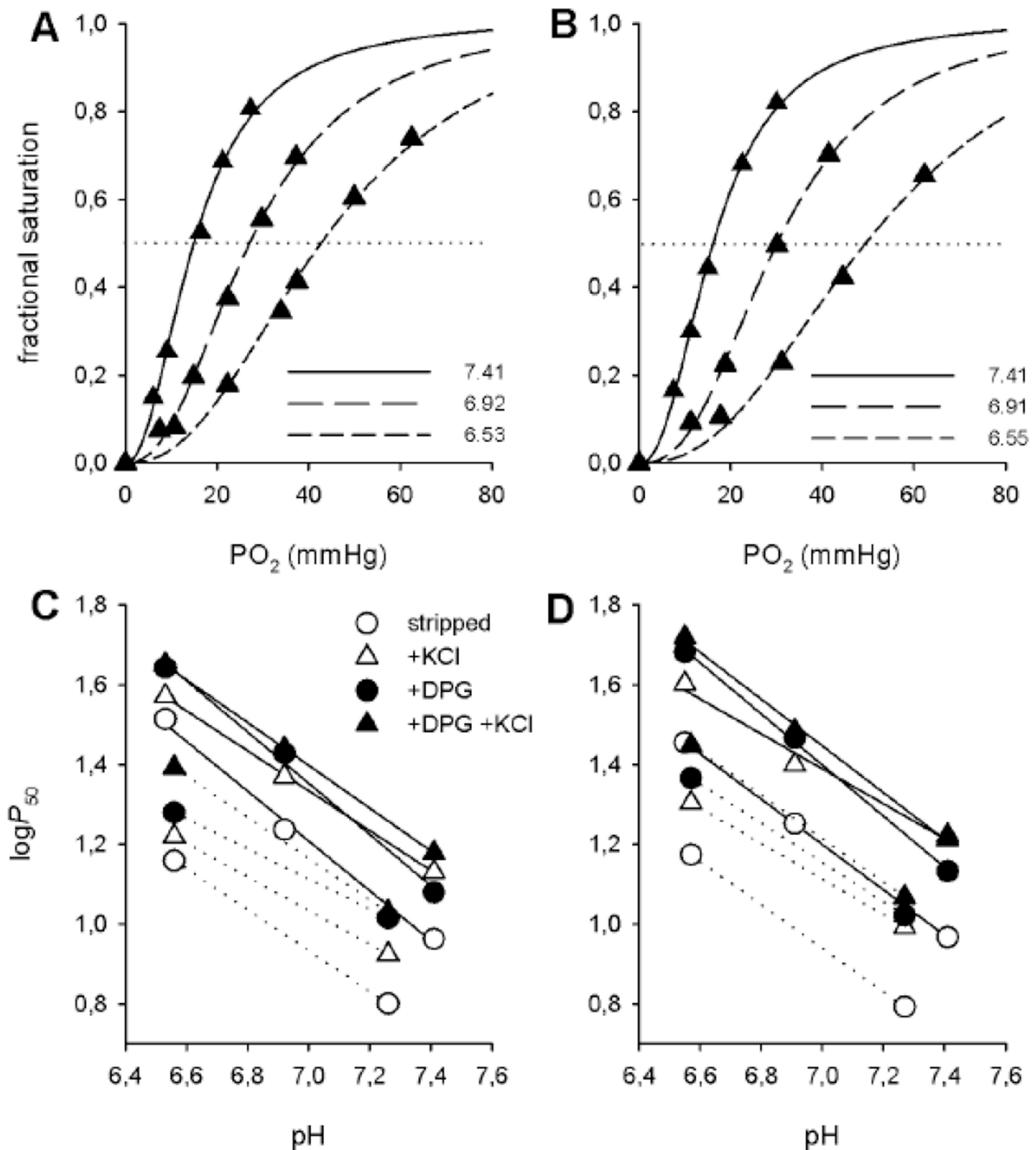
Figure 2. Similar Bohr effects in Hbs of high- and low-altitude deer mice

Representative O_2 equilibrium curves of high- (A) and low-altitude deer mice Hb (B) measured in 0.1 M Hepes buffer at different pH values at 37°C in the presence of KCl (0.09 ± 0.01 M) and DPG (2-fold molar excess over tetrameric Hb), as indicated. Fitting of the data according to the Hill's sigmoidal equation is shown. Bohr plots (logP_{50} vs pH) of high- (C) and low-altitude deer mouse Hb (D) in the absence (stripped) and presence of KCl and DPG, as indicated, at 37°C (continuous lines) and 25°C (dotted lines). The slope of each of

these linear plots corresponds to the magnitude of the Bohr effect (Bohr factor, Φ) and is reported in Table 2.
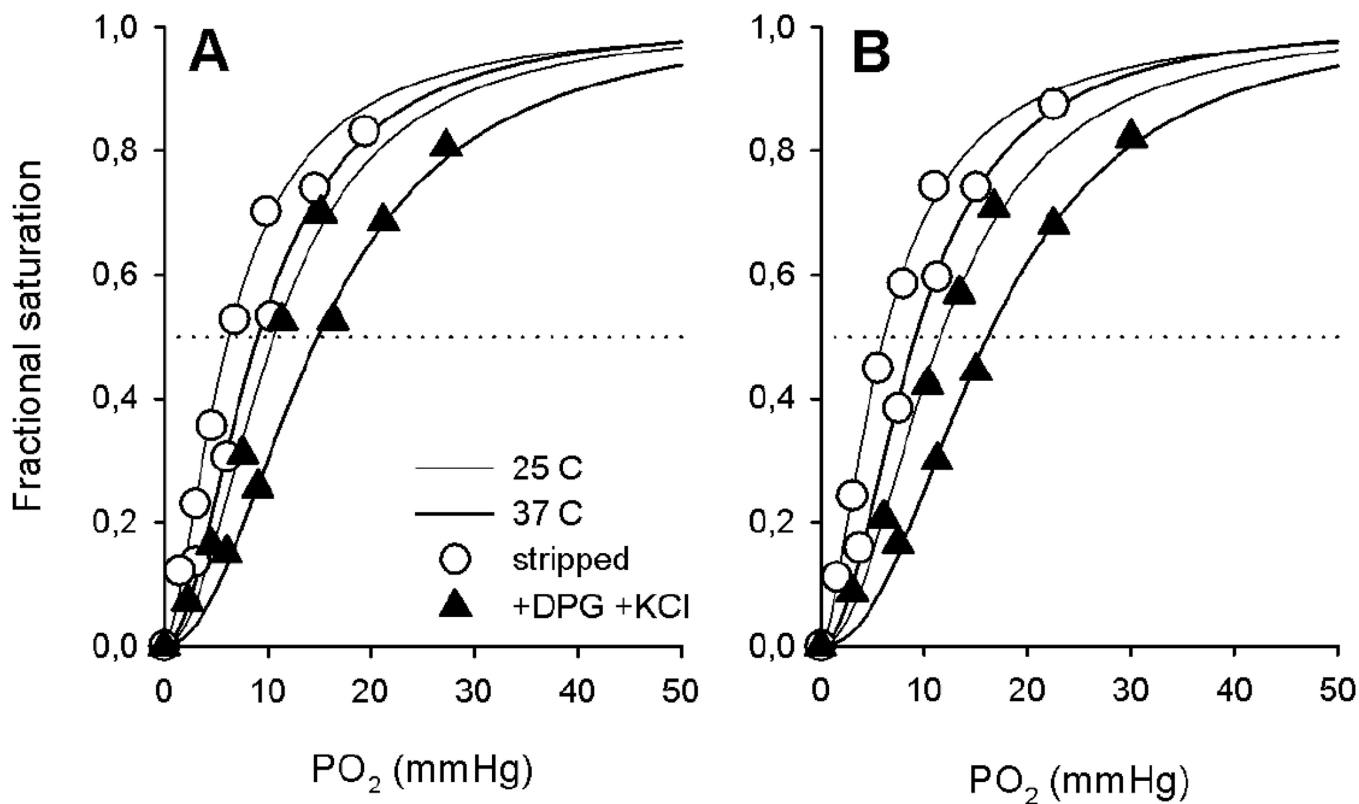
Figure 3. Similar temperature-dependent shifts of the O_2 equilibrium curves of high- and low-altitude deer mouse Hb

Representative O_2 equilibrium curves of high- (A) and low-altitude deer mice Hb (B) measured in 0.1 M Hepes buffer at 37°C (pH 7.41; thick lines) and 25°C (pH 7.26 in A and pH 7.27 in B; thin lines) in the absence (stripped) and presence of KCl (0.09 ± 0.01 M) and DPG (2-fold molar excess over tetrameric Hb) as indicated. Fitting of the data according to the Hill's sigmoidal equation is shown.

Table 1

O$_2$ affinities (P$_{50}$, mmHg) and Hill's cooperativity coefficients (n$_{50}$) for high- and low-altitude deer mouse Hbs measured at 37° and 25°C in 0.1 M Hepes buffer at varying pH values (heme 0.3 mM) and in the absence (stripped) and presence of KCl and DPG, as indicated. P$_{50}$ and n$_{50}$ values are derived from nonlinear regression of the sigmoidal Hill equation to 4–6 saturation points measured for each condition and are expressed as means±SEM. The corresponding overall enthalpy of oxygenation (kJ mol O$_2^{-1}$) calculated at pH 7.20 is indicated.

	High						Low					
temperature	**37 °C**			**25 °C**		**H**	**37 °C**			**25 °C**		**H**
pH	7.41	6.92	6.53	7.26	6.56	7.20	7.41	6.91	6.55	7.27	6.57	7.20
P$_{50}$ (mmHg)												
stripped	9.17±0.41	17.3±0.5	32.6±2.2	6.32±0.27	14.4±0.7	−11.0	9.2±0.22	17.8±0.5	28.5±1.1	6.20±0.20	14.9±0.4	−11.4
+KCl	13.5±0.5	23.5±0.9	37.3±3.2	8.40±0.33	16.6±1.0	−12.3	16.7±0.5	25.1±0.6	40.1±0.9	9.84±0.32	20.1±0.6	−12.3
+DPG	12.0±0.4	26.8±0.6	43.8±2.6	10.4±0.6	19.0±0.8	−7.9	13.6±0.4	29.3±1.3	48.0±1.5	10.5±0.3	23.2±0.6	−9.6
+KCl+DPG	15.1±0.5	27.5±0.7	44.7±1.8	10.7±0.4	24.7±1.3	−10.1	16.3±0.3	30.5±0.5	52.3±1.8	11.7±0.4	28.1±1.0	−9.6
Hill coeff. (n$_{50}$)												
stripped	1.94±0.14	1.75±0.07	1.79±0.18	1.63±0.11	1.46±0.10		2.03±0.09	1.86±0.09	1.76±0.09	1.61±0.08	1.02±0.01	
+KCl	2.02±0.13	2.04±0.13	1.92±0.26	1.87±0.13	1.96±0.21		2.03±0.11	2.04±0.09	2.43±0.12	2.06±0.12	1.87±0.10	
+DPG	2.00±0.13	2.48±0.12	2.11±0.22	1.81±0.20	1.95±0.15		2.20±0.14	2.30±0.22	2.51±0.17	2.11±0.15	2.26±0.13	
+KCl+DPG	2.11±0.11	2.32±0.13	2.33±0.19	2.05±0.15	1.72±0.13		2.27±0.08	2.53±0.10	2.40±1.76	2.08±0.14	2.00±0.13	

Table 2

Bohr factors for high- and low-altitude deer mouse Hbs measured at 37° and 25°C in 0.1 M Hepes buffer (heme 0.3 mM) and in the absence (stripped) and presence of KCl and DPG, as indicated. For each condition, values correspond to the linear regression of $\log P_{50}$ vs pH data shown in Fig. 2C,D.

	High		Low	
Bohr factor (Φ)	**37 °C**	**25 °C**	**37 °C**	**25 °C**
stripped	−0.62±0.04	−0.51	−0.57±0.00	−0.55
+KCl	−0.50±0.01	−0.42	−0.44±0.06	−0.44
+DPG	−0.64±0.05	−0.38	−0.64±0.02	−0.49
+KCl+DPG	−0.54±0.00	−0.52	−0.59±0.03	−0.54