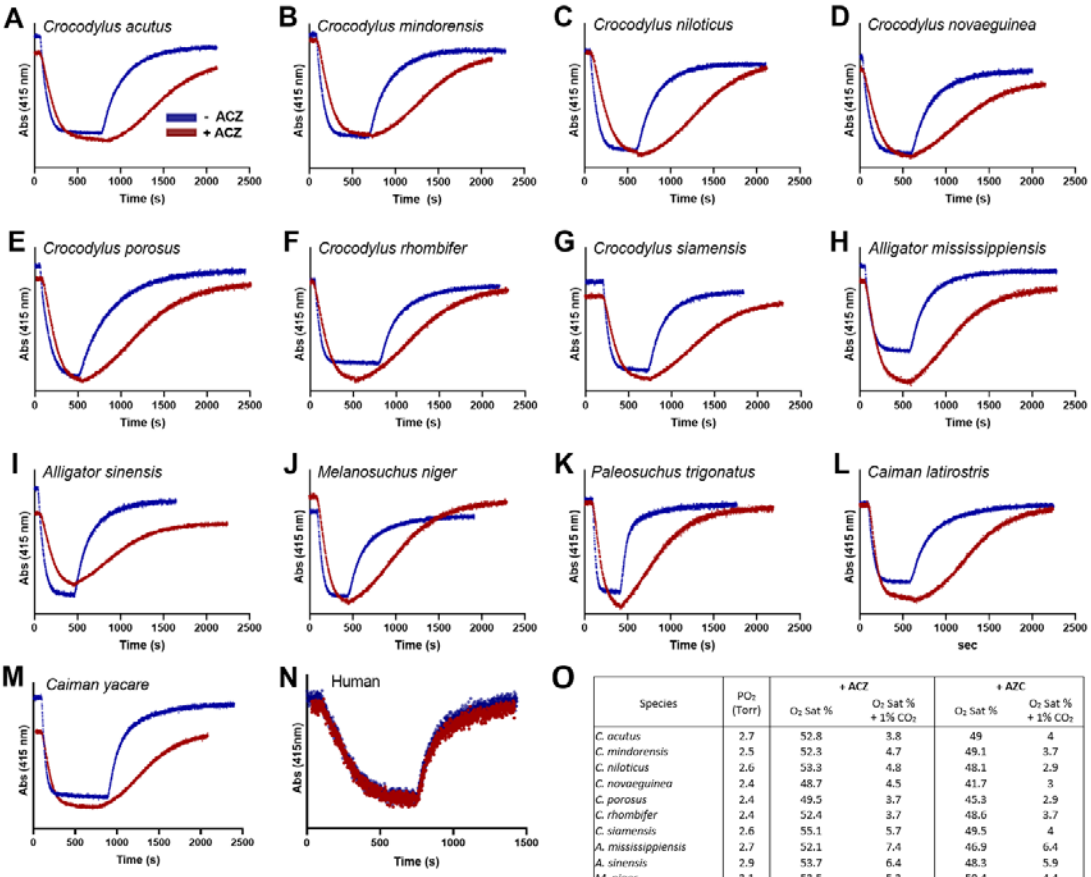

O

Species	PO_2 (Torr)	+ ACZ		+ AZC	
		O_2 Sat %	O_2 Sat % + 1% CO_2	O_2 Sat %	O_2 Sat % + 1% CO_2
C. acutus	2.7	52.8	3.8	49	4
C. mindorensis	2.5	52.3	4.7	49.1	3.7
C. niloticus	2.6	53.3	4.8	48.1	2.9
C. novaeguinea	2.4	48.7	4.5	41.7	3
C. porosus	2.4	49.5	3.7	45.3	2.9
C. rhombifer	2.4	52.4	3.7	48.6	3.7
C. siamensis	2.6	55.1	5.7	49.5	4
A. mississippiensis	2.7	52.1	7.4	46.9	6.4
A. sinensis	2.9	53.7	6.4	48.3	5.9
M. niger	3.1	53.5	5.3	50.4	4.4
P. trigonatus	4.8	51.7	8.5	50	7.8
C. latirostris	3.1	53.9	6.1	50.8	5.2
C. yacare	3.1	52.6	7	49.6	7.2
H. sapiens	2.3	51.3	37.2	47.2	33.6

Fig. S1. Absorbance traces showing the CO_2-mediated decrease in Hb-O_2 saturation in 13 species of crocodilians and in human. As shown in Figure 1, after equilibration with a PO_2 close to the P_{50} (~3.1 torr), 1% CO_2 (~7.5 torr) was added to the gas mixture equilibrating the samples and the decrease in absorbance was followed over time at 415 nm using the diffusion chamber technique. Absorbance traces are shown in the absence (blue trace) and presence (red trace) of the CA inhibitor acetazolamide (ACZ) for: A) *Crocodylus acutus,* B) *Crocodylus mindorensis,* C) *Crocodylus niloticus,* D) *Crocodylus novaeguinea,* E) *Crocodylus porosus,* F) *Crocodylus rhombifer,* G) *Crocodylus siamensis,* H) *Alligator mississippiensis,* I) *Alligator sinensis,* J) *Melanosuchus niger,* K) *Paleosuchus trigonatus,* L) *Caiman latirostris* and, M) *Caiman yacare*, N) Human. The corresponding % O_2 saturation before and after addition of 1% CO_2 for each species is summarized in panel O.

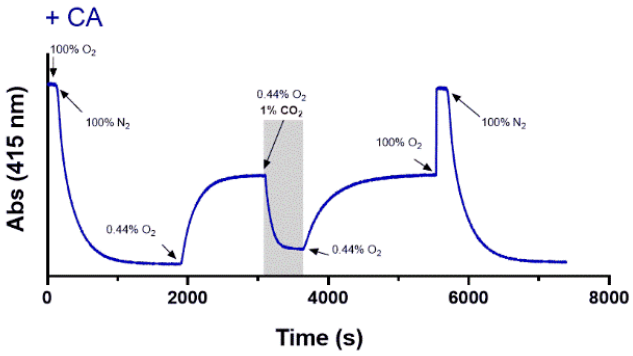

Fig. S2. Absorbance trace showing the CO_2-mediated decrease in Hb-O_2 saturation in the hemolysate of spectacled caiman in the presence of added bovine carbonic anhydrase II (1 µM). After equilibration with 100% O_2 (100% O_2 saturation) and 100% N_2 (0% O_2 saturation), the sample was equilibrated with 0.44% O_2 (PO_2 close to P_{50} = 3.1 torr, balance N_2) to achieve approximately 50% O_2 saturation and then with 1% CO_2, 0.44% O_2 (balance N_2) highlighted by the shaded area. Changes in the % gas in the mixture are indicated by arrows.

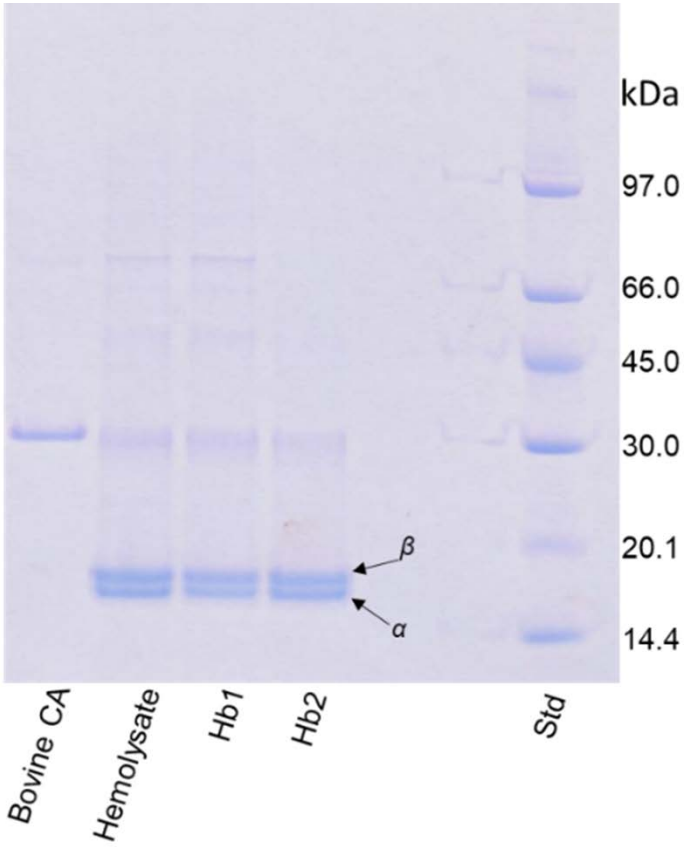

Fig. S3. SDS PAGE in the absence of DTT (non-reducing conditions) with bands corresponding to bovine CA II, spectacled caiman hemolysate, purified Hb1 and Hb2, and the protein standard (STD). In the lanes for the hemolysate and purified Hb fractions, bands representing the α and β-type chains of Hb are indicated.

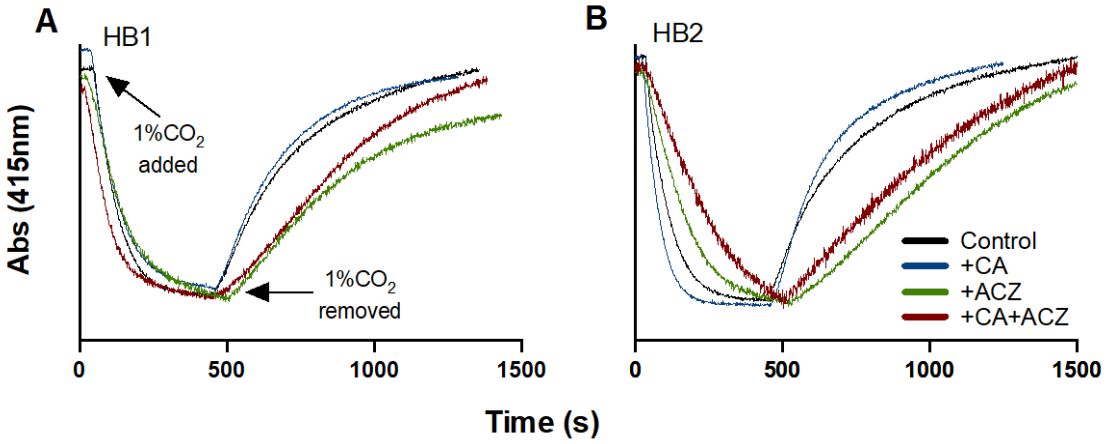

Fig. S4. Absorbance traces showing the reversible CO_2-mediated decrease in Hb-O_2 saturation of purified A) Hb1 and B) Hb2 fractions from spectacled caiman hemolysate, in the presence and absence of acetazolamide (1 mM, ACZ) or bovine carbonic anhydrase II (1 μM, CA), as indicated. Regardless of the presence or absence of carbonic anhydrase, the addition of acetazolamide delays CO_2 dissociation from Hb, after the removal of CO_2 gas. Conditions as in Figure S1.

Table S1. Non-linear regression fitting parameters of the CO_2 saturation curves of biological replicates shown in Fig. 3B,C and biological and technical replicates of P_{50} measurements of Hb1 and Hb2 (25°C, 0.3 mM heme, 0.1 M HEPES, pH 7.2, 0.5 mM EDTA). CI, confidence interval; Sy.x, standard error of estimate. The double hyperbola fitting produced a very large confidence interval for K'_{CO2} (the affinity constant for a second possible binding site), which rules out the possibility for two binding sites for CO_2.

	Single hyperbola $Y = \frac{SO_2min}{(X + KCO_2)}$		Double hyperbola $Y = SO_2\min \times (\frac{1}{(X + KCO_2)} + \frac{1}{(X + K'CO_2)})$	
	Hb1	Hb2	Hb1	Hb2
SO_2min	68.77	62.16	51.23	45.16
KCO_2 (torr)	1.464	1.323	1.174	1.043
$K'CO_2$ (torr)	-	-	13.62	10.94
95% CI for SO_2min	53.38-88.45	50.98-75.69	33.63-73.04	32.77- 60.61
95% CI for KCO_2	1.099-1.959	1.058-1.659	0.8728-1.620	0.8368-1.340
95% CI for $K'CO_2$	-	-	2.131-307.7	2.719-78.05
Degrees of freedom	16	16	15	15
r^2	0.9668	0.9808	0.9757	0.9879
Sum.Squares	140.3	83.32	102.6	52.57
Sy.x	2.961	2.282	2.615	1.872
Values for biological replicates (N=3)				
P_{50}, mean (torr)	3.10	3.22		
P_{50}, stdev (torr)	0.07	0.04		
P_{50}, s.e.m. (torr)	0.05	0.03		
Values for technical replicates (N=3)				
P_{50}, mean (torr)	3.15	3.19		
P_{50}, stdev (torr)	0.13	0.06		
P_{50}, s.e.m. (torr)	0.07	0.04		

Table S2. Non-linear regression fitting parameters of the bicarbonate saturation curves shown in Fig. 3E,F, and biological and technical replicates of final PO_2 measurements of Hb1 and Hb2 after addition of 2, 4, 7.7, 15.4, 23.1 and 30 mM bicarbonate in the Tucker chamber (0.05 mM heme, 0.1 M HEPES buffer pH 7.2, 0.5 mM EDTA). CI, confidence interval; Sy.x, standard error of estimate.

Single hyperbola $Y = \frac{\Delta PO_2 max}{(X + K_{bic})}$	Hb1	Hb2
ΔPO_2max	9.934	8.994
K_{bic} (mM)	3.313	2.473
95% CI for ΔPO_2max	8.770-11.38	8.346-9.716
95% CI for K_{bic}	1.887-5.489	1.722-3.450
Degrees of Freedom	16	16
r^2	0.7828	0.8728
Sum of Squares	15.14	5.82
Sy.x	0.9728	0.6031
Constraints	K_{bic} > 0	K_{bic} > 0
Values for biological replicates (N=3)		
PO_2, mean (torr)	4.42	4.26
PO_2, stdev (torr)	0.08	0.21
PO_2, s.e.m (torr)	0.05	0.12
Values for technical replicates (N=3)		
PO_2, mean (torr)	4.54	4.06
PO_2, stdev (torr)	0.02	0.03
PO_2, s.e.m (torr)	0.01	0.02