ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/15955773

Allosteric regulation of crocodilian haemoglobin

Article *in* Nature · July 1981

DOI: 10.1038/291682a0 · Source: PubMed

CITATIONS
54

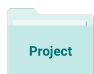
READS
32

9 authors, including:

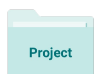
Gerolf Gros
Hannover Medical School
147 PUBLICATIONS **3,555** CITATIONS

SEE PROFILE

Some of the authors of this publication are also working on these related projects:



Diffusion of myoglobin in muscle cells View project



Gas Channels View project

All content following this page was uploaded by Gerolf Gros on 17 June 2016.

The user has requested enhancement of the downloaded file.

Allosteric regulation of crocodilian haemoglobin

M. F. Perutz*, C. Bauer†, G. Gros‡, F. Leclercq§, C. Vandecasserie§, A. G. Schnek§, G. Braunitzer‖, A. E. Friday¶ & K. A. Joysey¶

* MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, UK
† Institut für Physiologie, Universität, 8400 Regensburg, FRG
‡ Physiologisches Institut, Universitätsklinikum 4300 Essen, FRG
§ Chimie General I, Université Libre, Bruxelles, Belgium
‖ Max-Planck Institut für Biochemie, 8033 Martinsried, FRG
¶ University Museum of Zoology, Cambridge CB2 3EJ, UK

The oxygen affinity of most vertebrate haemoglobins in the absence of diffusible electrolytes is much higher than that of blood. In the red cell this affinity is lowered by organic phosphates, hydrogen ions, chloride ions and CO_2 (refs 1–5). Similarly, crocodilian haemoglobin also has a much higher oxygen affinity than crocodile blood, but this is due to bicarbonate ions[6], as neither phosphates nor carbamino CO_2^- lower its oxygen affinity, and chloride does so only weakly[7]. The complete sequences of the haemoglobins of the caiman, the Nile crocodile and the Mississippi alligator (to be reported elsewhere[8]) show 102 substitutions between human and caiman, and 123 between human and the other two crocodilian haemoglobins. Here we consider how these substitutions may explain the changes in allosteric control, and also their bearing on the phylogenetic relationships between the crocodilians and other groups of bony vertebrates. We propose that a few of the substitutions abolish or weaken the binding sites for the usual allosteric effectors and create a new pair of binding sites which are complementary to bicarbonate ions in the deoxy (T) structure, but not in the oxy (R) structure.

Consider first the loss of allosteric inhibition by organic phosphates, carbamino CO_2^- and chloride (Table 1). In human deoxyhaemoglobin, organic phosphates are bound by the four pairs of cationic residues that lie in the cavity between the two β-chains: Val NA1 (1), His NA2 (2), Lys EF6 (82) and His H21 (143)[9]. In the three crocodilian haemoglibins, the histidines are replaced by neutral residues. In two of the haemoglobins the α-amino groups are acetylated; in the third, a proline in position 2 forces a bend in the chain which places the α-amino groups out of reach of the phosphates. This leaves only the two lysines which are known from human fetal haemoglobins F_I to be insufficient for phosphate binding, although His NA2 is still present there[10]. In human deoxyhaemoglobin, carbamino CO_2^- bound to Val 1α accepts a hydrogen bond from Ser H14 (131) α (ref. 11). In the three crocodilian haemoglobins this is replaced by Ala. This replacement may also inactivate one of the strong chloride binding sites which probably coincides with the CO_2^- site[12]. In human deoxyhaemoglobin, another pair of CO_2 molecules competes with organic phosphates for binding to Val 1β; when bound, each carbamino CO_2^- is stabilized by a salt bridge to Lys 82β (ref. 11). In caiman haemoglobin, the bend in the chain forced by Pro 2 inhibits that bridge; in the other two crocodilian haemoglobins, the blocking of Val 1β inhibits binding of CO_2.

Two equivalents of bicarbonate ion per tetramer are bound to caiman deoxyhaemoglobin with an association constant of $2.0 \times 10^{-3}\ M^{-1}$, whereas oxyhaemoglobin shows no significant binding. In the presence of CO_2, discharge of oxygen produces no change in pH, because the protons taken up by haemoglobin are neutralized by those set free in the formation and binding of bicarbonate[6]. At pH 7.4 and 20 °C in 0.1 M NaCl, application to 40 torr CO_2 raises P_{50} ninefold from 3.4 to 30.0 torr; this is comparable to the 10-fold rise, from 4.35 to 44.0 torr, produced by the addition of 2 mM inositolhexaphosphate (IHP) to a similar solution of human haemoglobin A at 25 °C (ref. 13). In human haemoglobin A, IHP reduces Hill's coefficient from 2.9 to 2.4; in crocodile haemoglobin, CO_2 reduces it to the same value. In both cases the allosteric equilibrium is shifted too far towards the T structure for oxygen binding to be fully cooperative. At pH 7.0, addition of CO_2 to caiman methaemoglobin produces a large negative circular dichroism peak at 287 nm, diagnostic of the quaternary T structure; the same effect is produced by IHP in human methaemoglobin A[14,15].

We have examined all possible sites in the model of human deoxyhaemoglobin where the amino acid replacements indicated by the three crocodilian sequences could possibly cause oxygen-linked binding of bicarbonate ions, and have found only one pair of sites with the right stereochemistry. These lie in the cavity between the two β-chains where organic phosphates or carbamino CO_2^- are bound in other species, and are formed by Lys EF6(82) and Glu HC1(144) of one β-chain together with the N-terminal residue of its partner chain (Fig. 1, Table 1). In caiman haemoglobin, the N-terminal sequence is Ser-Pro-Phe-. A model of the bicarbonate binding site was first built manually by replacing the side chains in human deoxyhaemoglobin with those of caiman, and was then refined by computer. The main chain from the α-carbon of Pro 2 onwards was held in the same position as in human deoxyhaemoglobin. At this atom the chain is forced to turn a corner at an angle defined by the stereochemistry of the proline. This rigid turn brings the N-terminal serine within exact reach of the bicarbonate ion, so that one of the bicarbonate oxygens can form a salt bridge with the α-NH_3^+ and can also accept a rather long hydrogen bond from the serine OH. The second bicarbonate

Table 1 Amino acid replacements for allosteric control in crocodile haemoglobin

Position in			Species					
Structure		Sequence	Human	Other bony vertebrates	Caiman	Nile crocodile	Mississippi crocodile	Functions
NA1	β	1	Val	Val or Gly	Ser	Ac-Ala	Ac-Ala	In human and many other vertebrate deoxy-Hbs, the α-amino groups bind either organic phosphate or carbamino CO_2^-, which in turn forms a salt bridge with Lys 82 of the same β-chain. In crocodile Hb, blocking of α-NH_3^+ or Pro in position 2 inhibits these functions
NA2	β	2	His	Gln, Asn, Glu, His, Asp, Met	Pro	Ser	Ser	In human Hb, His, and in many other vertebrates Gln or Asn, bind organic phosphates
EF6	β	82	Lys	Lys	Lys	Lys	Lys	Lys binds diffusible anions in the internal cavity of deoxy-Hbs. Invariant in all vertebrate Hbs except trout I which lacks all oxygen-linked functions and where it is replaced by Leu*
H21	β	143	His	Lys, Arg, Ser, His	Ala	Ala	Ala	His, Lys or Arg form salt bridges with organic phosphates
HC1	β	144	Lys	Arg, Ala, Ser, Gln, Lys	Glu	Glu	Glu	In human Hb, Lys is free and the same is probably true of Arg, Ser or Gln in other species. Glu can accept an H-bond from HCO_3^- whose charge is compensated by Lys 82
H14	α	131	Ser	Very variable	Ala	Ala	Ala	In human Hb, OH of Ser forms an H-bond with carbamino CO_2^- or Cl^- bound to Val 1α, but this bond is absent in many species

* Unpublished data (D. Barra, F. Bossa and M. Brunori).

0028–0836/81/260682—03$01.00

© 1981 Macmillan Journals Ltd

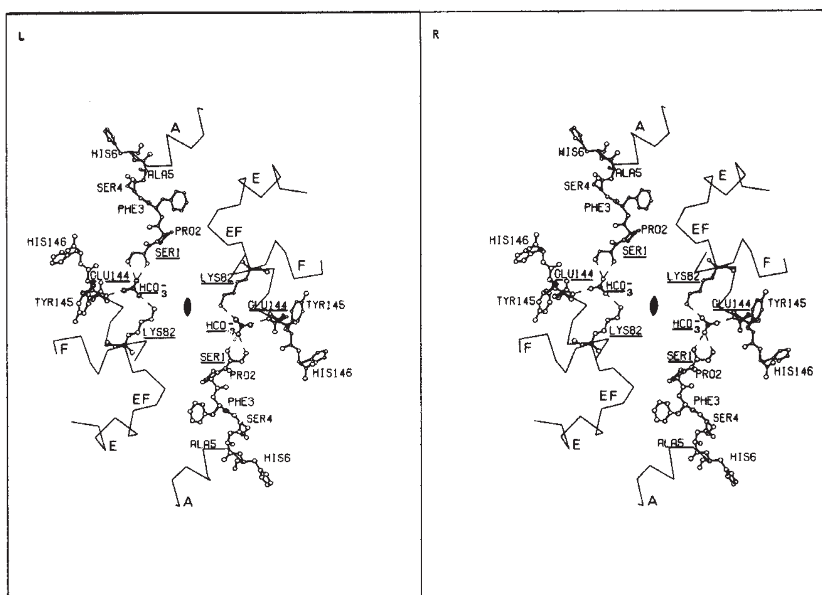


Fig. 1 Stereo drawing of proposed bicarbonate binding site between the two β-chains of caiman deoxyhaemoglobin. The central sign marks the dyad symmetry axis. Bicarbonates and their binding residues are underlined. Capital letters mark helical and inter-helical segments. The drawing was prepared using the PLUTO program and Fermi's refined atomic coordinates of human deoxyhaemoglobin[20]. Certain of the amino acid residues in the atomic model of this haemoglobin were replaced by those in caiman; the bicarbonate ions were attached to them and all the new atomic positions were then measured manually. These rough coordinates were then corrected to standard bond distances and angles using Diamond's BILDER program[21] on the Evans and Sutherland Picture System I linked to a DEC PDP 11/50 computer. The hydrogen bond distances quoted in the text were derived from this computer-generated model. The hydrogen bond from HCO_3^- to COO^- of Glu 144 looks straight in the picture, but in fact the two groups lie at different heights, making the bond angle COH 110°, as it should be.

oxygen can form a salt bridge with Lys EF6(82) and the third oxygen can donate a hydrogen bond to one of the carboxylate oxygens of Glu HC1(144). In this model all hydrogen bonds come out reasonably: Ser $N–O_1 = 2.8$ Å; Ser $O_\gamma–O_1 = 3.3$ Å; Lys $N_\zeta–O_2 = 2.9$ Å; Glu $O_\epsilon–O_3 = 2.7$ Å. All side chains are in the strain-free, straight, staggered conformation. Even so, the model should be checked by X-ray analysis. In the haemoglobins of the Nile crocodile and the Mississipi alligator, where the N-terminal sequence is acetyl-Ala-Ser-Phe, the α-NH of acetylalanine could donate a hydrogen bond to O_1 of the bircarbonate at the same distance as the α-NH_3^+ does in caiman, provided the chain turns a corner at Ser 2 at the same angle as it does at Pro 2 in caiman. Again, this should be checked by X-ray analysis.

The salt bridges and hydrogen bonds made by the two symmetry-related bicarbonate ions would stabilize the relative positions taken up by the two β-chains in the quaternary T structure; furthermore, by immobilizing Glu HC1(144), they would help to clamp the three C-terminal residues Glu-Tyr-His in their rigid deoxy conformation, with the tyrosine hydrogen-bonded to Val FG5β and the histidine salt-bridged to Asp FG1β and Lys C5α. On transition to the R structure, the two β-chains move relative to one another by several Ångstroms and their C-terminal residues become mobile, so that the bicarbonate binding sites are destroyed.

Other small anions also lower the oxygen affinity, but much less than bicarbonate. When the concentrations of chloride, nitrate, perchlorate or phosphate are increased from 0.01 M to 0.5 M at pH 7.0 and 25 °C, P_{50} is lowered by factors of 2.0, 1.6, 1.7 and 1.3, respectively, whereas the same increase in the concentration of bicarbonate lowers P_{50} 13-fold[6]. In human haemoglobin, chloride probably binds to Lys 82β (ref. 16). If the same is true of caiman haemoglobin, the binding of chloride and bicarbonate should be competitive; in fact, a rise in $[Cl^-]$ from 0.01 to 0.5 M approximately halves the effect of HCO_3^- on P_{50}. NO_3^- is a planar ion like HCO_3^-, and the NO distances are only 0.1 Å shorter than those of CO. NO_3^- could therefore bind to Ser 1 and Lys 82, but it has no hydrogen to donate to Glu 144; nor does ClO_4^-. HPO_4^{2-} could do so, but because this ion is tetrahedral, binding of one oxygen to Ser 1 and another to Lys 82 makes the OH bond of the third oxygen point away from the carboxylate of Glu 144, so that no hydrogen bond can be formed.

The decrease in oxygen affinity brought about by the interaction of crocodilian haemoglobin with bicarbonate ensures that oxygen is released from the blood to the tissues at a relatively high partial pressure of oxygen. If the haemoglobin were insensitive to bicarbonate, the venous P_{O_2} would be only 7 torr; interaction with bicarbonate raises this to 27 torr, thus creating a large enough pressure head for the flow of oxygen from the blood to the tissues[17]. In human haemoglobin, the same relative rise in P_{O_2}, from 19 to 40 torr, can be brought about only by the combined effects of CO_2 and diphosphoglycerate. It is surprising that the simple and direct reciprocating action between oxygen and one of the end products of oxidative metabolism has not been adopted by other vertebrates. We do not know what advantage it gives to the crocodilians.

Our results show that an entirely new function can evolve in a protein by no more than three amino acid substitutions, requiring only four nucleotide base changes; just two more amino acid substitutions, or three base changes, are needed for inhibition of the old functions (oxygen-linked phosphate, carbamino CO_2^- and Cl^- binding). Most of the other ~100 substitutions that distinguish crocodilian from human haemoglobins are conservative and would have little if any effect on the oxygen equilibrium. There are some significant substitutions in the haem

Table 2 Number of amino acid substitutions between the α-globin chains of different species

	Man	Caiman	Mississippi alligator	Nile crocodile	Goldfish	Trout	Tadpole	Chicken	Goose	Viper
1. Caiman	46									
2. Missipi alligator	47	20								
3. Nile crocodile	44	21	17							
4. Goldfish	65	68	68	64						
5. Trout	60	64	64	66	43					
6. Tadpole	61	65	67	66	70	63				
7. Chicken	35	46	49	45	72	70	70			
8. Goose	41	39	35	30	64	61	64	30		
9. Viper	50	64	66	62	76	70	69	57	59	
10. Kangaroo	27	43	43	43	67	60	60	41	39	54

1, *Caiman crocodilus*; 2, *Alligator mississippiensis*; 3, *Crocodylus niloticus*; 4, *Carassius auratus*; 5, *Salmo irideus*; 6, *Rana catesbeiana*; 8, *Anser anser*; 9, *Vipera aspis*; 10, *Macropus giganteus*.

©1981 Nature Publishing Group

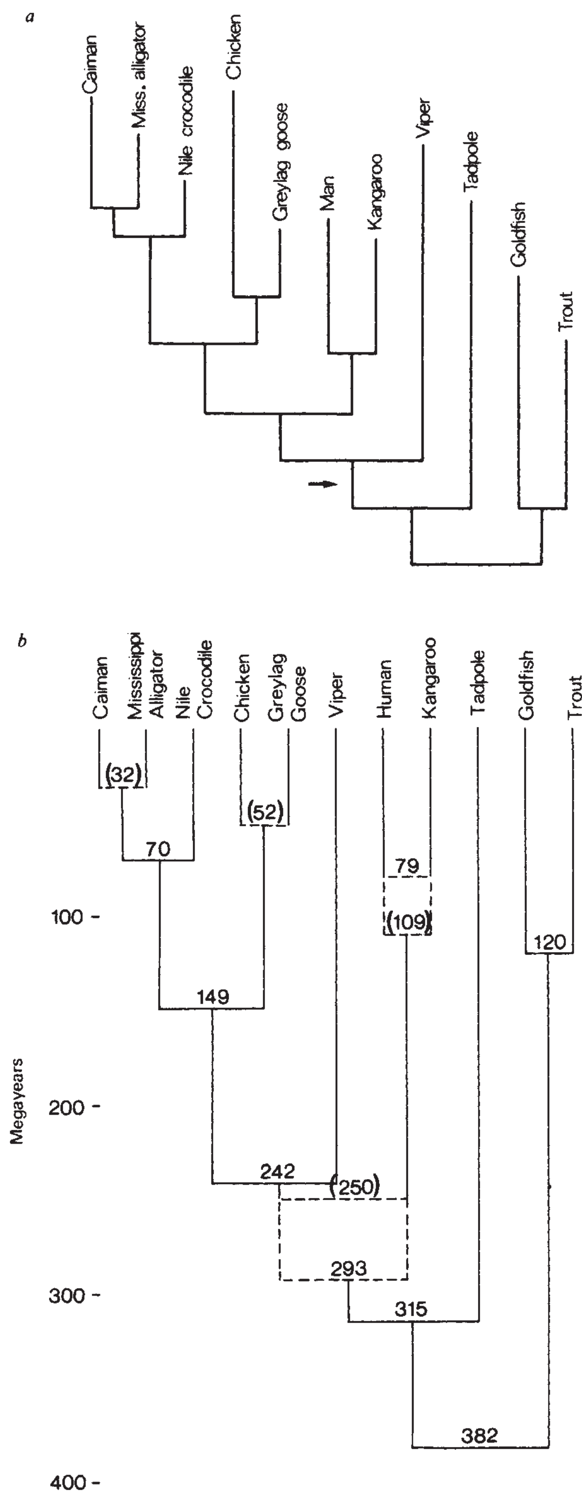


Fig. 2 *a*, Wagner network computed from Table 2 by the method of Farris[22]. The root has been placed midway along the branch separating the two fish from the other species, in accord with conventional zoological opinion. The arrow shows an alternative position of the root at the midpoint between the two species separated by the largest network distance. This network has a total length of 249.5; an alternative network, with the shortest length of 249.0, has a much poorer fit to the observed differences. *b*, Phylogenetic tree based on comparative anatomy and the fossil record. The number at each branching point is an estimate of the minimum date of divergence of the ancestors of the living forms, based on direct fossil evidence. Alternative possible dates, indicated by broken lines and numbers in parentheses, refer to less reliable dates. The justification for some of these dates is given in ref. 19 and the method of dating is further discussed in ref. 23.

pockets and at the subunit contacts, but none of these is unique or could play any part in the allosteric control by bicarbonate ions. All the residues which are essential for the formation of the characteristic T and R structures are either conserved or have been replaced by ones that can serve the same purpose equally well.

Do the crocodilian sequences tell us anything interesting about the phylogenetic relationships between these reptiles and other groups of bony vertebrates? The α-globin sequences of 21 mammals, 3 birds, 3 fish, 1 amphibian and 1 other reptile (viper) are now known, but not the β-globin sequence of the viper. We have selected two species from among each of the mammals, birds and fish and compared their α-globin sequences with those of the crocodilians, the bullfrog tadpole and the viper. Table 2 shows a matrix of amino acid differences between all possible pairs of sequences; the data in Fig. 2*a* have been calculated from this matrix by an algorithm that does not allow the difference between any pair of sequences to be less than the observed one; it attempts to minimize the total length of the network and to maximize its fit to the observed differences. It shows that the crocodilian sequences are most similar to those of the birds, consistent with fossil evidence suggesting that birds arose from archosaurian reptiles. As might be expected, the number of amino acid differences and the network distances between each of the crocodilians and the viper (a lepidosaur reptile) are much larger than between the crocodilians and the birds, but it is surprising that they are also much larger than between the crocodilians and the mammals. Figure 2*b* shows, for comparison, a zoologically conventional phylogenetic tree, derived from the evidence of comparative anatomy and the fossil record, which shows the crocodilians as more closely related to the viper than the mammals. For the rest, Fig. 2*a* and *b* are similar in pattern, although Fig. 2*b* has very unequal pairs of branches arising from the same node. These support the conclusions of Romero-Herrera *et al.*[18,19], based on myoglobin, that the rates of change differ on different branches of the phylogenetic tree. Without knowing more reptile sequences we cannot be sure, therefore, whether the large differences between the crocodilians and the viper suggest a divergence between the ancestors of archosaurian and lepidosaurian reptiles earlier than that between the archosaurian reptiles and mammals, or are due merely to accelerated fixation of mutations along one particular evolutionary lineage. This question is worth following up.

We thank Mr A. Stangl and Mrs B. Schrank for their help in determining the amino acid sequences, Professors K. Johansen and R. Coulson for gifts of crocodile blood, Drs S. E. V. Phillips and B. Shaanan for their help with Fig. 1 and Professor Howard Morris for checking an N-terminal sequence by mass spectrometry.

Received 24 March; accepted 8 May 1981.

1. Baldwin, J. M. *Prog. Biophys. molec. Biol.* **29**, 225–320 (1975).
2. Perutz, M. F. *Br. med. Bull.* **32**, 191–208 (1976).
3. Kilmartin, J. V. *et al. Biochim. biophys. Acta* **534**, 15–25 (1978).
4. Kilmartin, J. V. & Rossi-Bernardi, L. *Physiol. Rev.* **53**, 836–890 (1973).
5. Kilmartin, J. V. *Trends biochem. Sci.* **2**, 247–250 (1977).
6. Bauer, C. *et al. J. biol. Chem.* (in the press).
7. Bauer, C. & Jelkmann, W. *Nature* **269**, 825–827 (1977).
8. Leclercq, F., Schnek, A. G., Braunitzer, G., Stangl, A. & Schrank, B. *Hoppe-Seyler's Z. physiol. Chem.* (in the press).
9. Arnone, A. *Nature* **237**, 146–149 (1972).
10. Bunn, H. F. & Briehl, R. W. *J. clin. Invest.* **49**, 1088–1095 (1970).
11. Arnone, A., Rogers, P. H. & Briley, P. D. in *Biophysics and Physiology of Carbon Dioxide* (eds Bauer, C., Gros, G. & Bartels, H.) 67–74 (Springer, Berlin, 1980).
12. O'Donnell, S., Mandaro, R., Schuster, T. M. & Arnone, A. *J. biol. Chem.* **254**, 12204–12208 (1979).
13. Imai, K. *J. biol. Chem.* **249**, 7607–7612 (1974).
14. Perutz, M. F., Fersht, A. R., Simon, S. R. & Roberts, G. C. K. *Biochemistry* **13**, 2174–2186 (1974).
15. Fermi, G. & Perutz, M. F. *J. molec. Biol.* **114**, 421–431 (1977).
16. Perutz, M. F. *et al. J. molec. Biol.* **138**, 649–670 (1980).
17. Jelkmann, W. & Bauer, C. *Comp. Biochem. Physiol.* **A65**, 331–336 (1980).
18. Romero-Herrera, A. E., Lehmann, H., Joysey, K. A. & Friday, A. E. *Nature* **246**, 389–395 (1973).
19. Romero-Herrera, A. E., Lehmann, H., Joysey, K. A. & Friday, A. E. *Phil. Trans. R. Soc.* **B283**, 61–163 (1978).
20. Fermi, G. *J. molec. Biol.* **97**, 237–256 (1975).
21. R. Diamond in *Biomolecular Structure, Conformation and Evolution* Vol. 1 (ed. Srinivasan, R.) 567–588 (Pergamon, Oxford, 1980).
22. Farris, J. S. *Am. Nat.* **106**, 645–668 (1972).
23. Joysey, K. A. *Symp. zool. Soc. Lond.* **46** (in the press).

©1981 Nature Publishing Group

View publication stats