Evolution of an extreme hemoglobin phenotype contributed to the sub-Arctic specialization of extinct Steller's sea cows.

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Abstract

The extinct Steller's sea cow (*Hydrodamalis gigas*; †1768) was a whale-sized marine mammal that manifested profound morphological adjustments to exploit the harsh coastal climate of the North Pacific. Yet despite first-hand accounts of their biology, little is known regarding the physiological specializations underlying their evolution to this environment. Here, the adult-expressed hemoglobin (Hb) of this species is shown to harbor a fixed amino acid replacement at an otherwise invariant position (β/δ82Lys→Asn) that alters multiple aspects of Hb function. First, our functional characterization of recombinant sirenian Hb proteins demonstrate that the Hb–O_2 affinity of this sub-Arctic species was less affected by temperature than those of living (sub)tropical sea cows. This phenotype presumably safeguarded O_2 delivery to cool peripheral tissues and largely arises from a reduced intrinsic temperature sensitivity of the *H. gigas* protein owing to the β/δ82Lys→Asn substitution. Additional experiments on *H. gigas* β/δ82Asn→Lys mutant Hb further reveal this exchange renders Steller's sea cow Hb unresponsive to the potent intraerythrocytic allosteric effector 2,3-diphosphoglycerate, a radical modification that is the first documented example of this phenotype among mammals. Notably, β/δ82Lys→Asn moreover underlies the secondary evolution of a reduced blood–O_2 affinity phenotype that would have promoted heightened tissue and maternal/fetal O_2 delivery. Finally, this replacement is shown to increase protein solubility, which is consistent with an increased O_2 carrying capacity within both the adult and pre-natal circulations and may have further contributed to meeting the elevated metabolic (thermoregulatory) requirements and fetal growth rates associated with this species cold adaptation.

Main Text

Introduction

During periods of submergence apnea when atmospheric gas exchange has ceased, the underwater foraging time by mammals is dictated by onboard oxygen stores and the efficiency of their use. Thus, evolutionary increases in oxygen stores, in the form of increased hemoglobin (Hb) and myoglobin—located within erythrocytes and skeletal/cardiac muscle, respectively—are nearly ubiquitous among aquatic species (1). Notable exceptions to this rule are extant sirenians (sea cows), a group of strictly aquatic, (sub)tropical herbivores encompassing only four members, three species of manatee (family Trichechidae) and the dugong, *Dugong dugon* (family Dugongidae). While sirenians are proficient divers, they do not exhibit the strongly elevated body O_2 stores or an enhanced dive reflex common to other lineages of marine mammals (2, 3). Rather, previous work

53 revealed that the sirenian's secondary transition to aquatic life coincided with a rapid evolution of
54 their Hb encoding genes due, in part, to gene conversion events with a neighboring globin
55 pseudogene (4). The resulting high blood–O_2 affinity phenotype presumably allows extant sea cows
56 to maximize O_2 extraction from the lungs during submergence while curbing O_2 offloading, thus
57 lowering overall metabolic intensity and fostering a prolonged breath-hold capacity (4).
58 While the relatively weak thermoregulatory capacity of extant sea cows confine them to
59 (sub)tropical waters (5, 6), fossil evidence and first-hand accounts of the sub-Arctic Steller's sea
60 cow (*Hydrodamalis gigas*) provide insights into the biological and morphological adaptations of this
61 titanic sirenian to the harsh coastal conditions of the North Pacific, where they persisted from the
62 Miocene until their demise in 1768 (7–9). The retrieval of ancient genetic material from museum
63 specimens has since been instrumental in clarifying the phylogenetic affinities and population
64 history of this species, while providing additional details regarding the evolution of key
65 morphological and physiological attributes (4, 10–15) (Fig. 1A). For example, pilot experiments on
66 "resurrected" Steller's sea cow recombinant Hb demonstrated that the Hb–O_2 affinity of this lineage
67 secondarily decreased following their divergence from dugongs in the mid Oligocene (4). While
68 sirenians do not possess the capacity for non-shivering thermogenesis due to pseudogenization of
69 the *UCP1* gene (11), this affinity shift in Steller's sea cow Hb was speculated to have promoted
70 increased O_2 offloading to fuel increased thermogenesis to help cope with exposure to cold sub-
71 Arctic waters. Although the Hb of this species accumulated 11 amino acid replacements since its
72 divergence from the dugong, this decrease in Hb–O_2 affinity was hypothesized to arise from a highly
73 unusual 82Lys→Asn exchange in the chimeric β-type (β/δ) chain (4). Data mined from more recent
74 ancient DNA studies (10, 14) confirms that this substitution was fixed in the last remaining Steller's
75 sea cow population (Fig. 1B), though the specific functional effect(s) of this substitution have not
76 been characterized. This replacement is intriguing not only because β82Lys is invariant among
77 characterized mammalian Hbs, but because human variants with substitutions at this position
78 display profound alterations in both structural and functional properties (16–19). For example, the
79 human Hb Providence (β82Lys→Asn) variant exhibits a decreased inherent Hb–O_2 affinity and
80 markedly reduced sensitivity to the allosteric effectors 2,3-diphosphoglycerate (DPG), Cl^-, and H^+
81 (16, 17, 20–23), which preferentially bind and stabilize the (low O_2 affinity) deoxy-state conformation
82 of the protein. However, opposite to what was suggested for Steller's sea cows (4), this exchange
83 causes the whole blood O_2 affinity of Hb Providence carriers to be noticeably higher than that of
84 the general population (20,23). It is thus unclear if and how the Steller's sea cow β/δ82Lys→Asn
85 replacement underlies the lower Hb–O_2 affinity of this extinct species relative to other sirenians, or
86 whether this attribute arises from one (or more) of the other 10 residue replacements that evolved
87 in this lineage.

The β/δ82Lys→Asn exchange also raises other evolutionary significant questions, as it is predicted alter multiple aspects of Hb function that may lead to antagonistic pleotropic effects. Notably, this exchange may detrimentally increase the effect of temperature on Hb–O_2 binding and release (4). The formation of the weak covalent bond between O_2 and the heme iron requires free energy, thus dictating an inverse relationship between Hb–O_2 affinity and temperature (23). In temperate and Arctic endotherms this inherent attribute of Hb potentially impedes O_2 delivery to the limbs and flukes, which are maintained at substantively lower temperatures to minimize heat loss and hence energy requirements (24). Accordingly, heterothermic mammals generally possess Hbs whose O_2 binding properties are less sensitive to temperature than the Hbs of non-cold-adapted species, thereby maintaining sufficient O_2 offloading in the face of decreasing tissue temperatures. This reduction in thermal sensitivity (the overall enthalpy of oxygenation, ΔH'), appears to predominantly arise from an increased interaction between allosteric effectors and the Hb moiety (an exothermic process), which releases additional heat to assist with deoxygenation (23). Hence, the *H. gigas* β/δ82Lys→Asn replacement, which deletes integral binding sites for the heterotropic ligands Cl^- and DPG (17), is puzzling in that it is expected to maladaptively increase the effect of temperature on O_2 uptake and release in the blood of the sub-Arctic Steller's sea cow.

Taken together, it remains unknown whether the *H. gigas* β/δ82Lys→Asn residue exchange contributed adaptively the species biology or is instead linked to small population sizes (e.g., genetic drift) over the past half million years (10, 14). To unravel the combined effects of evolved amino acid replacements on hemoglobin function in relation to the extreme thermal biology of the extinct Steller's sea cow, we synthesized recombinant Hb proteins of this species together with those of the dugong (*Dugong dugon*) and Florida manatee (*Trichechus manatus latirostris*), and measured their O_2 binding properties, relative solubility, responses to allosteric effectors, and thermal sensitivities. We also synthesized a *H. gigas* β/δ82Asn→Lys Hb mutant to assess the specific effects of this exchange, together with the Hb of the last common ancestor ('ancestral dugongid') shared between the dugong and Steller's sea cow (Fig. 1A) in order to assess the directionality of evolved physicochemical changes in Hb function.

Results and Discussion

O_2 Affinity of Sirenian Hbs. Measured O_2-equilibrium curves of the five examined Hbs revealed marked differences in intrinsic O_2 affinity (Figs. 2A, S1, S2 and Table S1). In the absence of allosteric effectors (pH 7.2, 37°C), the P_{50} (the O_2 tension resulting in 50% Hb–O_2 saturation) of Steller's sea cow Hb (P_{50} = 8.8 mmHg) is ~2 times higher than that of dugong (3.5 mmHg), ancestral dugongid (4.3 mmHg), and manatee (5.4 mmHg) Hbs under the same conditions (Figs. 2A, S1 and Table S1). Site directed mutagenesis experiments reveal that the increased intrinsic P_{50} of Steller's

124 sea cow Hb predominantly arises from the β/δ82Asn substitution, as the β/δ82Asn→Lys mutant
125 exhibits an intrinsic P_{50} similar to that of the ancestral dugongid (Fig. 2A, S2). Of note, the O_2 affinity
126 of dugong, ancestral dugongid, and manatee Hbs was reduced in the presence of Cl^- and DPG (P_{50}
127 = 10.2, 9.9, and 10.9 mmHg, respectively) by a similar degree to that of Asian elephant Hb (25).
128 This finding extends previous studies conducted on sirenian Hbs (4, 26, 27), and reveals that the
129 high O_2 affinity of dugong and manatee blood is not attributable to decreased allosteric effector
130 sensitivity. Conversely, Steller's sea cow Hb was shown to be markedly less responsive to allosteric
131 effectors, as only a moderate reduction in O_2–affinity was observed in the presence of Cl^- and DPG
132 (P_{50} = 14.3 mmHg). When the effects of these allosteric effectors were measured individually,
133 Steller's sea cow Hb exhibits markedly reduced DPG, Cl^-, and H^+ (Bohr) effects relative to those of
134 the ancestral dugongid and β/δ82Asn→Lys mutant (Fig. 2C-E; Table S1). These data confirm that
135 a high intrinsic (i.e. in the absence of allosteric effectors) Hb–O_2 affinity is an ancient sirenian trait
136 that likely aided the transition of the group to the aquatic environment, and that Hb–O_2 (and hence
137 whole blood) affinity was secondarily reduced in the Steller's sea cow lineage (4). This latter finding
138 contrasts with allometric expectations for mammals—whereby blood O_2 affinity and body mass are
139 inversely correlated (28)—and thus further suggests this modification serves an adaptive function.
140 The single most distinct feature of *H. gigas* Hb is the lack of a discernable effect of DPG
141 on P_{50} ($\Delta\log P_{50}^{(0.2\text{ mM DPG} - 0.0\text{ mM DPG})}$ = 0.02 at 37°C and pH 7.2; Fig. 2C and Table S1), relative to
142 the Hbs of the ancestral dugongid (0.27) and the extant manatee and dugong (0.14 and 0.30,
143 respectively). This intracellular effector generally occurs in equimolar concentrations to Hb (29) and
144 strongly decreases the O_2 affinity of most mammalian Hbs via direct electrostatic interactions with
145 β2His and β82Lys (Fig. 3A), together with potential water-mediated interactions with β143His and
146 the α-NH_2 group of 1Val of the β_2 chain (30). However, unlike the other (ionizable) residues whose
147 ability to interact with DPG is highly pH dependent, β82Lys is strongly cationic and thus is able to
148 bind DPG across the entire physiological pH range. Presumably arising from this indispensable role
149 in DPG binding, this residue is uniformly conserved in mammalian Hbs (Fig. 3A), with the exception
150 of several heterozygous adult human HbA carriers with substitutions at this position (18, 19, 31).
151 Given that none of the other six β/δ-chain replacements that evolved on the Steller's sea cow
152 branch (Figs. 3B and S3) are implicated in DPG binding, the deletion of the integral DPG binding
153 site at β/δ82 in Steller's sea cow Hb is consistent with its inability to bind DPG (Fig. 3B). This
154 conclusion is further supported by our measurements on the Steller's sea cow β/δ82Asn→Lys
155 mutant, which show that reversion to the ancestral state restores the DPG effect to the same level
156 observed in ancestral dugongid Hb (Fig. 2C). Notably, and despite possessing the identical DPG
157 binding site residues, the *H. gigas* protein exhibits a distinctly lower DPG effect than Hb Providence
158 (0.08; 17,20). The stronger reduction of DPG sensitivity in the former thus implicates an epistatic
159 contribution from other amino acids in the vicinity of the DPG pocket. Importantly, the Lys→Asn

160 replacement in the DPG binding pocket causes the O_2 affinity of human HbA to increase in the
161 presence of allosteric cofactors (20,23), whereas results presented in Fig. 2A show Steller's sea
162 cow Hb–O_2 affinity is reduced relative to its ancestors carrying β/δ82Lys under all test conditions.
163 This observation highlights a growing body of research indicating that both the direction and overall
164 phenotypic effect of specific amino acid substitutions may be conditional on the genetic background
165 in which they occur (32).
166 The insensitivity of *H. gigas* Hb to DPG it also notable as it would have markedly reduced
167 their capacity to modulate blood–O_2 affinity *in vivo* (e.g., seasonally), and is the first demonstrated
168 example of a genuine DPG insensitive Hb phenotype among mammals. While eastern moles
169 (*Scalopus aquaticus*) are a possible exception (33), feliformid carnivores, ruminants, and two
170 species of lemurs have also traditionally been placed in the 'DPG insensitive' category (29) despite
171 the fact their Hbs are moderately responsive to DPG in the absence of Cl^- (17, 34–36). Nonetheless,
172 red blood cell DPG concentrations of species with suppressed DPG sensitivities are markedly
173 reduced relative to mammals whose Hb–O_2 affinity is regulated by DPG (<0.1-1.0 mM vs. 4-10 mM,
174 respectively;(29)). While the potential benefits of a low DPG sensitivity phenotype have been
175 debated (33, 37) approximately 20% of glucose uptake by human erythrocytes is diverted to DPG
176 synthesis (38), thereby bypassing production of both ATP molecules generated via anaerobic
177 glycolysis (29, 39, 40). Accordingly, since each molecule of DPG produced comes at the expense
178 of producing an ATP molecule, the probability that Steller's sea cow blood similarly contained low
179 levels of this organophosphate is high.
180
181 **Hb Solubility.** Ectopic expression of human Hb Providence β82Asp mutants in *E. coli* has been
182 shown to improve soluble protein production by 47-116% relative to the expression of human Hb
183 variants not carrying this substitution (22). Consistent with this observation, we found that Steller's
184 sea cow Hb is more soluble than those of other sirenians and the engineered *H. gigas*
185 β/δ82Asn→Lys mutant (Fig. 2F and S4). While the precise mechanism underlying this
186 phenomenon is unknown, Hb Providence variants have been shown to strongly reduce irreversible
187 oxidative damage of nearby β93Cys that initiates Hb denaturation (16, 41, 42). These β82
188 replacements thereby presumably decrease the rate of Hb turnover and increase the half-life of the
189 protein (42), and may be the underlying cause of mild polycythemia in humans carrying this
190 substitution (20, 31). Blood with an elevated O_2 carrying capacity is typical of most mammalian
191 divers, where it increases onboard O_2 stores and extends dive times (1), but is not observed in
192 extant sirenians (27, 43, 44). However, solubility driven increases in blood Hb levels resulting from
193 the β82Lys→Asn exchange would have allowed Steller's sea cows to maintain an elevated rate of
194 tissue O_2 delivery to meet their metabolic demands during extended underwater foraging intervals.
195 Although this species was presumably unable to completely submerge (9, 45), this conjecture is

196 corroborated by Steller's account that, "*they keep their heads always under water* [foraging]*, without*
197 *regard to life and safety*" (9).
198
199 **Thermal Sensitivity.** The invariant energy change associated with forming the weak covalent bond
200 between O_2 and the heme iron (i.e. the enthalpy of heme oxygenation; ΔH^{O2}) is exothermic (-59 kJ
201 mol^{-1} O_2) (46), and only moderately opposed by the endothermic solubilization of O_2 (ΔH^{H2O}; 12.55
202 kJ mol^{-1} O_2), resulting in an inverse relationship between temperature and Hb–O_2 affinity. However,
203 the heat of the T→R conformational change ($\Delta H^{T\rightarrow R}$), and the oxygenation-linked dissociation of H^+
204 (ΔH^{H+}), Cl^- (ΔH^{Cl-}), and DPG (ΔH^{DPG}) may offset this relationship, such that the overall enthalpy of
205 Hb oxygenation (ΔH') can become greatly minimized or even endothermic (23, 47). By facilitating
206 adequate oxygenation of cool peripheral tissues, Hbs with numerically low ΔH' values are
207 interpreted to be adaptive for cold-tolerant, regionally heterothermic mammals. The evolution of
208 this phenotype has predominantly been attributed to the formation of additional heterotropic ligand
209 binding sites on the protein moiety, as has previously been demonstrated for the woolly mammoth,
210 *Mammuthus primigenius* (23, 25). Conversely, Steller's sea cow Hb lacks heterotropic binding of
211 DPG and displays lower Bohr (H^+) and Cl^- effects than all other sirenian Hbs measured (Fig. 2;
212 Table S1), yet exhibits a ΔH' value (-18.8 kJ mol^{-1} O_2; Fig. 2B) that is close to that of mammoth Hb
213 (-17.2 kJ mol^{-1} O_2) (25). This striking convergence largely arises from the inherently low ΔH of
214 stripped Steller's sea cow Hb (-34.2 kJ mol^{-1} O_2) relative to dugong, ancestral dugongid, and
215 manatee Hbs (range: -50.1 to -58.6 kJ mol^{-1} O_2) at pH 7.8—where oxygenation-linked binding of
216 protons is minimal—and indicates that structural differences modifying the T→R transition largely
217 underlie the low thermal sensitivity of *H. gigas* Hb. Recent studies have shown that a large positive
218 $\Delta H^{T\rightarrow R}$ may similarly contribute to the low ΔH' of deer mouse, cow, shrew, and mole Hbs (33, 48–
219 51) suggesting that this mechanism of temperature adaptation may be more widespread than
220 previously appreciated. Our experiments with the Steller's sea cow β82Asn→Lys mutant further
221 implicate substitutions at this position as a key factor underlying the inherently low ΔH of the protein,
222 as this modified protein displays a greatly increased ΔH in the absence of allosteric effectors
223 relative to the wild-type *Hydrodamalis* protein (Fig. 2B). Interestingly, despite these inherent ΔH
224 differences between the mutant and wild-type Steller's sea cow Hbs, their ΔH' values are
225 indistinguishable in the presence of allosteric effectors (Fig. 2B). These data suggest that β82Asn
226 uncouples thermal sensitivity from DPG concentration, permanently conferring the *H. gigas* protein
227 with a numerically low ΔH' by genetic assimilation while simultaneously eliminating the energetic
228 cost of DPG production within the red blood cells.
229
230

Paleophysiology of Steller's sea cows. The posthumously published behavioral and anatomical accounts of the last remaining *H. gigas* population by naturalist Georg Wilhelm Steller while stranded on Bering Island (55°N, 166°E) in 1741/1742 provide a rich tapestry to interpret the paleophysiology of this colossal marine herbivore. For example, their protective thick bark-like hide and extensive blubber layer give credence to the extreme nature of their shallow rocky and (during winter) ice strewn habitat (10). Here, as Steller (9) remarked, they used fingerless, bristle-covered forelimbs for support and to shear "*algae and seagrasses from the rocks*", which they masticated "*not with teeth, which they lack altogether, but with*" large, ridged keratinized pads located on the upper palate and lower mandible. Although they became visibly thin during winter when "*their spinous processes can be seen*", Steller (9) noted that "*(t)hese animals are very voracious, and eat incessantly*" such that their stupendous stomach ("*6 feet* [1.8 m] *long, 5 feet* [1.5 m] *wide*") and enormous intestines—which measured a remarkable 5,958 inches (~151.5 m) from esophagus to anus, equivalent to "*20½ times as long as the whole animal*"—are constantly "*stuffed with food and seaweed*". The proportionally larger gut (45) is consistent with Steller's sea cow's higher energetic requirements relative to extant manatees, which, owing to their low metabolic intensity become cold stressed and die if chronically exposed to water temperatures below 15°C (52). The reduction in insulative blubber thickness during the winter months would likely have compounded the rate of heat loss to sub-zero degree Celsius air and water, though may have been compensated for by arteriovenous anastomoses that regulated blood flow to the skin, and by countercurrent rete supplying the flippers and tail flukes, the latter of which are well developed in manatees and presumably other sirenians (53, 54). These structures conserve thermal energy by promoting profound cooling at the appendages and periphery (26), and presumably underlie the low thermal dependence of Steller's sea cow Hb relative to those of extant sea cows.

Reductions in blood–O_2 affinity accompanying the *H. gigas* β/δ82Lys→Asn substitution is expected to have further augmented tissue O_2 delivery without tangible effects on lung O_2 uptake, thereby helping to fuel thermogenesis and maintain a stable core temperature. The latter was presumably supplemented by a substantive heat increment arising from fermentation and other post-prandial processes (54). Although the attendant increase in the rate of O_2 consumption would have mandated a reduction in breath-hold endurance—likely reflecting the relatively short submergence times (4 to 5 minutes) observed by Steller (9)—our results suggests this may be been partially counteracted by an elevated blood–O_2 carrying capacity that was potentially coupled to a greater lung volume (45). Underwater foraging times were presumably further defended by key components of the dive reflex, namely bradycardia and peripheral vasoconstriction. Indeed, Steller inadvertently was the first to (indirectly) describe this phenomenon as he observed his crew hunting the animals with spears and knives, "*the blood from the wounded back spurted up like a fountain.*

As long as he kept his head under water the blood did not flow out, but as soon as he raised his head to breathe the blood leaped forth anew".

A final compelling aspect of Steller's sea cow evolution was their immense increase in size—up to 11,000 kg in mass and 10 m in length—relative to extant sirenians (7). While Steller does not provide measurements of "*their tender little offspring*", Gerhard Friedrich Müller, who edited Steller's manuscript prior to publication, noted calves "*weighed 1200 pounds* [544 kg] *and upwards*" (55). This value is ~10 to 50 times the mass of new born manatees and dugongs (~10-50 kg) (56) and is suggestive of rapid prenatal growth during the ~1 year gestational period indicated by Steller (9). Given that all mammals except ruminants and anthropoid primates express the same Hb isoform during both the fetal and post-natal stages of development (57), the apparently unique DPG insensitive phenotype of *H. gigas* is particularly notable in this regard owing to its potential impact on maternal/fetal O_2 exchange. Presumably to assist in this process, fetal blood cells express only trace amounts of DPG and hence the O_2 affinity of fetal blood is elevated relative to that of the maternal circulation in the vast majority of mammalian species (29, 57). By contrast, and owing to the inability of Steller's sea cows Hb to respond to DPG in either the fetal or adult circulations, this species would represent a rare example (feloids and eastern moles are others) in which fetal and maternal blood have the same (relatively low) O_2 affinity. However, placental O_2 delivery in these species will still be defended by the well-known double Bohr effect, whereby CO_2 transport from the fetal to maternal circulation increases blood O_2 affinity in the former while lowering it in the latter (57). More importantly, the evolved reduction in blood O_2 affinity of Steller's sea cows would have stipulated that equilibrium between umbilical and uterine blood was reached at a higher PO_2, a condition that is expected to substantially improve O_2 delivery to the fetal circulation (57). The lower fetal blood O_2 affinity and concomitant higher fetal blood-to-tissue PO_2 gradients are further expected to have augmented O_2 delivery to the developing tissues of this species. As such, these attributes, together with increases in Hb solubility/reduced susceptibility to oxidative damage arising from β/δ82Lys→Asn that conceivably also elevated the O_2 carrying capacity of fetal blood, may have been important contributors to the enhanced fetal growth rate of these immense sirenians. The resulting increase in thermal inertia and relatively low surface-area-to-volume ratio following birth, together with an adaptively reduced Hb thermal sensitivity and thick a 'bark-like' skin arising from inactivation of lipoxygenase genes (10), were presumably central components of Steller's sea cows successful exploitation of the harsh sub-Arctic marine environments of the North Pacific.

Materials and Methods

302 **Sequence Collection and Analyses.** The adult-expressed Hb genes (HBA and HBB/HBD) of the
303 Florida manatee, dugong, and Steller's sea cow, and the most recent common ancestor shared by
304 Steller's sea cow and the dugong ('ancestral dugongid') have previously been determined (4). As
305 the *H. gigas* β/δ82Lys→Asn exchange is not known to occur in any living species, we mined
306 recently deposited genomes for 13 additional Steller's sea cows (PRJNA484555, PRJEB43951) to
307 test for the prevalence of this replacement in the population. Briefly, we first searched SRA files of
308 each specimen using the megablast function against a previously determined *H. gigas HBB/HBD*
309 gene sequence (GenBank accession #: MK562081). All hits were then downloaded, trimmed of
310 adapters and low-quality regions using BBDuk (Joint Genome Institute), and assembled to *H. gigas*
311 *HBB/HBD* using Geneious Prime 2019 software (Biomatters Ltd, Auckland, New Zealand).
312 Assemblies generated using genome reads that were not pre-treated with uracil-DNA glycosylase
313 and endonuclease VIII to reduce C→T and G→A damage artifacts (10) were examined to ensure
314 these deamination artifacts did not affect the consensus sequences.
315
316 **Construction of Recombinant Hb Expression Vectors.** Coding sequences for the above four
317 Hb proteins were optimized for expression in *E. coli* and synthesized *in vitro* by GenScript
318 (Piscataway, NJ). The resulting gene cassettes were digested with restriction enzymes and
319 tandemly ligated into a custom Hb expression vector (58) using a New England BioLabs Quick
320 Ligation Kit as recommended by the manufacturer. Chemically competent JM109 (DE3) *E. coli*
321 (Promega) were prepared using a Z-Competent *E. coli* Transformation Kit and Buffer Set (Zymo
322 Research). We also prepared a *H. gigas* β/δ82Asn→Lys Hb mutant via site-directed mutagenesis
323 on the Steller's sea cow Hb expression vector by whole plasmid amplification using mutagenic
324 primers and Phusion High-Fidelity DNA Polymerase (New England BioLabs), phosphorylation with
325 T4 Polynucleotide Kinase (New England BioLabs), and circularization with an NEB Quick Ligation
326 Kit (New England BioLabs). All site-directed mutagenesis steps were performed using the
327 manufacture's recommended protocol.
328 Hb expression vectors were co-transformed into JM109 (DE3) chemically competent *E.*
329 *coli* alongside a plasmid expressing methionine aminopeptidase (58), plated on LB agar containing
330 ampicillin (100 µg/ml) and kanamycin (50 µg/ml), and incubated for 16 hours at 37°C. A single
331 colony from each transformation was cultured in 50 ml of 2xYT broth for 16 hours at 37°C while
332 shaking at 200 rpm. Post incubation, 5 ml of the culture was pelleted by centrifugation and plasmid
333 DNA was isolated using a GeneJET Plasmid Miniprep Kit (Thermo Scientific). The plasmid
334 sequence was verified using BigDye 3.1 sequencing chemistry and an ABI3130 Genetic Analyzer.
335 The remainder of the culture was supplemented with glycerol to a final concentration of 10%,
336 divided into 25 ml aliquots and stored at -80°C until needed for expression.
337

338 **Expression and Purification of Recombinant Hb.** 25 ml of starter culture (above) was added to
339 1250 ml of TB media containing ampicillin (100 µg/ul) and kanamycin (50 µg/ul) and distributed
340 evenly amongst five 1 L Erlenmeyer flasks. Cultures were grown at 37°C while shaking at 200 rpm
341 until the absorbance at 600 nm reached 0.6-0.8. Hb expression was induced by supplementing the
342 media with 0.2 mM isopropyl β-D-1-thiogalactopyranoside, 50 µg/ml of hemin and 20 g/L of glucose
343 and the culture was incubated at 28°C for 16 hours while shaking at 200 rpm. Once expression had
344 completed, dissolved O_2 was removed by adding sodium dithionite (1 mg/ml) to the culture, which
345 was promptly saturated with CO for 15 minutes. Bacterial cells were then pelleted by centrifugation
346 and Hb purified by ion exchange chromatography according to Natarajan et al. (58)
347 It should be noted that the β82Asn residue of human Hb Providence is relatively uncommon
348 in that it slowly undergoes post-translational deamidation *in vivo* to form aspartic acid, with the latter
349 residue (β82Asp) comprising ~67-75% in mature mixed blood (20, 59). While it is unknown to what
350 degree *H. gigas* β/δ82Asn was catalyzed into Asp in nature, O_2 binding data (see below) of this
351 species was collected from freshly purified recombinant samples for which only one peak—
352 presumably β/δ82Asn—was resolved during chromatography (data not shown). Additionally, this
353 reaction is dependent on the local protein environment (60), specifically two nearby residues
354 β143His and β83Gly (59). Importantly, the latter residue was replaced by β/δ83Ser on the Steller's
355 sea cow branch (Figs. 1B and 3B), which is expected to slow (but not stop) the rate of deamidation
356 (60). Regardless, since the two Hb Providence isoforms have similar O_2 affinities and functional
357 properties (17, 20, 21) it is unlikely that presence of β/δ82Asp in Steller's sea cow blood would
358 meaningfully alter the results and interpretations presented herein.
359
360 **Functional Analyses of Hbs.** O_2-equillibrium curves for Hb solutions (0.25 mM heme in 0.1 M
361 HEPES buffers) were measured at 25 and 37°C using the thin film technique (61). Hb solutions
362 varied in their pH (6.9, 7.4, and 7.9), chloride concentration (0 or 0.1 M KCl), and organic phosphate
363 concentration (0 or 2-fold molar excess of DPG relative to tetrameric Hb concentrations) in order
364 to test the influence of these cofactors on Hb function. Each Hb solution was sequentially
365 equilibrated with three to five different oxygen tensions (PO_2) that result in Hb–O_2 saturations
366 between 30 to 70%. Hill plots (log[fractional saturation/[1-fractional saturation]] vs logPO_2)
367 constructed from these measurements were used to determine the PO_2 (P_{50}) and the cooperativity
368 coefficient (n_{50}) at half saturation, from the χ-intercept and slope of these plots, respectively. By this
369 method, the r^2 determination coefficients for the fitted curves exceed 0.995 and the standard errors
370 (SEM) are less than 3% of the P_{50} and n_{50} values (51). P_{50} values at 25 and 37°C were used to
371 assess the thermal sensitivity of sirenian Hbs by calculating the apparent enthalpy of oxygenation
372 using the van't Hoff isochore:
373

$$\Delta H = 2.303R \times \Delta \log P_{50} \times (1/T_1 - 1/T_2)^{-1}$$

where R is the universal gas constant and T_1 and T_2 are the absolute temperatures (°K) at which the P_{50} values were measured. All ΔH values were corrected for the heat of O_2 solubilization (12.55 kJ mol^{-1} O_2).

Solubility assay. Ammonium sulfate was added to Hb solutions (0.074±0.004 mM Hb_4) to generate final concentrations that ranged from 0 to 3.5 M. These solutions were incubated for 60 minutes at 37°C and the remaining soluble Hb was measured via Drabkin's reagent, according to the manufacturer's instructions (Sigma-Aldrich).

Homology modelling. To assess the structural effect of the *H. gigas* specific mutations on the DPG binding site, homology models of ancestral dugongid and Steller's sea cow Hb were constructed using the SWISS-MODEL server (62) using the three-dimensional human deoxy structure with DPG bound (PDB: 1B86) as a template (30). The sequence conservation of amino acid residues implicated in DPG binding were calculated by the ConSurf Server (63) from a subsample of 51 mammalian beta-type hemoglobin chains downloaded from GenBank (Table S2). Homology models were visualized with UCSF Chimera (64).

Acknowledgments

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561 **Figures and Tables**
562

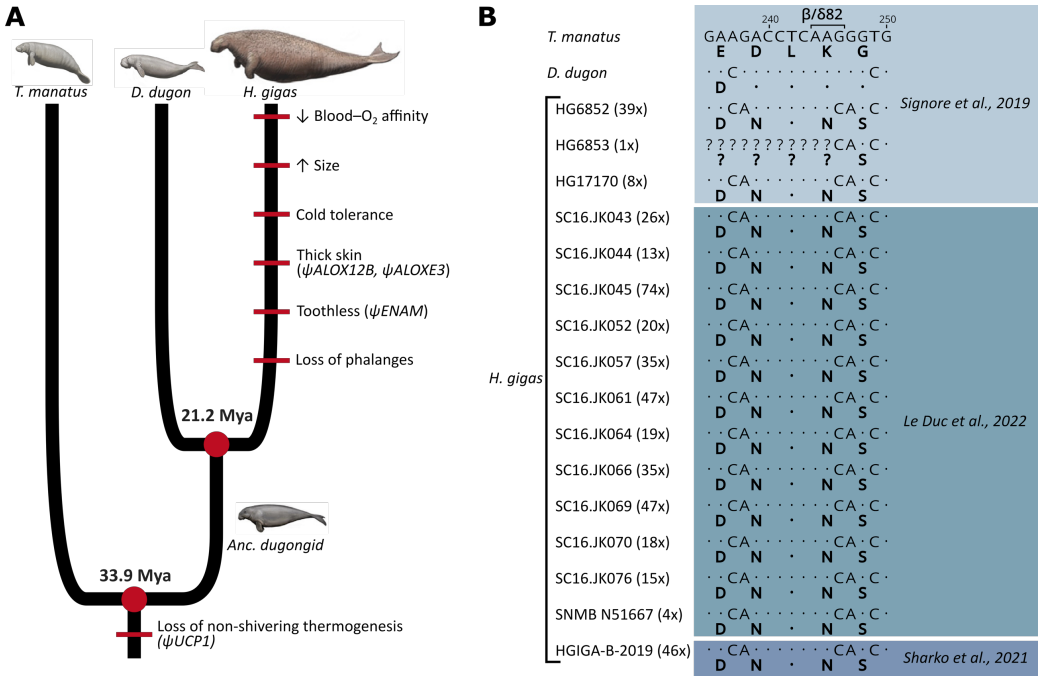

Figure 1. Evolution of notable morphological and genetic attributes within Sirenia. A) Phenotypic innovations contributing to the unique biology of Steller's sea cows are mapped along the sirenian phylogeny (red bars) with the underlying genetic causes shown in brackets where known. Note that the red bars do not represent the dating of these traits and that their placement order is arbitrary. Divergence dates in millions of years (Mya) are based on Heritage and Seiffert (65). The ancestral dugongid (Anc. dugongid) is represented by a late-Oligocene *Metaxytherium spp*. Images by Carl Buell, and are used with permission. B) Partial nucleotide alignment of the sirenian β/δ-globin gene encoding the central region of the 2,3-diphosphoglycerate binding pocket of hemoglobin; corresponding amino acid residues (bolded) are provided below each sequence. The G→C nucleotide mutation underlying the otherwise invariant amino acid substitution (β/δ82Lys→Asn; K→N) of Steller's sea cow hemoglobin is apparent in all 16 individuals for which sequence data is available. Values in brackets next to each *H. gigas* specimen represent the depth of sequence coverage for this nucleotide. Dots represent sequence identity with *T. manatus*.

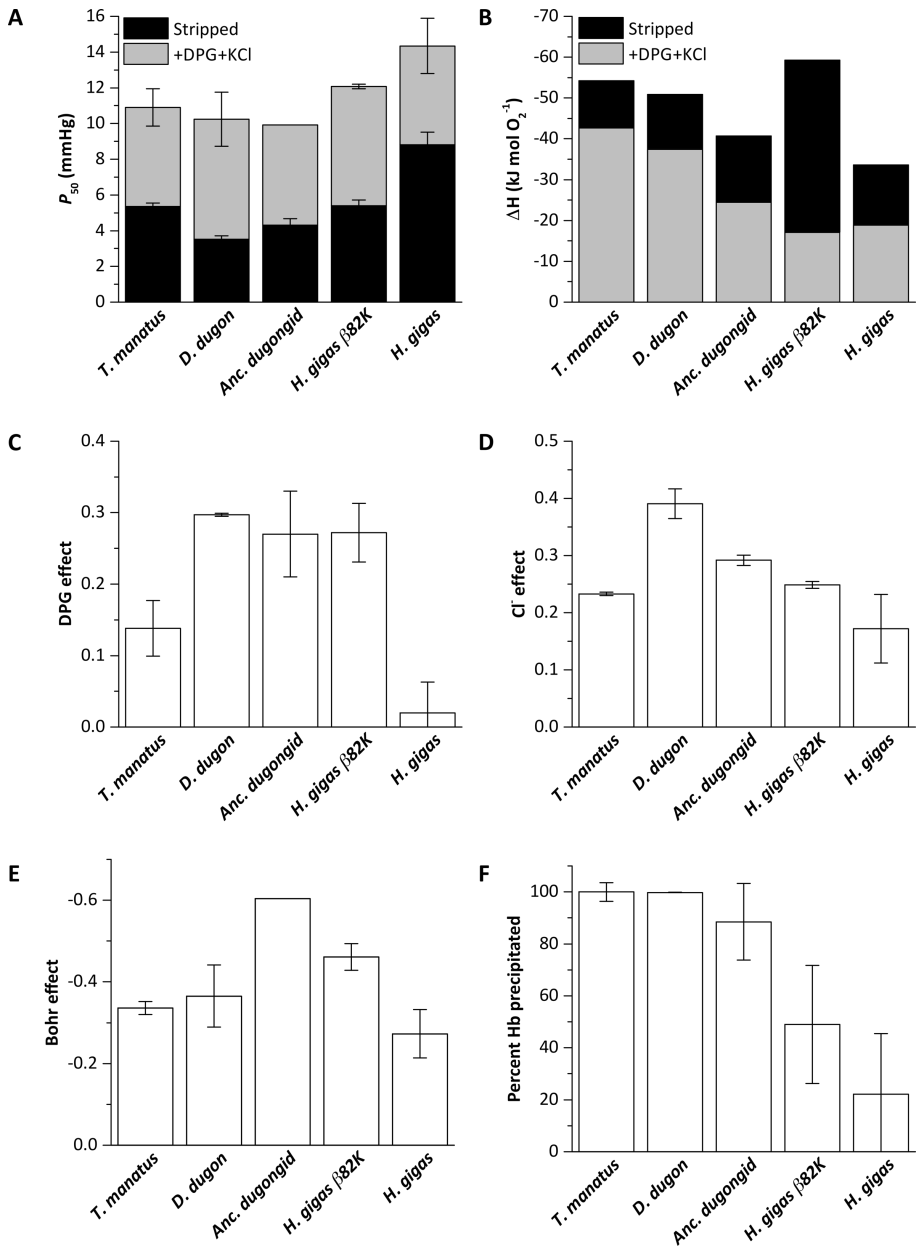

Figure 2. Biochemical properties of hemoglobins (Hbs) from manatee (*T. manatus*), dugong (*D. dugon*), ancestral dugongid (Anc. dugongid), Steller's sea cow β/δ82Asn→Lys mutant (*H. gigas* β82K), and wild-type Steller's sea cow (*H. gigas*). All values were measured at 37°C and corrected to pH 7.2, unless otherwise noted. A) Oxygen tensions at half O_2 saturation (P_{50}) in the absence (stripped) and presence of allosteric cofactors (0.2 mM 2,3-diphosphoglycerate (DPG) and 0.1 M KCl). B) The enthalpy of oxygenation (ΔH) between 25 and 37°C in stripped Hb and in the presence of allosteric cofactors (0.2 mM DPG and 0.1 M KCl). C) The effect of DPG on sirenian Hbs determined from $\log P_{50}^{(0.2\ mM\ DPG)} - \log P_{50}^{(stripped)}$. D) The effect of chloride on sirenian Hbs determined from $\log P_{50}^{(0.1\ M\ KCl)} - \log P_{50}^{(stripped)}$. E) The Bohr effect of sirenian Hbs in the presence of allosteric cofactors (0.2 mM DPG and 0.1 M KCl), as calculated from $\Delta \log P_{50}/\Delta pH$ over the pH range 6.9 and 7.8. F). The relative solubility of sirenian Hbs is denoted by the percentage of Hb protein precipitated by the addition of 3 M ammonium sulfate.

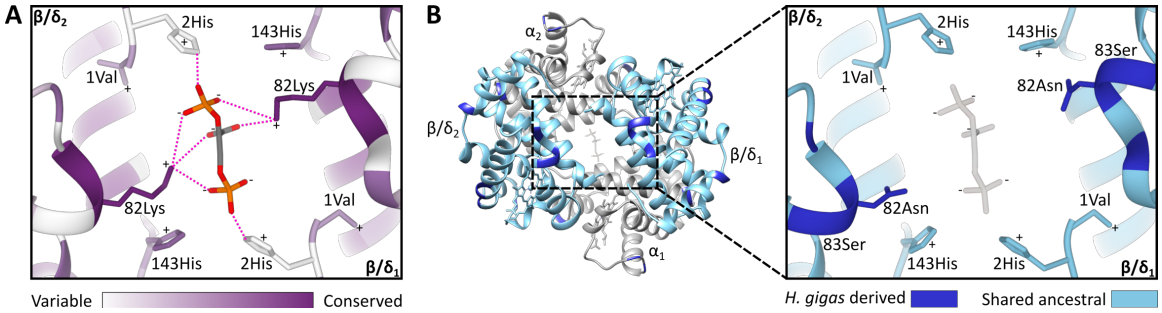

Figure 3. Homology models of the DPG binding site in ancestral dugongid and Steller's sea cow (*H. gigas*) hemoglobin. A) Model of the ancestral dugongid DPG binding site. Amino acids are colored according to the degree of sequence conservation. Notably, β/δ82Lys shows the highest level of sequence conservation, as it is able to bind to multiple sites on the DPG molecule (indicated by dashed pink lines), whereas β/δ2His is only able to directly interact with DPG in the protonated state. B) Model of Steller's sea cow hemoglobin (left) and a close-up of the DPG binding pocket (right). Dark blue colored residues represent the 11 *H. gigas* specific substitutions, while those in light blue denote the ancestral state. Homology modelling illustrates how the replacement of β/δ82Lys with neutral Asn inhibits DPG binding to the hemoglobin molecule.