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ORIGINAL PAPER

Molecular and physicochemical characterization of hemoglobin from the high-altitude Taiwanese brown-toothed shrew (*Episoriculus fumidus*)

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Abstract Red-toothed shrews (subfamily Soricinae) exhibit the highest mass-specific rates of O_2 consumption recorded among eutherian mammals, though surprisingly no data appears to be available on the functional characteristics of their hemoglobin (Hb). As a first step in addressing this shortcoming, we investigated the O_2 binding characteristics of Taiwanese brown-toothed shrew (*Episoriculus fumidus*) Hb and its temperature and pH dependence in the absence and presence of anionic red blood cell effectors. Although comparative data regarding the intrinsic O_2 affinity of other shrew species are currently unavailable, our data suggest that the sensitivity of this high-elevation endemic species' Hb to allosteric effector molecules is similar to that of the two lowland species of white-toothed (crocidurine) shrews examined to date. The efficient exploitation of blood O_2 reserves by *E. fumidus* appears to be achieved via synergistic modulation of O_2 affinity by Cl^- and organic phosphates that moreover dramatically lowers the overall enthalpy of oxygenation of their Hb. Oxygen unloading is presumably further enhanced by a relatively high Bohr effect (ΔLog P_{50}/ΔpH $= -0.69$) and marked reduction in the titratable histidine content (predicted low proton buffering value) of the component globin chains relative to human HbA. Notably, however, the limited data available suggest these latter attributes may be widespread among shrews and hence likely are not adaptations to chronic altitudinal hypoxia per se.

Keywords Shrew · Hemoglobin · High-altitude · Oxygen affinity · Enthalpy of oxygenation

Abbreviations

DPG	2,3-Diphosphoglycerate
Hb	Hemoglobin
IEF	Isoelectric focusing
P_{50}	O_2 tension at 50 % Hb–O_2 saturation
pK_a	Logarithmic acid dissociation constant
n_{50}	Hill's cooperativity coefficient at half saturation
T_b	Body temperature
pI	Isoelectric point
PO_2	Partial pressure of oxygen
ΔH	Enthalpy of oxygenation

Introduction

Soricid shrews are among the largest and most successful of the mammalian families, comprising more than 300 described species distributed on all continents save Australia and Antarctica (Hutterer 2005; Nowak 1999). The family Soricidae is commonly subdivided into two living subfamilies (but see Hutterer 2005) with distinct physiological traits and evolutionary histories: the Crocidurinae

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('white-toothed shrews') and Soricinae ('red-toothed shrews'). White-toothed shrews are believed to have diversified in paleotropical climates and are characterized by labile body temperatures (T_b), the ability to enter daily torpor, and basal rates of metabolism only slightly higher than predicted from allometry (McNab 1991). Conversely, red-toothed shrews are not known to enter torpor and are believed to be Holarctic in origin. Members of this clade further possess relatively high, stable core body temperatures, and exhibit the highest mass-specific rates of basal O_2 consumption recorded among eutherian mammals (Gusztak et al. 2005; McNab 1991). In this light, it is surprising that—apart from a single O_2 affinity measurement (oxygen half-saturation pressure or $P_{50} = 32.8$ mmHg at 37 °C, pH 6.8) of a dilute hemolysate solution from a short-tailed shrew, *Blarina brevicauda* (Foreman 1954)—no information appears to be available on the functional properties of the hemoglobin (Hb) of any red-toothed shrew. In fact, oxygen binding data are only currently available for hemolysates of two lowland species of crocidurine shrews (Etruscian shrew, *Suncus etruscus* and greater white-toothed shrew, *Crocidura russula*) at pH 7.2 (Bartels et al. 1979; Jürgens et al. 1981). These studies suggest that a high Bohr factor ($\Delta\text{Log } P_{50}/\Delta\text{pH} = -0.61$ to -0.66) coupled with a relatively low blood O_2 affinity ($P_{50} = \sim$34–35 mmHg) integrally complements high ventilatory diffusion and circulatory transport capabilities to fuel the high aerobic metabolic rates of shrews. However, an elevated P_{50} may drastically impede Hb saturation at low ambient PO_2's, thus potentially placing a strain on the hypoxia tolerance of shrews. This may be especially true during bouts of activity as the low myoglobin concentration and low anaerobic capacity of shrew muscles (Emmett and Hochachka 1981; Peters et al. 1999; Stewart et al. 2005) necessitate an uninterrupted delivery of O_2 to the tissue mitochondria via the bloodstream. Despite these constraints, numerous species of red-toothed shrew readily exploit elevations ranging from 3,000 to at least 4,500 m (Hutterer 2005), where oxygen tensions are ~60–70 % of that at sea level. As most physiological traits (i.e. cardiac output, respiration rate, blood oxygen carrying capacity) and morphological traits (i.e. mitochondrial capacity, diffusion distance, surface area for gas exchange) are presumably already operating at or near their upper limits (Bartels et al. 1979; Gehr et al. 1980; Morrison et al. 1959), how (if at all) do the functional properties of the blood contribute to the adaptation of these species to high elevation?

As a first step in addressing this question, we investigated the intrinsic O_2 affinity—that is of pervasive importance for mammalian existence at high altitude (Storz 2007; Weber 2007; Storz et al. 2010)—of the major Hb component of the Taiwanese brown-toothed shrew (*Episoriculus fumidus* Thomas 1913). This 6 to 8 g shrew is a member of the 'red-toothed' (soricine) subfamily, and is endemic to subtropical temperate forests, grasslands and dwarf bamboo stands in the central highlands of Taiwan where it is found at elevations ranging from 2,000 to 3,700 m (Jameson and Jones 1977; Yu 1993). Mean monthly temperatures within this zone range from −1.7 to 14.2 °C (Yu 1993), suggesting that this species is routinely exposed to thermally challenging conditions. Thus, in addition to assessing the physiological properties of this gas-binding protein to the conflicting demands of fueling a high mass-specific metabolic rate in face of limited O_2 availability, we also investigated the temperature and pH dependence of O_2 binding to *E. fumidus* Hb in the absence and presence of the intraerythrocytic cofactors Cl^- and 2,3-diphosphoglycerate (DPG). Finally, as globin chain sequence data for shrews are sparse, we sequenced the α- and β-like (δ) globin genes encoding their Hb subunits to gain insight into possible structural mechanisms underlying the observed functional characteristics of the protein.

Materials and methods

Hemoglobin collection and preparation

Blood samples were collected from two *E. fumidus* specimens (1 ♀, 1 ♂) collected at an elevation of 2,200 m in Tengchih, Kaohsiung county, Taiwan (23°20′N, 120°50′E), and immediately stored at −80 °C. Blood samples were thawed, diluted with 1 volume distilled water and 0.1 volume 1 M HEPES buffer (pH ~7.5), and centrifuged for 15 min at 14,000 rpm (19,000 g); this and all additional Hb purification steps were carried out at 5 °C. The supernatant (1–2 ml) was stripped from organic phosphates by isoelectric focusing (IEF) in a 110-ml sucrose gradient column (LKB model 8100) containing a 1 % solution of CO-saturated Pharmacia ampholines (0.2 % pH 7–9, 0.8 % pH 6.7–7.7). Eluted fractions were collected in 1 ml samples for absorption (540 nm) and pH measurements. Fractions containing the major component were pooled (Fig. 1), dialyzed for 24–36 h against 3 changes of CO-equilibrated 10 mM l^{-1} HEPES buffer, pH ~7.5, and concentrated by ultrafiltration. To verify the surprisingly high isoelectric points obtained from the direct pH measurements, the constituent Hb components were also assessed using a thin Phast System IEF (20-min runs at 15 °C in pH ranges 3–9 and 5–8) (Amersham BioSciences, NJ, USA) and pH calibration standards followed by Coomassie blue staining of 1 mm-thick acrylamide gels.

Final tetrameric (Hb_4) concentrations were measured spectrophotometrically using standard extinction coefficients for human HbA, and the samples frozen at −80 °C in small (e.g. 200 µl) volumes. Spectrophotometric evidence

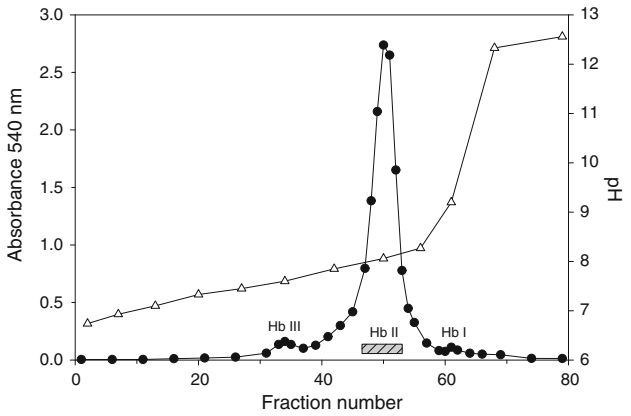

Fig. 1 Preparative isoelectric focusing of Taiwanese brown-toothed shrew Hb. *Circles*, absorbance at 540 nm; *triangles*, pH values of eluted fractions at 5 °C. *Horizontal bar* denotes fractions pooled and dialyzed for O_2 equilibrium measurements (see Fig. 2)

suggested minimal oxidation (<5 %) was evident for the purified male Hb sample equilibrated to air, while the female Hb sample was moderately (~18 %) oxidized. As the oxygen affinity values of this latter sample were consistently higher than those obtained for the non-oxidized sample, while the Hill's cooperativity coefficient values at 50 % saturation (n_{50}) were slightly lower, the functional data from this sample were omitted from further analyses.

Oxygen binding studies

Appropriate volumes of water, 1 M HEPES buffer, and when applicable, standard 1 M KCl and/or 0.1 M 2,3-DPG solutions were added to 104 µl aliquots of Hb solution (final Hb_4 and buffer concentrations were 0.05 mM and 0.1 M, respectively) immediately before O_2 equilibrium determinations. Oxygen-binding data for each treatment were continuously measured (occasionally in duplicate to access measurement variability; see below) at 25 and 37 °C via absorbance changes at 436 nm using a modified diffusion chamber technique (Weber 1992). Ultrathin layers of Hb solutions (3 µl) were first equilibrated with pure (>99.998 %) N_2 and O_2 to establish deoxygenated and oxygenated baselines, respectively, then subjected to stepwise mixes of these and air prepared with precision (Wösthoff, Bochum, Germany) gas-mixing pumps (Weber 1992). This method exhibits high reproducibility among replicate measurements on a single Hb sample (mean $P_{50} = 4.73$ mmHg, SE = 0.04, $n = 6$) at 25 °C and pH 7.4 (Weber 1992). Similarly, we found small variability between duplicate measurements of five different treatments conducted at 25 °C (mean differences in calculated P_{50} values were 0.041 mmHg ± 0.014 SE; data not shown). Following binding measurements, Cl^- levels for each sample were verified using a CMT19 chloride titrator (Radiometer, Copenhagen, Denmark), and pH assessed at both 25 and 37 °C using a Radiometer BMS2 Mark 2 Blood Micro system and PHM 64 Research pH meter. Stock solutions of DPG added to Hb samples were assayed using Sigma enzymatic test chemicals. In experiments employing this organophosphate, the final DPG concentration was 0.75 mM (i.e. ~15:1 DPG/Hb_4 ratio).

For each trial, values of P_{50} and n_{50} were interpolated from linear Hill plots (Log ([OxyHb]/[Hb]) vs. Log PO_2) based on at least 4 equilibration steps between 30 and 70 % saturation. As expected, Hill plots showed high correlation coefficients within this range (0.998 ± 0.003 SD, $n = 32$). In cases where duplicate measurements were obtained, mean P_{50} and n_{50} values are presented. For each treatment, Bohr coefficients were calculated using P_{50} measurements obtained within the pH range 6.8–7.3. The overall enthalpy of oxygenation (ΔH, kJ mol^{-1} O_2), corrected for the solubilization heat of O_2 (−12.55 kJ mol^{-1}), was calculated following Signore et al. (2012).

Gene sequencing

Genomic DNA was prepared from 10 to 25 mg of ethanol-preserved liver tissue from two shrew specimens (EFT6955 and EFT7324) using a DNeasy® Blood and Tissue Kit (Qiagen). PCR Primers (supplementary Table S1) were designed using areas of high-sequence identity in the coding, and 5′ and 3′ flanking regions of shrew (*Sorex araneus*; see below) and mole *HBA* (α) and *HBD* (δ) globin nucleotide sequences (unique among mammals, shrews, moles and hedgehogs all appear to lack an ortholog of the *HBB* (β) globin gene; Campbell et al. 2010a; Opazo et al. 2008). *HBD* genes were amplified in 20 µl PCRs comprising 50 ng of template DNA, 1.5 mM $MgCl_2$, 1× PCR buffer (Invitrogen), 200 µM of each dNTP, 0.5 µM of each primer and 2 U of *Taq* DNA polymerase (Invitrogen). Due to their very high GC content (>70 %), *HBA* genes were amplified in 15 µl PCRs comprising 50 ng of template DNA, 1× KAPA2G Robust HotStart ReadyMix (Kapa Biosystems), 0.5 µM of each primer and 5 % DMSO. Following a 150 s denaturation period at 95 °C, a standard three-step PCR protocol was used (95 °C for 30 s; 50–65 °C for 15 s; 72 °C for 60 s; 30–35 cycles) followed by a final extension at 72 °C for 5 min. Gel electrophoresis of small aliquots (2 µl) of PCR product was conducted on 1 % agarose gels and visualized. PCRs containing bands of the expected size range were purified with a Montage PCR Filter Unit (Millipore) and used as template in a subsequent PCR with nested primers, under the conditions listed above. Nested PCR products of the desired size range were excised from 1 % agarose gels and purified using a MinElute Gel Extraction Kit (Qiagen). These products were either sequenced directly or cloned into Qiagen

pDrive cloning vectors. The plasmids from positive clones were purified with the QIAprep Spin Miniprep Kit (Qiagen). Sequencing was conducted in both directions using an ABI 3130 Genetic Analyzer with BigDye 3.1 sequencing chemistry (Applied Biosystems, Foster City, USA). Gene sequences were deposited into GenBank under accession #'s JQ582441–JQ582446.

Results

Thin layer IEF revealed the presence of one major (Hb II) component with a CO-derivative isoelectric point (p*I*) near 8.0 at 15 °C, and two minor (Hb I and Hb III) components with p*I*'s ranging from ~7.6 to 8.2 (data not shown). The notably high p*I* estimate of the major component, which comprised ~95 % of the hemolysate in both animals (Fig. 1), corresponded well with the value obtained via preparative IEF (8.06 at 5 °C).

Hill's cooperativity coefficients at 50 % O_2 saturation ranged from 2.0 to 2.7 and tended to increase with pH (Fig. 2). The P_{50} (7.7 mmHg, pH 7.2, 37 °C) and Bohr coefficient (–0.30) of co-factor free *Episoriculus* Hb (Fig. 2; Table 1) increased both in the presence of 100 mM Cl^- (14.8 mmHg and −0.41, respectively) and saturating DPG (21.6 mmHg and −0.69, respectively). Notably, the presence of both these cofactors further increased P_{50}

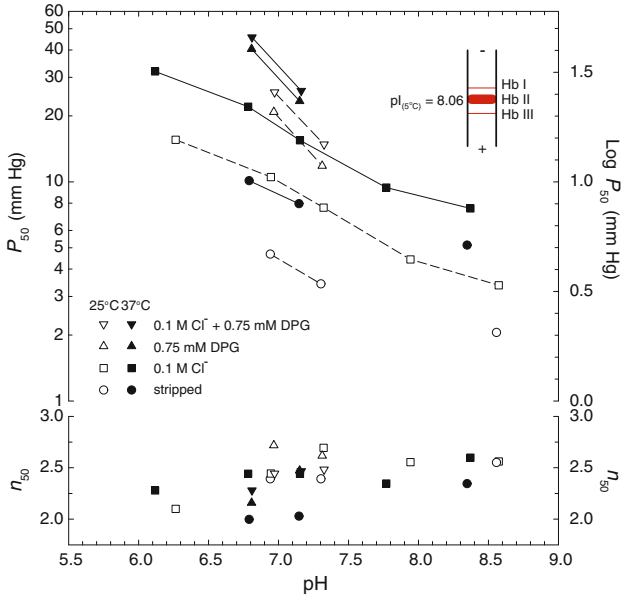

Fig. 2 Values of PO_2 and Hill's cooperativity coefficient at half oxygen saturation (P_{50} and n_{50}) for the major hemoglobin component ([Hb_4] = 0.05 mM) of the Taiwanese brown-toothed shrew at 25 and 37 °C, and its pH dependence in the absence and presence of 0.1 M Cl^- and/or 0.75 mM 2,3-DPG. *Inset*: diagram of isoelectric focusing column at the end of focusing illustrating the relative abundance and p*I* of the major (Hb II) component

values (24.5 mmHg) but not the Bohr factor (−0.69). Similar to the trend found for oxygen affinity, the presence of DPG leads to a greater reduction in ΔH than Cl^- (Table 1). Thus at pH 7.2, the ΔH of stripped Hb (−33.4 kJ mol^{-1} O_2) numerically decreased to −23.3 and −14.3 kJ mol^{-1} O_2 in the presence of Cl^- and DPG, respectively, and by a further 50–70 % (to −7.2 kJ mol^{-1} O_2) when both these anions were present.

Two α-like and two *β*-like globin paralogs were amplified and sequenced. Conceptual translations of these nucleotide sequences indicated that one of the α-like and both *β*-like genes encoded a protein of the expected length; the second partial α-like sequence possessed two premature stop codons and was hence assumed to be a pseudogene. Phylogenetic reconstructions of intron 2 sequences following previously established procedures (Campbell et al. 2010a; Opazo et al. 2008) confirmed that both *β*-like genes were orthologous to the *HBD* (*δ*-globin) loci of the European shrew (*Sorex araneus*) and other eutherian mammals (data not shown). Notably, the coding sequence of these two loci only differed at a single synonymous nucleotide position in exon 2, and thus encoded identical polypeptide products. The deduced amino acid sequence of the single complete *Episoriculus* α-chain was found to differ from that of European shrews and musk shrews (*Suncus murinus*) at 11 and 14 positions, respectively, while the (*β*-type) *δ*-chain sequence differed at 17 and 19 positions, respectively (Fig. 3). This information was used to estimate the number of titratable His residues (in the physiological pH range) of known shrew Hb sequences following the criteria outlined in Berenbrink (2006). Briefly, the Hbs of *E. fumidus* and *S. araneus* are predicted to possess 14 titratable His residues, while the two Hb isoforms of *S. murinus* Hb (whose *β*-type chains differ at four residue positions including a His → Lys exchange at position 117; Maita et al. 1981) are predicted to possess 14 and 16 titratable His residues, respectively (Fig. 3).

Discussion

Mass-specific metabolic rate varies inversely with body mass, while pulmonary transit time, muscle oxygen storage capacity (i.e. [Mb]), and the ability to exploit anaerobic pathways vary directly with this variable (Emmett and Hochachka 1981; Lindstedt 1984; McNab 1991). Hence, small homeothermic mammals must employ a suite of morphological and physiological adjustments to continually supply tissue mitochondria with sufficient O_2 to fuel oxidative metabolism. These include reduced diffusion distances, high circulatory and ventilatory gas transport capacities, and blood with relatively low affinity for O_2 (Bartels et al. 1979; Gehr et al. 1980; Morrison et al. 1959).

Table 1 Effects of temperature, anionic allosteric effectors and pH on the half-saturation oxygen pressure (P_{50}), Bohr coefficient (ϕ, ΔLog P_{50}/ΔpH), enthalpy of oxygenation (ΔH) and temperature sensitivity (ΔLog $P_{50}/\Delta T$) of the major hemoglobin component (Hb II) of *E. fumidus* in 0.1 M HEPES buffer

pH	37 °C			25 °C			25–37 °C			
	6.8	7.2	ϕ	6.8	7.2	ϕ	6.8		7.2	
	P_{50} (mmHg)			P_{50} (mmHg)			ΔH (kJ mol^{-1})	ΔLog $P_{50}/\Delta T$	ΔH (kJ mol^{-1})	ΔLog $P_{50}/\Delta T$
Stripped	10.06	7.67	−0.30	5.29	3.74	−0.38	−28.7	0.023	−33.4	0.026
0.1 M Cl^-	21.67	14.81	−0.41	11.89	8.46	−0.37	−25.9	0.022	−23.3	0.020
0.75 mM DPG	40.85	21.63	−0.69	27.34	14.22	−0.71	−13.2	0.015	−14.3	0.015
0.1 M Cl^- + 0.75 mM DPG	46.41	24.51	−0.69	33.45	18.00	−0.67	−8.4	0.012	−7.2	0.011

Values for each treatment have been standardized to pH 6.8 and pH 7.2 using linear equations derived from Fig. 2

Moreover, the level of carbonic anhydrase (which catalyzes the hydration of CO_2 to HCO_3^- and H^+) within the erythrocytes of mammals increases with decreasing size (Larimer and Schmidt-Nielsen 1959), presumably to ensure that carbon dioxide exchange equilibria are maintained in the systemic and pulmonary capillaries. These authors further suggested that the high rate of proton generation at the tissues arising from this reaction, coupled with a relatively high Bohr factor, may allow the blood O_2 stores of small mammals to be more fully exploited. Consistent with these predictions, measured Bohr coefficients of shrew Hb and blood (−0.61 to −0.69) are at the upper range for mammals (Bartels et al. 1979; Jürgens et al. 1981; this study). However, our findings regarding the dual modulation of *Episoriculus* Hb by phosphate and chloride ions, together with its low thermal sensitivity and predicted low proton buffering capacity, suggest that the functional control of O_2 uptake and delivery in these diminutive insectivores may be more complex than previously suspected.

Oxygen affinity

Under presumed intracellular conditions (pH 7.2 and 37 °C) the P_{50} of *Episoriculus* Hb devoid of anions (7.7 mmHg) is ~75 % higher than that recorded for human HbA (4.4 mmHg; Perutz and Imai 1980) under the same conditions, and only half of that reported for 'stripped' hemolysates of *S. etruscus* and *C. russula* (13.1 and 17.4 mmHg respectively; Bartels et al. 1979). Notably, however, these latter samples were eluted in 0.16 M Cl^- Tris buffer (Jürgens et al. 1981) and thus are likely more comparable to values obtained from *E. fumidus* Hb in the presence of 0.1 M Cl^- (14.8 mmHg; Table 1). Hence, we are presently unable to determine whether or not the intrinsic O_2 affinity of the high-elevation Taiwanese shrew Hb is consistent with selection pressures acting to ensure adequate Hb saturation at hypobaric oxygen tensions approaching 100 mmHg; this will have to await comparative determinations from lowland white- and red-toothed shrew Hbs.

A reduction in Hb phosphate sensitivity is traditionally considered an 'adaptation' for living at altitude (e.g. llama; Bauer et al. 1980) or underground (e.g. European mole; Jelkmann et al. 1981). In this regard it is significant that the response of *E. fumidus* Hb to saturating concentrations of this organophosphate (Log $P_{50\ (0.75\ mM\ DPG\ +\ 0.1\ M\ Cl^-)}$ − Log $P_{50\ (0.1\ M\ Cl^-)} = 0.22$; Table 2) is within the lower range of that reported for semi-aquatic and fossorial moles (0.23–0.26; Campbell et al. 2010a; Jelkmann et al. 1981; Signore et al. 2012). Jelkmann et al. (1981) speculated that the low interaction of European mole Hb with DPG results from changes in the β-type chain contacts, conferred by amino acid substitutions at helical positions A1 (δ4Thr → Ser) and A2 (δ5Pro → Gly), compared to human HbA. It is of note that these same two residues (δ4Ser, δ5Gly) are present in both crocidurine (*Suncus murinus*) and soricine (*Sorex araneus* and *E. fumidus*) shrews (Fig. 3), suggesting that Hb with a reduced DPG sensitivity may be an ancestral trait in the family Soricidae (and as such does not represent an adaptation to altitude in the Taiwanese brown-toothed shrew). Consistent with this suggestion, hemolysates of the hitherto studied white-toothed shrews, *Crocidura russula* and *Suncus etruscus*, possess notably low DPG sensitivities (0.16 and 0.22, respectively) in the presence of Cl^- (Jürgens et al. 1981).

As with DPG, we found the chloride sensitivity (Log $P_{50\ [0.1\ M\ Cl^-]}$ − Log $P_{50\ [stripped]}$) of *E. fumidus* Hb at 37 °C (0.29; Table 2) to be ~30 % less than for human HbA (0.42; Perutz et al. 1993), but nearly identical to Hb components of coast, star-nosed and American shrew moles (0.29–0.33; Campbell et al. 2010a; Signore et al. 2012) and recombinant human embryonic Gower II ($\alpha_2\varepsilon_2$) Hb (0.31; Hofmann et al. 1995). It has been shown that the difference between the two human Hbs (i.e. HbA and Hb Gower II) largely resides in the replacement of a single positive residue (His) in the β-chain of HbA with a neutral one (Asn) at position 77 of the ε-chain (Zheng et al. 1999). This change was suggested to reduce the cationic excess

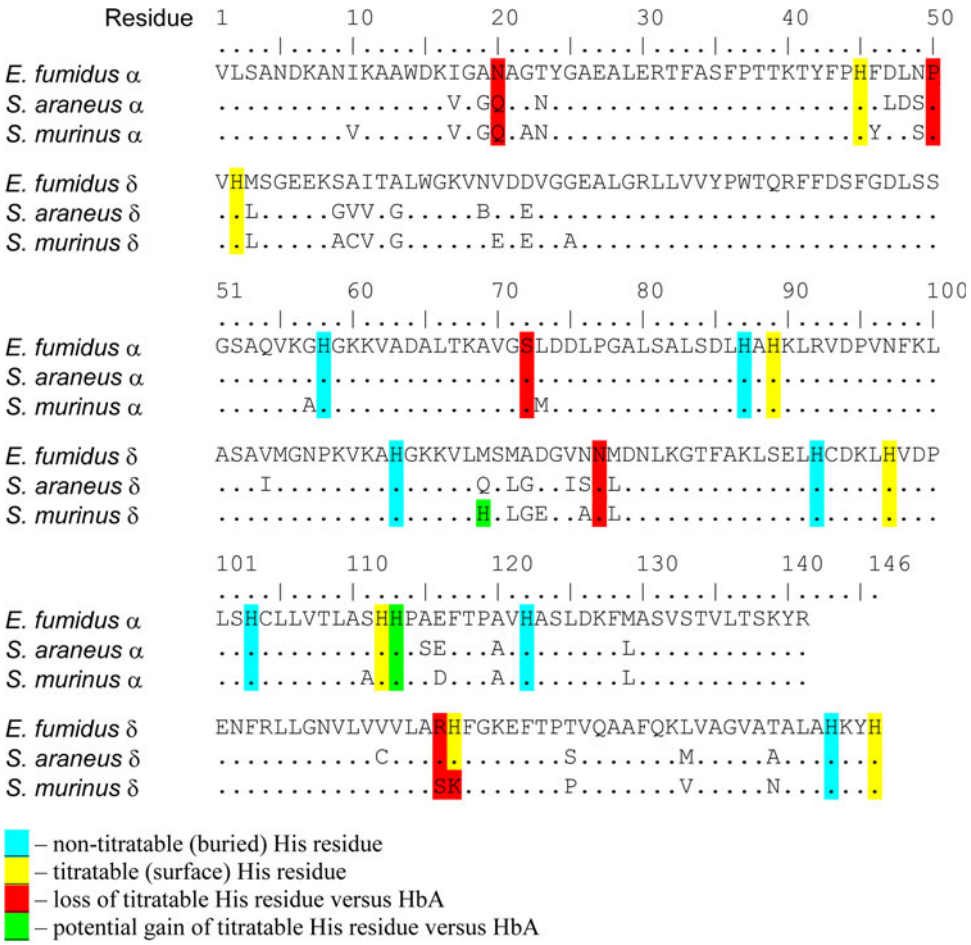

Fig. 3 Alignment of shrew α- and β-type (δ) globin chains. Amino acid residues of the musk shrew (*S. murinus*) protein were determined directly (Maita et al. 1981) and were found to differ at a single position (α_I15Asp → α_{II}15Glu) in the α-chains and at four positions in the "β-globin" chains (116Arg → Ser, 117His → Lys, 125Pro → 125Met, 126Val → Thr); only the α_I (GenBank accession P01950) and "β_I" chain (P02060) data are presented here. European shrew (*S. araneus*) primary sequences were deduced from globin gene sequences identified within working draft genomic sequences available on GenBank (AC166625.2 and AC166888.2; see Opazo et al. 2008). Residues for the latter two species are shown only at positions where they differ from those of the Taiwanese brown-toothed shrew. Residue positions highlighted in *blue* and *yellow* denote buried and surface (titratable) His residues, respectively, that are also found in human HbA, while residues highlighted in *green* and *red* represent potential gain and loss of titratable His residues, respectively, relative to human HbA (Berenbrink 2006). Note that the Hbs of all three shrew species are predicted to have up to eight less titratable His residues relative to the human protein (color figure online)

within the central cavity of Gower II (Zheng et al. 1999), thereby reducing the Cl^- effect as less chloride ions would diffuse into the central cavity upon deoxygenation (Perutz et al. 1993). It is unlikely, however, that a similar mechanism is operating in *E. fumidus* Hb as no charge altering substitutions are found in the central cavity relative to HbA. Moreover, unlike human and bovine Hbs in which the P_{50} is lower in the joint presence of DPG and chloride than with DPG alone (Table 2), the most significant characteristic of *E. fumidus* Hb is the additive effect that these anions exert on P_{50} (Fig. 2; Table 1). Notably, this attribute is also shared by deer mice and talpid moles (Campbell et al. 2010a; Storz et al. 2010; Signore et al. 2012), which indicates the presence of discrete non-overlapping DPG and Cl^- binding sites in these species. In this regard it is interesting that the above noted human Gower II ε77Asn → His mutant (Zheng et al. 1999) forms a pocket of cationic residues (ε8Lys, ε76Lys and ε77His) on the external surface of the protein that has been implicated in creating an 'additional' (in relation to HbA) Cl^- dependent regulatory site in fetal human, bovine, pig, horse and bear Hbs (De Rosa et al. 2004). However, a comparable binding site in this region can be excluded for *E. fumidus* Hb, as neutral Asn residues are found at both δ76 and δ77 (Fig. 3), precluding bridging of this cluster by Cl^-.

The fixed-acid Bohr coefficient of *Episoriculus* Hb at 37 °C and in the presence of allosteric effectors (−0.69) is similar to that determined for the blood of white-toothed

Table 2 Heterotropic effects at half saturation (P_{50}) for *E. fumidus* (shrew), coast mole, human and bovine hemoglobin at pH 7.2

	37 °C		25 °C		
	Shrew	Coast mole[c]	Shrew	Human[d]	Bovine[d]
Chloride effect[a]					
[DPG] = 0	0.29	0.31–0.33	0.35	0.48	0.42
[DPG] = 0.75 mM	0.05	–	0.10	−0.05	−0.06
DPG effect[b]					
$[Cl^-]$ = 0	0.45	–	0.58	0.95	0.56
$[Cl^-]$ = 0.1 M	0.22	0.23–0.26	0.33	0.42	0.08

[a] Log $P_{50\ (0.1\ M\ Cl^-)}$ − Log $P_{50\ (stripped)}$

[b] Log $P_{50\ (0.75\ mM\ DPG)}$ − Log $P_{50\ (stripped)}$

[c] Data from Campbell et al. (2010a)

[d] Data from Perutz et al. (1993)

shrews (range −0.61 to −0.66; Bartels et al. 1979), but somewhat higher than for human HbA (−0.41 to −0.42; Hofmann et al. 1995; Zheng et al. 1999). While the residues primarily responsible for proton binding have been debated, it is widely accepted that His side chains are the most likely candidates on the basis that the logarithmic acid dissociation constant (pK_a) of their imidazole groups is close to physiological pH (Berenbrink 2006; Perutz et al. 1980). The four sites implicated as being primarily responsible for this effect, viz., β146 His, α122 His, β143 His and the C_α-NH_2 group of α1 Val (the latter two of which are chloride dependent) (Perutz et al. 1980; Perutz 1983; Van Beek and De Bruin 1980), are present in shrews (Fig. 3). It is thus possible that the elevated Bohr effect in shrew Hbs may arise from the presence of surface His residues not found in HbA. A potential candidate in this regard is α113 His (Fig. 3), which is also found in the Hbs of hedgehogs and moles, and whose Hbs also possess comparably high fixed-acid Bohr effects (−0.52 to −0.62; Campbell et al. 2010a; Jelkmann et al. 1981; Kramm et al. 1975; Signore et al. 2012). In this respect it is notable that α113 lies close to δ116Arg (His in human HbA) of the neighboring chain, conceivably forming a proton-linked Cl^- binding site. However, the alkaline Bohr coefficient of *Episoriculus* Hb is not altered by the addition of chloride at 25 °C and is only marginally increased at 37 °C (Table 1). This finding contrasts sharply with human HbA, where nearly half of the Bohr effect is chloride dependent, a trait that has been attributed to the binding of this halide to sites at α1 Val-α131 Ser and β1 Val-β82 Lys (Perutz et al. 1980). Together, these observations suggest that different mechanisms may be at play in human and shrew Hbs.

It has recently been suggested that a large number of His residues contribute to the overall Bohr effect (Lukin and Ho 2004). Specifically, these authors have demonstrated that the pK_a of numerous His residues in human HbA—most notably β146 His—increases in the T-state (heightening the likelihood that the basic nitrogen of the imidazole ring will become protonated), while that of others decrease. Importantly, the protonation of His residues upon deoxygenation contributes to lowering oxygen affinity only if they are able electrostatically interact with nearby anionic residues or the C-termini of the globin chains, thus stabilizing the T-state protein. The net effect of these interactions, however, appears to be countered by the concurrent destabilization of salt bridges resulting from the deprotonation of other His residues (whose pK_a decreases in the T-state). Hence, the overall Bohr factor is dictated by the overall balance of 'positive' and 'negative' contributing His residues, and by the magnitude of the pK_a shift of these residues over the course of the R ↔ T transition. In short, this model predicts that the overall Bohr coefficient may be increased via gene mutations that lower the number of 'negative' contributing His residues in the protein, and vice versa. The substitution of β77 His (which contributes negatively to the alkaline Bohr effect; Lukin and Ho 2004) for Asn in many bird Hbs has been forwarded as a potential residue exchange that may increase the Bohr effect in this regard (Berenbrink 2006). Interestingly, δ77 Asn is also found in shrew Hbs (Fig. 3), which moreover exhibit high Bohr coefficients (Table 1; Bartels et al. 1979; Jürgens et al. 1981). However, as noted above, this same substitution is also found in embryonic human Gower II Hb, in which the Bohr effect is unaltered relative to HbA (Hofmann et al. 1995; Zheng et al. 1999). Functional and structural data from additional species of shrews, perhaps combined with studies on site-directed Hb mutants, will be required to delineate the specific residues modifying the magnitude of the Bohr coefficient within this group.

In addition to their allosteric role in stabilizing the deoxy-state protein via the formation of inter- and intra-subunit salt bridges, solvent-exposed His residues are the primary buffer groups of Hb (Berenbrink 2006). In this light it is noteworthy that *E. fumidus* Hb is predicted to possess eight less titratable His residues per tetramer than the 22 present on human HbA. Thus, assuming identical Bohr coefficients, a given acid load should result in a larger drop in pH (greater arterio-venous pH difference) of shrew blood relative to human HbA (Berenbrink 2006), and hence a correspondingly larger increase in the P_{50} of the shrew protein. However, the Bohr coefficient (~ -0.7) of Taiwanese brown-toothed shrew Hb is also substantially higher than that of HbA (~ -0.4), a trait which would further augment the exploitation of available blood oxygen stores and enhance O_2 offloading at the tissues. Notably, this mechanism fosters a stronger link between the

products of aerobic metabolism ($CO_2 \rightarrow HCO_3^- + H^+$) and O_2 delivery than that found in other (larger) mammal species, and may thus represent an important strategy to fuel the high mass-specific rates of metabolism found within this group of diminutive mammals. It must be stressed, however, that available data suggest these attributes (i.e. high Bohr effect and low predicted proton buffering capacity) may be widespread among shrews and thus do not appear to be adaptations to chronic altitudinal hypoxia per se.

Thermal sensitivity

Because oxygenation of the heme iron is exothermic, the P_{50} of mammalian Hb tends to increase in parallel with temperature. The binding of H^+, DPG, chloride and CO_2 to deoxyHb, however, may mitigate this effect to varying degrees by numerically lowering the overall ΔH (Weber and Campbell 2011). Interestingly, this compensatory mechanism appears to be most pronounced in heterothermic (namely polar marine, arctic and hibernating) species, where it has been argued that the ensuing reduction in temperature sensitivity ensures adequate O_2 delivery to cool (often peripheral) tissues (De Rosa et al. 2004; Weber and Campbell 2011). In this context, it is noteworthy that the overall ΔH of *Episoriculus* Hb in the presence of allosteric effectors (−7 kJ mol^{-1} O_2 at pH 7.2; Table 1) is even less than that of Hbs of 'cold-tolerant' mammals (range −10 to −29 kJ mol^{-1} at pH 7.4; Campbell et al. 2010b; De Rosa et al. 2004). De Rosa et al. (2004) suggested that a specific pocket of basic residues, namely β8 Lys, β76 His and β77 His/Asn constitutes an 'additional' oxygenation-linked chloride binding site in human fetal, ruminant and bear Hbs that lowers the ΔH with respect to human HbA. As noted above, although *E. fumidus* Hb possesses an 'extra' Cl^- binding site (Fig. 2), the expressed δ-chains of this species possess uncharged Asn residues at δ76 and δ77 (Fig. 3) precluding Cl^- binding within the above proposed cleft (i.e. an alternate Cl^- binding site must be operating in brown-toothed shrew Hb). We recently presented evidence supporting this same conclusion for talpid mole Hbs (Signore et al. 2012), which share a close phylogenetic affinity with shrews. Finally, it is of note that the ΔH of stripped *Episoriculus* Hb above pH 8.3 ($\sim$−46 kJ mol^{-1}; data not shown), where the Bohr effect of Hb should be abolished, is markedly lower than pig Hb (–67 kJ mol^{-1}; Weber et al. 1987) and human HbA (−59 kJ mol^{-1}; Atha and Ackers 1974). While the structural basis for this phenomenon is unknown, it may be associated with inherent oxygenation-linked conformational differences (e.g. a lowered $\Delta H^{T \rightarrow R}$) not related to effector dissociation as has been suggested for bovine and elephantid Hbs (Campbell et al. 2010b; Weber and Campbell 2011).

As previously noted, evolution of a numerically low ΔH is generally considered to be an adaptation whereby regionally and temporally heterothermic mammals can match oxygen offloading with (changing) tissue oxygen requirements (Weber and Campbell 2011). While this trait would clearly be beneficial to white-toothed shrews (many of which enter torpor; McNab 1991), it is difficult to imagine that the extremities of a soricine shrew like *E. fumidus* can be chronically cooled, thereby significantly impeding O_2 offloading (i.e. the legs are too short for an effective counter-current heat exchanger to operate). In this regard it is of note that numerically high negative ΔH values dictate elevated rates of outward heat flow relative to those in which Hb oxygenation is less exothermic (Weber and Wells 1989). Consequently, a reduced ΔH may represent an important heat conservation mechanism for small-bodied shrew species that exhibit both a high surface area to mass ratios and high respiratory rates (up to 1,080 breaths min^{-1}!; Morrison et al. 1959).

In summary, we conclude that the ability of the Taiwanese brown-toothed shrew to colonize cool montane environments does not appear to be linked to structural modifications that lowered the sensitivity of their Hb to anionic effectors and temperature. Indeed, available sequence data suggest these traits are probably ancestral, and likely prevalent among shrews as a whole. The limited sequence and oxygen-binding data available suggest that in general, shrews are able to sustain their high metabolic requirements via a synergistic modulation of Hb–O_2 affinity by chloride and DPG, and by a high Bohr coefficient coupled to a reduced predicted buffering capacity of their Hb. These attributes should foster increased arterial blood-to-tissue PO_2 gradients in shrew blood (i.e. O_2 offloading at a relatively high PO_2), hence promoting a more efficient exploitation of red cell O_2 stores required to fuel their high mass-specific rates of metabolism.

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References

Atha DH, Ackers GK (1974) Calorimetric determination of the heat of oxygenation of human hemoglobin as a function of pH and the extent of reaction. Biochemistry 13:2376–2382

Bartels H, Bartels R, Baumann R, Fons R, Jürgens KD, Wright P (1979) Blood oxygen transport and organ weights of two shrew species (*S. etruscus and C. russula*). Am J Physiol 236:R221–R224

Bauer C, Rollema HS, Till HW, Braunitzer G (1980) Phosphate binding by llama and camel hemoglobin. J Comp Physiol B 136:67–70

Berenbrink M (2006) Evolution of vertebrate haemoglobins: histidine side chains, specific buffer value and Bohr effect. Respir Physiol Neurobiol 154:165–184

Campbell KL, Storz JF, Signore AV, Moriyama H, Catania KC, Payson A, Bonaventura J, Stetefeld J, Weber RE (2010a) Molecular basis of a novel adaptation to hypoxic-hypercapnia in a strictly fossorial mole. BMC Evol Biol 10:214

Campbell KL, Roberts JEE, Watson LN, Stetefeld J, Sloan AM, Signore AV, Howatt JW, Tame JRH, Rohland N, Shen T-J, Austin JJ, Hofreiter M, Ho C, Weber RE, Cooper A (2010b) Substitutions in woolly mammoth hemoglobin confer biochemical properties adaptive for cold tolerance. Nat Genet 42:536–540

De Rosa MC, Castagnola M, Bertonati C, Galtier A, 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

Emmett B, Hochachka PW (1981) Scaling of oxidative and glycolytic enzymes in mammals. Respir Physiol 45:261–272

Foreman CW (1954) A comparative study of the oxygen dissociation of mammalian hemoglobin. J Cell Comp Physiol 44:421–429

Gehr P, Sehovic S, Burri PH, Claassen H, Weibel ER (1980) The lung of shrews: morphometric estimation of diffusion capacity. Respir Physiol 40:33–47

Gusztak RW, MacArthur RA, Campbell KL (2005) Bioenergetics and thermal physiology of the American water shrew (*Sorex palustris*). J Comp Physiol B 175:87–95

Hofmann O, Carrucan G, Robson N, Brittain T (1995) The chloride effect in the human embryonic haemoglobins. Biochem J 309:959–962

Hutterer R (2005) Order Soricomorpha. In: Wilson DE, Reeder DA (eds) Mammal species of the world: a taxonomical reference, 3rd edn. John Hopkins University Press, Baltimore, pp 220–311

Jameson EW, Jones GS (1977) The Soricidae of Taiwan. Proc Biol Soc Wash 90:459–482

Jelkmann W, Oberthür W, Kleinschmidt T, Braunitzer G (1981) Adaptation of hemoglobin function to subterranean life in the mole, *Talpa europaea*. Respir Physiol 46:7–16

Jürgens KD, Bartels H, Bartels R (1981) Blood oxygen transport and organ weights of small bats and small non-flying mammals. Respir Physiol 45:243–260

Kramm Ch, Sattrup G, Baumann R, Bartels H (1975) Respiratory function of blood in hibernating and non-hibernating hedgehogs. Respir Physiol 25:311–318

Larimer JL, Schmidt-Nielsen K (1959) A comparison of blood carbonic anhydrase of various mammals. Comp Biochem Physiol 1:19–23

Lindstedt SL (1984) Pulmonary transit time and diffusing capacity in mammals. Am J Physiol 246:R384–R388

Lukin JA, Ho C (2004) The structure–function relationship of hemoglobin in solution at atomic resolution. Chem Rev 104:1219–1230

Maita T, Matsuda G, Takenaka O, Takahashi K (1981) The primary structure of adult hemoglobin of musk shrew (*Suncus murinus*). Hoppe Seylers Z Physiol Chem 362:1465–1474

McNab BK (1991) The energy expenditure of shrews. In: Findley JS, Yates TL (eds) The biology of the Soricidae. The Museum of Southwestern Biology, University of New Mexico, Albuquerque, pp 35–45

Morrison P, Ryser FA, Dawe AR (1959) Studies on the physiology of the masked shrew *Sorex cinereus*. Phys Zool 32:256–271

Nowak RM (1999) Walker's mammals of the world, 6th edn. Johns Hopkins University Press, Baltimore

Opazo JC, Hoffmann FG, Storz JF (2008) Differential loss of embryonic globin genes during the radiation of placental mammals. Proc Natl Acad Sci USA 105:12950–12955

Perutz MF (1983) Species adaptation in a protein molecule. Mol Biol Evol 1:1–28

Perutz MF, Imai K (1980) Regulation of oxygen affinity of mammalian haemoglobins. J Mol Biol 136:183–191

Perutz MF, Kilmartin JV, Nishikura K, Fogg JH, Butler PJG, Rollema HS (1980) Identification of residues contributing to the Bohr effect of human haemoglobin. J Mol Biol 138:649–668

Perutz MF, Fermi G, Poyart C, Pagnier J, Kister J (1993) A novel allosteric mechanism in haemoglobin: structure of bovine deoxyhaemoglobin, absence of specific chloride-binding sites and origin of the chloride-linked Bohr effect in bovine and human haemoglobin. J Mol Biol 233:536–545

Peters T, Kubis HP, Wetzel P, Sender S, Asmussen G, Fons R, Jürgens KD (1999) Contraction parameters, myosin composition and metabolic enzymes of the skeletal muscle of the Etruscan shrew *Suncus etruscus* and of the common European white-toothed shrew *Crocidura russula* (Insectivora: Soricidae). J Exp Biol 202:2461–2473

Signore AV, Stetefeld J, Weber RE, Campbell KL (2012) Origin and mechanism of thermal insensitivity in mole hemoglobins: a test of the 'additional' chloride binding site hypothesis. J Exp Biol 215:518–525

Stewart JM, Woods AK, Blakely JA (2005) Maximal enzyme activities, and myoglobin and glutathione concentrations in heart, liver and skeletal muscle of the Northern Short-tailed shrew (*Blarina brevicauda*; Insectivora: Soricidae). Comp Biochem Physiol B Biochem Mol Biol 141:267–273

Storz JF (2007) Hemoglobin function and physiological adaptation to hypoxia in high-altitude mammals. J Mammal 88:24–31

Storz JF, Runck AM, Moriyama H, Weber RE, Fago A (2010) Genetic differences in hemoglobin function between highland and lowland deer mice. J Exp Biol 213:2565–2574

Van Beek GGM, De Bruin SH (1980) Identification of the residues involved in the oxygen-linked chloride-ion binding sites in human deoxyhemoglobin and oxyhemoglobin. Eur J Biochem 105:353–360

Weber RE (1992) Use of ionic and zwitterionic (Tris/BisTris and HEPES) buffers in studies on hemoglobin function. J Appl Physiol 72:1611–1615

Weber RE (2007) High-altitude adaptations in vertebrate hemoglobins. Respir Physiol Neurobiol 158:132–142

Weber RE, Campbell KL (2011) Temperature dependence of haemoglobin–oxygen affinity in heterothermic vertebrates: mechanisms and biological significance. Acta Physiol 202: 549–562

Weber RE, Wells RGM (1989) Hemoglobin structure and function. In: Wood C (ed) Comparative pulmonary physiology: current concepts. Marcel Dekker, New York, pp 279–310

Weber RE, Kleinschmidt T, Braunitzer G (1987) Embryonic pig hemoglobins Gower I ($\zeta_2\varepsilon_2$), Gower II ($\alpha_2\varepsilon_2$), Heide I ($\zeta_2\theta_2$) and Heide II ($\alpha_2\theta_2$): oxygen-binding functions related to structure and embryonic oxygen supply. Respir Physiol 69:347–357

Yu H-T (1993) Natural history of small mammals of subtropical montane areas in central Taiwan. J Zool (Lond) 231:403–422

Zheng T, Zhu Q, Brittain T (1999) Origin of the suppression of chloride ion sensitivity in human embryonic hemoglobin Gower II. IUBMB Life 48:435–437