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Enthalpic partitioning of the reduced temperature sensitivity of O_2 binding in bovine hemoglobin

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ABSTRACT

The oxygenation enthalpy of the heme groups of hemoglobin (Hb) is inherently exothermic, resulting in decreased Hb-O_2 affinity with rising temperature. However, oxygenation is coupled with endothermic dissociation of allosteric effectors (e.g. protons, chloride ions and organic phosphates) from the protein, which reduces the overall oxygenation enthalpy. The evolution of Hbs with reduced temperature sensitivity ostensibly safeguards O_2 unloading in cold extremities of regionally-heterothermic vertebrates permitting energy-saving reductions in heat loss. Ungulate (e.g. bovine) Hbs have long served as a model system in this regard in that they exhibit numerically low oxygenation enthalpies that are thought to correlate with the presence of an additional Cl^- binding site (compared to human Hb) comprised of three cationic residues at positions 8, 76 and 77 of the β-chains of Hb. However, ungulate Hbs also exhibit distinctive amino acid exchanges at the N-termini of the β-chains that stabilize the low-affinity deoxystructure of the Hb, mimicking the action of organic phosphates. In order to assess the relative contributions from these two effects, we measured the temperature sensitivity of Hb-O_2 affinity in bovine and human Hbs in the absence and presence of Cl^- ions under strictly controlled pH conditions. The data indicate that Cl^--binding accounts for a minority (~30%) of the total reduction in the oxygenation enthalpy manifested in bovine compared to human Hb, whereas the majority of this reduction is ascribable to structural differences, including increased β-chain hydrophobicity that would increase the heat of oxygenation-linked conformational change in bovine Hb.

1. Introduction

The oxygenation of vertebrate hemoglobin (Hb) is associated with a concerted transition of the two α and two β chain subunits from 'tense' (T) to 'relaxed' (R) states. The shift is coupled to the liberation of heat and of the ionic allosteric effectors that modulate Hb-O_2 affinity by binding to and stabilizing the low-affinity T structures (Benesch et al., 1969; Brix et al., 1990; Coletta et al., 1992; Weber and Campbell, 2011). In mammalian Hbs, the major effectors are protons and CO_2 (that enhance O_2 release in metabolizing issues via the Bohr effect), the red cell organic phosphate BPG (2,3-bisphosphoglycerate) and chloride ions (Perutz, 1983).

Based on the exothermic nature of heme oxygenation ($\Delta H < 0$), increasing temperature decreases Hb-O_2 affinity (increases the half-saturation O_2 tension, P_{50}) thus acting as an 'allosteric effector' (Weber and Campbell, 2011) that promotes O_2 unloading in warm tissues that consume O_2 at a higher rate (Barcroft and King, 1909). Since the oxygenation-linked release of the effectors is endothermic ($\Delta H > 0$), this process reduces overall heat liberation upon oxygenation (i.e. the temperature sensitivity of Hb-O_2 affinity) resulting in less negative enthalpy values. In addition to the heat of solvation of O_2 (ΔH^{sol}), the intrinsic heat of O_2 binding by the heme groups (ΔH^0) and the heat of conformational change ($\Delta H^{T\rightarrow R}$), the overall oxygenation enthalpy ($\Delta H'$) thus comprises the heats of dissociation of protons, Cl^-, BPG and of other effector ions (ΔH^{H+}, ΔH^{Cl-}, ΔH^{BPG}, ΔH^{X}, respectively) (Weber and Campbell, 2011) — each of which include contributions from the reactions of these effectors with partner ions (e.g. the heats of ionization of buffers). Changes in blood levels of allosteric factors thus modulate blood-O_2 affinity directly as well as indirectly by altering the temperature sensitivity of the Hb-O_2 affinity.

Although the reduction in Hb-O_2 affinity observed with increasing temperature favors Hb-O_2 unloading in warm tissues – that have higher O_2 metabolic rates – it may also compromise O_2 delivery, in cold tissues.

Abbreviations: φ, Bohr factor ($\Delta\log P_{50}/\Delta pH$); BPG, 2,3-bisphosphoglycerate; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; Hb, hemoglobin; P_{50}, O_2 tension that saturates 50% of the Hb; n_{50}, Hill's cooperativity coefficient at 50% oxygenation of the Hb; $\Delta H'$, apparent enthalpy of oxygenation.

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Table 1
Schematic representation of the α and β chain amino acid residues [cf. (Kleinschmidt and Sgouros, 1987)] implicated in oxygenation-linked binding of protons (H), chloride ions (Cl^-) and BPG (D) in human, bovine and horse Hb.

	α-Chain		β-Chain							
Effector	H Cl[a]	Cl[a]	D[b] Cl[c]	D[d] H	Cl[e]			D[d] H Cl[c]	D[d] H[e]	H
Residue no. in human Hb (helical position)	1(NA1)	131(H14)	1(NA1)	2(NA2)	8(A5)	76(E20)	77(EF1)	82(EF6)	143(H21)	146(HC3)
Human HbA	Val	Ser	Val	His	Lys	Ala	His	Lys	His	His
Bovine Hb	Val	Asn	–	Met	Lys	Lys	His	Lys	His	His
Horse Hb	Val	Ser	Val	Gln	Lys	His	His	Lys	His	His

[a] Shared Cl^- binding between Val-1 and Ser-131 of one α chain and Arg 141 (data not shown) of the opposite α-chain (O'Donnell et al., 1979).
[b] BPG binding to one β-chain.
[c] Shared β-chain Cl^- binding site (Riggs, 1988).
[d] BPG binding to both β-chains (Richard et al., 1993).
[e] 'Additional' Cl^--binding site (Lys and His residues at positions 8, 76 and 77) in bovine Hb.

Presumably to compensate for this shortfall, ΔH′ (i.e. the overall temperature sensitivity of O_2 binding) has convergently been reduced in 'regionally heterothermic' vertebrates. Examples are cold-tolerant mammals, where low temperature sensitivity may safeguard O_2 unloading in cold extremities (Giardina et al., 1989; Brix et al., 1990; Coletta et al., 1992; Clementi et al., 1994; De Rosa et al., 2004). Analogously, in the case of fast-swimming fish like tuna, sharks and spearfish that have warm muscles, brains or eyes (Rossi-Fanelli et al., 1960; Block and Carey, 1985; Block, 1986), low thermal sensitivities of Hb-O_2 affinity will reduce the premature O_2 release and thus decrease the risk of short-circuiting of O_2 from arterial to venous blood in counter-current exchanger systems, by lowering the O_2 tension gradients between the blood vessels (Larsen et al., 2003; Weber et al., 2010). Given that the normal temperature sensitivity (i.e. heat uptake by Hb upon deoxygenation in the tissues, and heat liberation upon oxygenation at the respiratory surfaces) constitutes outward heat transport (that accounts for 9% of the heat produced by glucose metabolism), a reduction in temperature sensitivity will decrease Hb-mediated heat transfer, thus contributing to maintain warm tissues in regionally heterothermic animals (Weber and Wells, 1989; Campbell et al., 2012).

In cold-tolerant mammals (including reindeer, horse and cattle) the low temperature sensitivities have been suggested to correlate with the presence of the additional Cl^- binding site compared to human Hb. In contrast to human Hb, where chloride ions predominantly bind at one α-chain site (involving Val1α and Ser131α) and one β-chain site (involving Val1β and Lys82β) (O'Donnell et al., 1979; Riggs, 1988), Hbs from these species have a cluster of positively charged (His and Lys) amino acid residues at positions 8, 76 and 77 of the β-chains (Fronticelli, 1990) (Table 1), that potentially increases the endothermic contribution of Cl^- release upon O_2 binding. In accordance with this contention, a human Hb mutant constructed to contain 'bovine', β-chain amino acid substitutions including Ala76β→Lys, displayed a heightened Cl^- sensitivity (Fronticelli et al., 1995) — although these substitutions did not reduce O_2 affinity to the level found in bovine Hb (Baudin-Creuza et al., 2002). Moreover, computational analyses identified this site as energetically most favorable for Cl^- binding in horse, bovine and fetal human Hb (De Rosa et al., 2004). Finally, Hbs with the 'additional' Cl^- binding cluster show synergistic Cl^- and BPG effect, i.e. O_2 affinity is depressed more by both anions than when only one is present (Coletta et al., 1994; De Rosa et al., 2004). However, the 'additional' site is not uniquely correlated with increased Cl^- binding. For instance, although Asian elephant Hb contains the β8Lys–β76Lys–β77His motif and shows a lower temperature sensitivity, it exhibits a distinctly lower Cl^--sensitivity (i.e. it releases fewer Cl^- ions upon oxygenation) than bovine and human Hbs. Moreover, identical slopes of Cl^- titrations reflect the same number of Cl^- ions released upon oxygenation in human and bovine Hbs (Perutz et al., 1993); also see Supplementary Fig. 12 in Campbell et al. (2010). Furthermore, the Cl^--sensitivity and the variability in ΔH′ observed amongst talpid mole Hbs do not correlate with amino acid exchanges at this site (Signore et al., 2012).

It thus is feasible that the low temperature sensitivities in ungulate Hbs may correlate with other structural attributes, such as the difference in hydrophobicity in the β-chains between bovine and human Hbs (Fronticelli et al., 1995). As is well documented [cf. (Bunn, 1980)] mammalian Hbs can be divided into two groups in regard to O_2 affinity modulation: (a) those with high intrinsic O_2 affinities and high BPG sensitivities (e. g. adult human Hb), and (b) those with low intrinsic O_2 affinities and low BPG sensitivities (e.g. ruminant Hb). In contrast to the solvent-exposed, hydrophilic His residue found at position NA2 in human Hb (Table 1), bovine Hb has Met, whose hydrophobic side chains adhere to the hydrophobic interior of the β-chains, locking the A helices tightly to neighboring segments of the polypeptide chains,

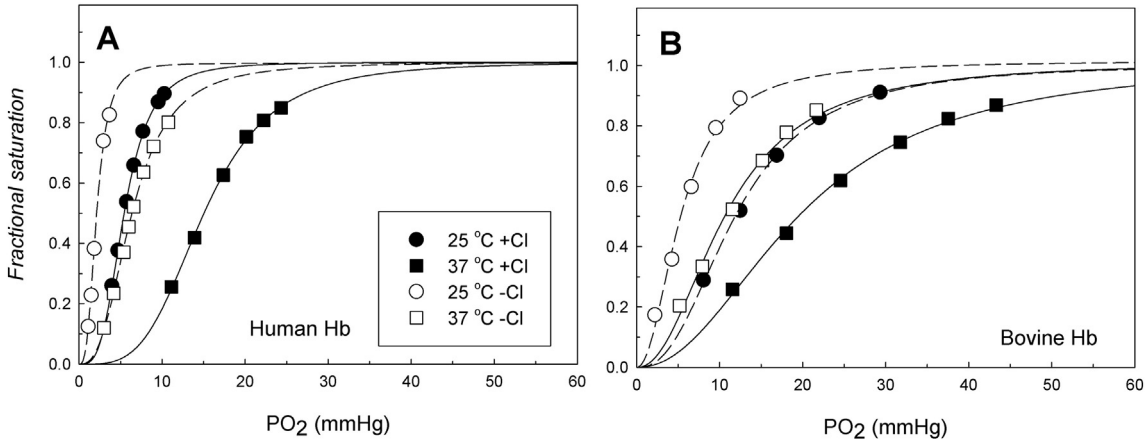

Fig. 1. O_2 equilibrium curves of stripped A, human Hb at pH 7.41 ± 0.01 and B, bovine Hb at pH 7.42 ± 0.03 measured at 25 °C (circles) and 37 °C (squares) in the absence (open symbols) and presence (solid symbols) of 0.1 M Cl^-.

Table 2
O_2-affinities (indexed as P_{50}, an inverse measure of Hb-O_2 affinity), cooperativity coefficients (n_{50}) and r^2 determination coefficients for O_2 equilibria of stripped bovine and human Hbs measured at 25 and 37 °C, pH values near 6.8, 7.4 and 8.1, and in the absence (−) and presence (+) of 0.1 M Cl^-.

Hb	°C	Cl^-	pH	P_{50} ± SEM (mm Hg)	n_{50} ± SEM	r^2
Human	25	−	6.818	3.50 ± 0.05	2.52 ± 0.09	0.999
	37	−	6.806	9.18 ± 0.13	2.75 ± 0.09	0.994
	25	+	6.808	10.72 ± 0.22	3.08 ± 0.22	0.998
	37	+	6.803	23.55 ± 0.26	3.28 ± 0.14	1.000
	25	−	7.411	2.14 ± 0.04	3.08 ± 0.12	0.999
	37	−	7.420	6.39 ± 0.05	2.75 ± 0.05	0.998
	25	+	7.406	5.44 ± 0.02	3.38 ± 0.04	1.000
	37	+	7.418	15.06 ± 0.11	3.66 ± 0.08	1.000
	25	−	8.050	1.44 ± 0.003	3.18 ± 0.03	1.000
	37	−	8.028	4.86 ± 0.06	3.07 ± 0.09	1.000
	25	+	8.045	2.78 ± 0.04	2.80 ± 0.10	0.999
	37	+	8.026	9.03 ± 0.05	3.70 ± 0.07	1.000
Bovine	25	−	6.822	7.83 ± 0.12	2.17 ± 0.09	0.999
	37	−	6.788	15.76 ± 0.34	2.04 ± 0.09	0.999
	25	+	6.805	25.92 ± 0.37	2.07 ± 0.05	1.000
	37	+	6.824	38.12 ± 1.21	2.02 ± 0.14	0.999
	25	−	7.395	5.40 ± 0.29	2.11 ± 0.21	0.997
	37	−	7.425	10.73 ± 0.38	2.18 ± 0.14	0.998
	25	+	7.402	11.94 ± 0.16	2.44 ± 0.08	0.999
	37	+	7.460	19.79 ± 0.41	2.16 ± 0.09	0.999
	25	−	8.128	3.74 ± 0.24	2.19 ± 0.26	0.995
	37	−	8.094	9.95 ± 0.23	2.59 ± 0.15	0.999
	25	+	8.124	5.92 ± 0.11	2.37 ± 0.09	0.999
	37	+	8.109	11.65 ± 0.27	2.16 ± 0.09	0.999

which stabilizes the deoxygenated structure, mimicking the effect of BPG (Perutz and Imai, 1980; Perutz et al., 1993) and predictably perturbs the heat of conformational structural changes (Fronticelli et al., 1988; Razynska et al., 1990). Additionally the synergism between Cl^- and BPG binding may be affected by asymmetric binding of BPG in bovine Hb that widens the central ('phosphate-binding') cavity allowing the entry of chloride ions (Marta et al., 1998).

Although temperature sensitivity and its molecular underpinnings in bovine and human Hbs have been extensively investigated (Fronticelli, 1990; Razynska et al., 1990; Coletta et al., 1994; Fronticelli et al., 1994, 1995; Marta et al., 1998; De Rosa et al., 2004; Sasagawa et al., 2006), the oxygenation enthalpies of Hbs that, respectively, possess and lack the 'additional' Cl^--binding site have not been accurately examined over a wide range of strictly-controlled (temperature-independent) pH values, using the same experimental technique. The present study aims to remedy this short-coming, by evaluating the relative contributions of 'additional' Cl^--binding vs. structural differences to the reduced temperature sensitivity observed in bovine compared to human Hbs.

2. Materials and methods

Human blood was obtained from a non-smoking donor. Bovine blood was obtained from Aarhus Slagtehus (Aarhus Abattoir). Hbs were prepared by freezing and osmotic lysis of washed red cells, as previously described (Weber et al., 2010). Human Hb was stripped of effectors by column chromatography using Sephadex G25 fine gel. Bovine Hb was stripped using a mixed-bed resin AG 501-X8. Control measurements revealed the same P_{50} values in Hbs stripped by either method.

The stripped Hb solutions were frozen at −80 °C in 100 μL aliquots that were thawed individually for admixture of HEPES buffers of various pH and standard KCl solutions. Cl− concentration in the Hb samples was measured using a Sherwood MKII 926S Analyzer. pH was measured at the same temperature as O_2 equilibrium measurements, using a thermostatted microelectrode (G 299) coupled to a BMS2 mk2 Blood Micro System and a PHM 64 pH Meter (Radiometer, Copenhagen).

O_2 equilibrium curves were measured on 4-μL, ultrathin Hb samples using a modified gas diffusion chamber, connected via fiber optics to a Cary 50 Probe UV–visible spectrophotometer and coupled to an Environics 4040 Gas Dilution system for mixing ultrapure (>99.998%) N_2 and air. Absorption ratios at 430 nm/421 nm were recorded continuously while O_2 tension in gas mixes perfusing the chamber was increased stepwise from zero (deoxygenated Hb) to ~150 mm Hg, followed by perfusion with pure O_2 to record absorption of the fully

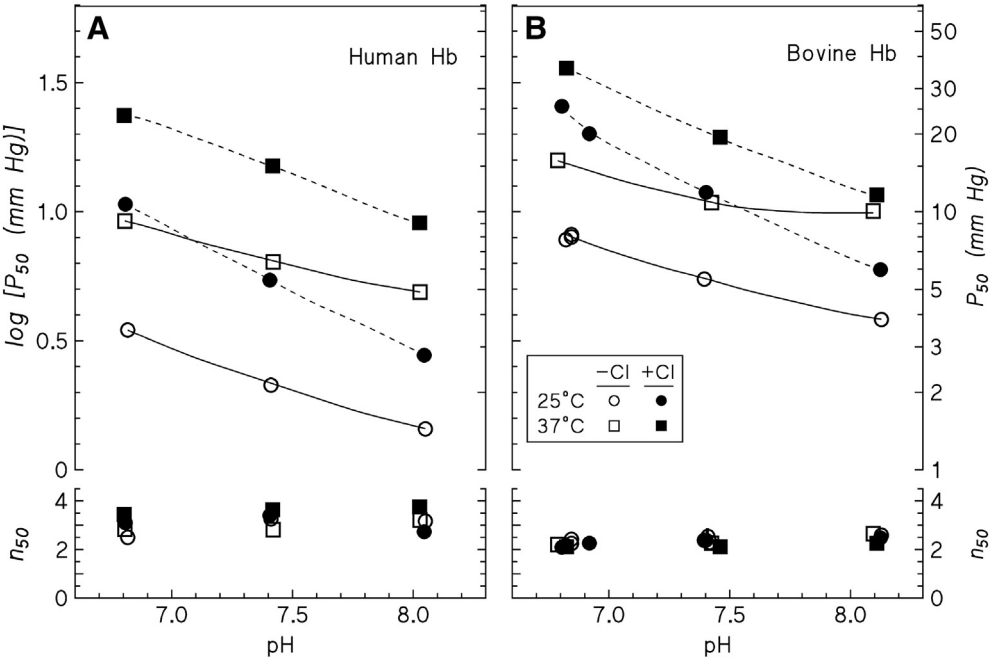

Fig. 2. pH dependence of O_2 tensions and Hill's interaction coefficients at half-saturation (P_{50} and n_{50}) of human Hb (A) and bovine Hb (B), measured in 0.1 M HEPES buffer at 25 °C (circles) and 37 °C (squares) and in the absence (open symbols) and presence (solid symbols) of 0.10 M chloride. Heme concentration, 0.62 mM.

Table 3
O_2 affinities of human and bovine Hbs (expressed as log P_{50} values) at pH 7.40, and their sensitivities to pH (Bohr factors, φ, derived from linear regressions in Fig. 2), Cl^- ions (expressed as the shifts induced by 0.1 M Cl^-, $\Delta_{(0.10-0)}$) and temperature (expressed as ΔH′, the overall heats of oxygenation excluding the heat of solution of O_2 ($^-$12.5 kJ·mol^{-1})).

	Human Hb			Bovine Hb		
[Cl^-] (M)	0	0.10	$\Delta_{(0.10-0)}$	0	0.10	$\Delta_{(0.10-0)}$
log P_{50} (25 °C)	0.35	0.75	0.39	0.73	1.07	0.34
log P_{50} (37 °C)	0.81	1.17	0.36	1.04	1.31	0.27
Δlog P_{50} (37–25 °C)	0.46	0.43		0.31	0.24	
φ (25 °C)	−0.31	−0.47	0.16	−0.24	−0.46	0.22
φ (37 °C)	−0.23	−0.34	0.11	−0.15	−0.38	0.23
ΔH′ (kJ·mol^{-1})	−55.2	−50.7	−4.5	−32.8	−22.9	−9.9

oxygenated Hb. Typically 5–8 O_2 steps between 20 and 70% saturation (Y) were measured for each O_2 equilibrium curve. Values of P_{50} and n_{50} (Hill's cooperativity coefficient at 50% O_2 saturation) were obtained by nonlinear regressions ($r^2 > 0.994$) of Hill plots, $Y = PO_2^n / (P_{50}^n + PO_2^n)$. Control experiments showed no systematic difference between values recorded by this method and by using a diffusion chamber mounted on an Eppendorf 1100 M photometer (without the use of fiber optic connections) and linked to Wösthoff gas pumps for preparing gas mixtures (Weber et al., 1976).

In order to assess the O_2 affinities and enthalpies at exactly defined pH values, O_2 equilibria of Hbs suspended in 0.1 M HEPES buffers were measured at 25 and 37 °C, at pH values close to target values (of 6.80, 7.40 and 8.00). P_{50} values at these exact values were then calculated from the Bohr factors (Δlog P_{50}/ΔpH) applicable to each specific Hb sample. The apparent enthalpy of oxygenation was calculated from the temperature sensitivity of P_{50} at constant pH, using the van't Hoff isochore: $\Delta H' = 2.303 \cdot R (\Delta \ln P_{50}) \cdot [(1/T_1 - 1/T_2)]^{-1}$, where R is the gas constant and T_1 and T_2 are the absolute temperatures (298 and 310 K). The use of P_{50} for the derivation of ΔH and the use of these values for the analysis of allosteric effects is justified by the correspondence between P_{50} and P_m (median O_2 tension) values (highly symmetric O_2 equilibrium curves) of bovine and human Hbs (Clementi et al., 1996) (Amiconi et al., 1981).

3. Results and discussion

Human Hb exhibited distinctly higher O_2 affinities (lower P_{50} values and left-shifted O_2 equilibrium curves) compared to bovine Hb at both measured temperatures and in the absence and presence of 0.1 M Cl^- at pH ~7.4 (Fig. 1, Table 2).

3.1. O_2 affinity and its pH and Cl^- sensitivities

The lower intrinsic O_2 affinity in bovine Hb is manifested over a wide pH range (~6.8–8.1) (Figs. 1 & 2, Table 3). Assessed as Δlog P_{50}, the shifts in O_2 affinity induced by 0.10 M Cl^- at pH 7.4 are smaller in bovine Hb (0.34 and 0.27, at 25 and 37 °C, respectively) than in human Hb (0.39 and 0.36, respectively) (Table 3). These results are at variance with the view (Fronticelli et al., 1984) that human and bovine Hbs have similar intrinsic O_2 affinities and that the difference at physiological Cl^- levels is largely accounted for by the different Cl^- sensitivities. The Bohr coefficient of Hbs from both species is lower at 37 than at 25 °C, and increased upon addition of Cl^- ions (Table 3) consistent with the increased protonation of Cl^--binding sites at low pH.

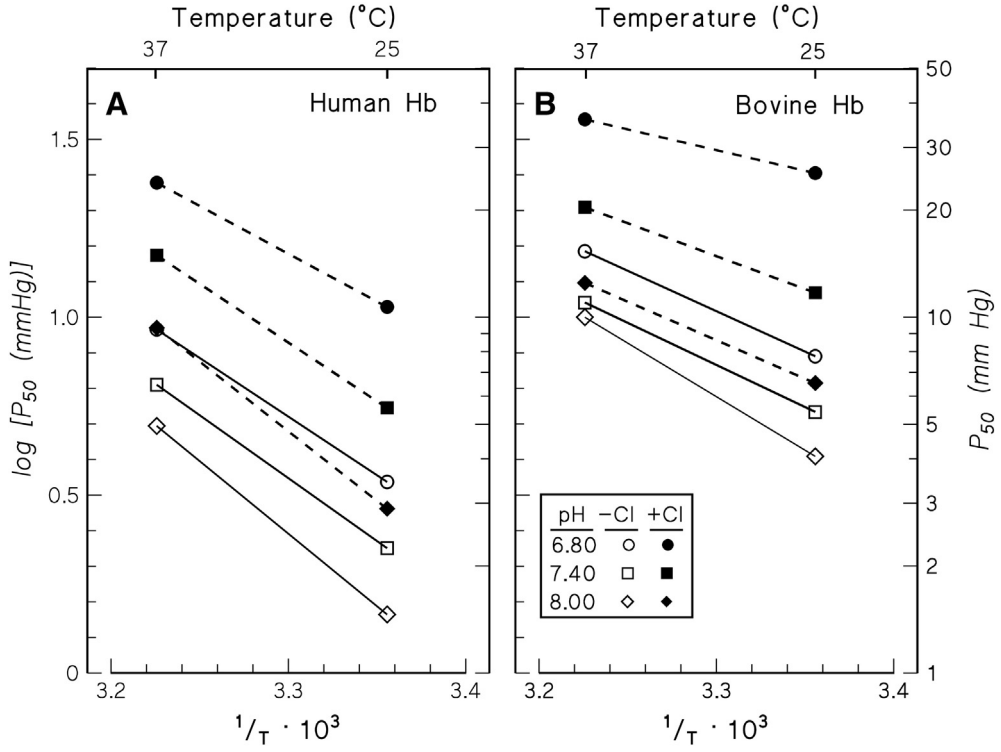

Fig. 3. Van't Hoff isochores of the temperature dependence of P_{50} values of (A) human and (B) bovine Hb at pH 6.80 (circles), 7.40 (squares) and 8.00 (diamonds) in the absence (open symbols) and presence of 0.10 M Cl^- (closed symbols).

3.2. Oxygenation enthalpies

As illustrated by the temperature-induced log P_{50} shifts (difference between solid circles and squares in Fig. 2), bovine Hb exhibits a markedly lower temperature sensitivity than human Hb in the presence of 0.1 M Cl^- ($\Delta H' = -22.9$ and -50.7 kJ·mol^{-1}, respectively, at pH 7.4; Table 3; Fig. 4). However, stripped bovine Hb also shows markedly lower $\Delta H'$-values than human Hb in the absence of Cl^- ($\Delta H'$ at pH 7.4 $= -32.8$ and -55.2 kJ·mol^{-1}, respectively; Table 3) over the entire pH range tested (Fig. 4). This indicates that the majority of the reduction in temperature sensitivity of bovine Hb cannot be ascribed to Cl^- binding. Moreover the lower temperature sensitivity in bovine Hbs cannot be attributed to differences in endothermic dissociation of Bohr protons, given that the Bohr factors of bovine Hb in the absence of Cl^- are smaller than those of stripped human Hb (Table 3, Fig. 3). Additional evidence for minority enthalpic contribution from Cl^--binding to bovine Hb is illustrated by the observation that the difference in ΔH between bovine and human Hbs is only slightly larger in the presence of Cl^- than in its absence (27.8 and 22.4 kJ, respectively at pH 7.4; Table 3). The greater difference in $\Delta H'$ at pH 6.8 than at higher pH (cf. Fig. 4) suggests greater physiological relevance of Cl^--mediated reductions in Hb-O_2 affinity under acidotic spells. The correspondence between the present $\Delta H'$ values and data points from literature (Fig. 4) attests to the reproducibility of the data.

Compared to the $\Delta H'$ value of Cl^--free human Hb at pH 7.4 ($\Delta H' = -55.2$ kJ·mol^{-1}), the $\Delta H'$ values obtained for bovine Hb in the absence and presence of Cl^- (-32.8 and -22.9 kJ·mol^{-1}, respectively) (Table 3), indicate that only ~30% [(22.8–32.8)/(55.2–22.9)] of the reduction in $\Delta H'$ observed in bovine Hb in the presence of Cl^-, is attributable to Cl^--binding, whereas the majority ~70% [(55.2–32.8)/(55.2–22.9)] of this difference is attributable to other causes. A primary cause may be the amino acid exchanges that increase hydrophobicity and lock the N-terminus of bovine β-chains to neighboring segments, thereby mimicking the action of BPG in stabilizing the tense structure (Perutz and Imai, 1980) and increasing the heat of quaternary structural change ($\Delta H^{T\rightarrow R}$). The pH invariance of the difference in enthalpy between human and bovine Hbs in the absence of Cl^- [$\Delta(\Delta H) = 19.7$, 22.4 and 20.4 kJ·mol^{-1} at pH 6.8, 7.4 and 8.0, respectively] is consistent with the non-ionic nature of the hydrophobic interactions.

Although accounting for a relatively small part of the reduced temperature sensitivity of bovine Hb, the Cl^--induced enthalpy change in bovine Hb (-32.8 to $-22.9 = 9.92$ kJ·mol^{-1}) remains twice as large as that for human Hb (-55.2 to $-50.7 = 4.5$ kJ·mol^{-1}) (Table 3). However, this difference may not be directly correlated with Cl^--binding at the 'additional' site (i.e. at Lys8β, Lys76β, His77β), given the evidence that yet other amino groups within the central cavity of bovine Hb (including Lys103β that is at a homologous position to Arg104β of human Hb) may be considered functional Cl^--binding sites (Ueno and Manning, 1992). Regardless, the negative correlation between the overall number of Cl^--binding sites and the temperature sensitivity of Hb-O_2 affinity remains valid, and is supported — and extended to lower vertebrates — by the distinctly higher temperature dependence of O_2 affinity encountered in Andean frog *Telmatobius peruvianus* Hb that lacks the α-chain Cl^--binding site, compared to that found in lowland toad *Xenopus laevis* Hb that has retained this Cl^--binding site (Weber, 2014).

The results of our study indicate that the oxygenation-linked reaction of Cl^- ions accounts for only ~30% of the reduction in the temperature sensitivity of O_2 affinity observed in bovine Hb (compared to that in Cl^--free human Hb), despite its possession of the 'additional' Cl^--binding site, whereas the majority of this reduction may be ascribed to other, structural attributes — notably the greater hydrophobicity in the N-terminal residues of the bovine β chains that increases the heat of conformational change.

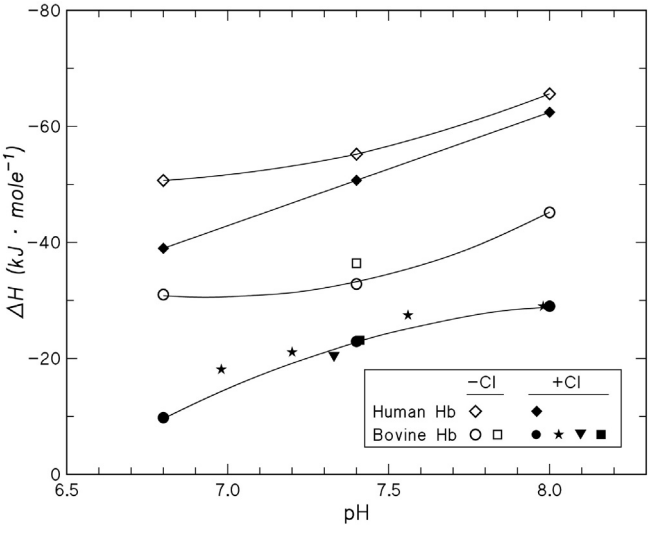

Fig. 4. $\Delta H'$ values for human Hb (diamonds) and bovine Hb (circles) in the absence (open symbols) and presence (solid symbols) of 0.10 M Cl^-, derived from P_{50} measurements at 25 and 37 °C. Also included are values from Razynska et al. (1990) (stars), Clementi et al. (1996) (triangles) and Sasagawa et al. (2006) (squares).

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