TABLE 2 Opportunities for mating with wild-type and experimental males in the second experiment

	Number of wild-type male matings			
	Days 2–11		Days 14–23	
(a)	Taken	Not taken	Taken	Not taken
(1) 'Full' main-cell products	28	117	9	41
(2) 'Reduced' main-cell products	28	120	15	54
(3) 'Greatly reduced' main-cell products	26	129	12	75
(4) 'No' main-cell products	30	115	15	99
Non-mating control (1)	19	136	22	90
Non-mating control (2)	17	141	19	101
Non-mating control (3)	17	139	15	109
Non-mating control (4)	25	111	16	80
	Number of experimental male matings			
	Days 3–12		Days 15–24	
(b)	Taken	Not taken	Taken	Not taken
'Full' main-cell products	6	136	1	40
'Reduced' main-cell products	16	127	3	60
'Greatly reduced' main-cell products	14	137	7	72
'No' main-cell products	2	151	5	103

There were no significant differences in remating rates with wild-type males during the first or last 4 remating samples combined (days 2 to 11, $\chi^2=13.08$, 7d.f., $P>0.05$; days 14 to 23, $\chi^2=5.40$, 7d.f., $P>0.05$). In the first 4 remating samples of the experiment (days 3–12) there were differences in experimental male remating rate ($\chi^2=15.18$, $P<0.01$) attributable mostly to higher than expected mating rates by 'reduced' and 'greatly reduced' and lower than expected mating rates by 'no' and 'full' main-cell product males. There were no significant differences over the last 4 samples of the experiment (days 15–24; $\chi^2=2.68$, $P>0.05$). Note that mating frequencies in *b* were for only one of the 2 days for which the females were exposed to experimental males. Methods as for Table 1, except that rematings were observed for 4 h instead of 3 h. To check that matings of the different types of males were qualitatively similar, their mating durations and those of Dahomey males were measured with Dahomey virgin females. Single 4-day-old males, at least 22 per genotype, were aspirated into vials each containing a single virgin female, and the time of the onset and ending of mating was recorded. There were no significant differences in mating duration ($F_{4,4}=4.16$, $P>0.05$).

by females may have evolved under conditions of low nutrition. The high level of nutrition in these experiments may therefore have made the cost of mating more apparent than it would be in nature. Nonetheless, the results imply that female remating rate under natural conditions would evolve to an intermediate optimum determined by the need to maintain sperm supplies and the effect of mating on female mortality. It will be important now to identify the molecules responsible for the cost of mating and to discover how the increase in death rate is caused. □

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Transplanting a unique allosteric effect from crocodile into human haemoglobin

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CROCODILES are able to remain under water for more than one hour without surfacing to breathe[1,2] and often kill their prey by drowning it. How do crocodiles stay under water for a long time? When they hold their breath, bicarbonate ions, the final product of respiration, accumulate and drastically reduce the oxygen affinity of haemoglobin, releasing a large fraction of haemoglobin-bound oxygen into the tissues[3,4]. We have now located the bicarbonate-ion-binding site at the $\alpha_1\beta_2$-subunit interface by making various human–crocodile chimaeric haemoglobins. Furthermore, we have been able to transplant the bicarbonate effect into human haemoglobin by replacing only a few residues, even though the amino-acid sequence identity between crocodile (*Crocodylus niloticus*) and human haemoglobins is only 68% for the α- and 51% for the β-subunit[5]. These results indicate that an entirely new function which enables species to adapt to a new environment could evolve in a protein by a relatively small number of amino-acid substitutions in key positions[6].

During vertebrate evolution the amino-acid sequence of haemoglobins (Hbs) has diverged, and Hbs have acquired the ability to respond to various metabolic stimuli such as 2,3-bisphosphoglycerate (BPG), inositol pentaphosphate, ATP, CO_2 and H^+. In the absence of these effectors, the oxygen affinity of Hb is too high to deliver oxygen efficiently to the tissues[6,7] but these metabolites aid the unloading of oxygen by lowering the oxygen affinity of Hb. The oxygen affinity of crocodile Hb is markedly reduced by physiological concentration of CO_2 (40 torr)[3], and this unique allosteric effect is due to the binding of two bicarbonate ions, rather than CO_2 itself, to each molecule of deoxyhaemoglobin[4]. At physiological pH, CO_2 dissolves into water and is present predominantly as bicarbonate ions. The muscle myoglobin content of the crocodilians is nearly 100 times lower than that of diving mammals such as whales and seals[8] (N.H.K., unpublished results), so the bicarbonate effect, rather than oxygen-storing myoglobin, enables them to stay under water for a long time. It was proposed that the BPG-binding site in the central cavity between two β-subunits may have evolved into a bicarbonate-ion-binding site in crocodile Hb by substitutions of several amino-acid residues[9]. We introduced these amino-acid substitutions into human Hb but observed no bicarbonate effect[10].

To locate the bicarbonate-ion-binding site, we produced various human–crocodile chimaeric Hbs and investigated their oxy-

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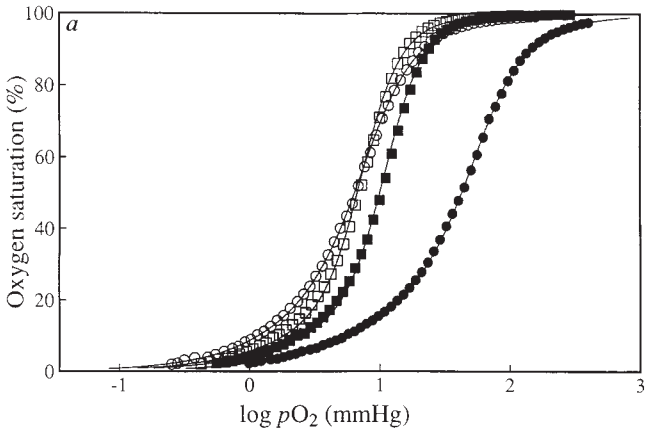

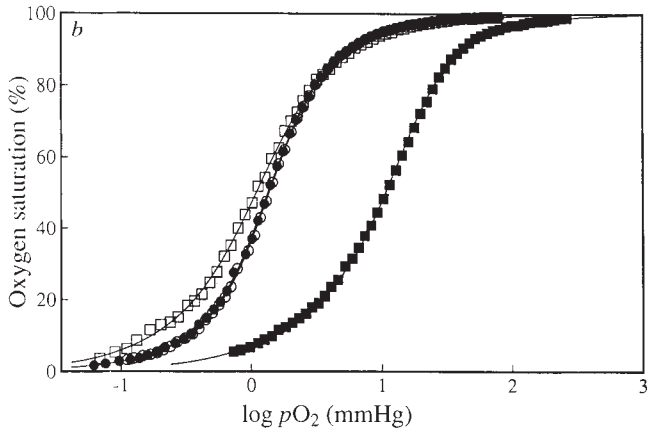

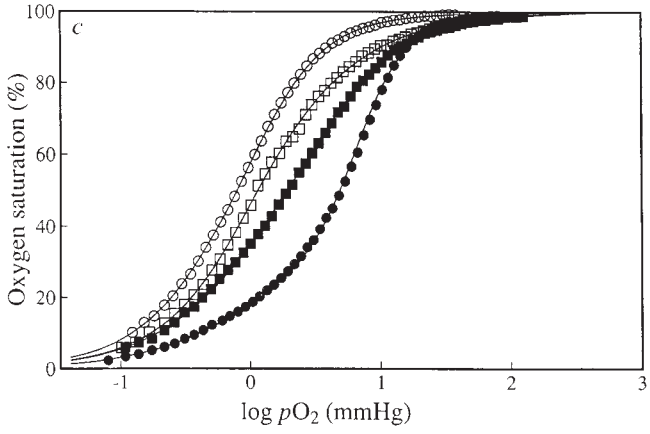

FIG. 1 *a*, The oxygen-binding curves of recombinant crocodile Hb (circles) and human Hb (squares) determined in the absence (empty symbols) and presence (filled symbols) of 5% CO_2. *b*, The oxygen-binding curves of α(crocodile)$_2\beta$(human)$_2$ (circles) and α(crocodile)$_2\beta$(SC4)$_2$ (squares) determined in the absence (empty) and presence (filled) of 5% CO_2. β(SC4) chain is human β-chain with the Gly 29β→Ser, Leu 31β→Met, Thr 38β→Lys, Gln 39β→Arg and Phe 41β→Tyr mutations. *c*, The oxygen-binding curve of Hb Scuba, which is human Hb with the Leu 34α→Cys, Ser 35α→Ala, Phe 36α→Tyr, Thr 37α→Gln, Thr 41α→Ile, Leu 100α→Phe, His 103α→Gln, Gly 29β→Ser, Lys 31β→Met, Thr 38β→Lys, Gln 39β→Arg, Phe 41β→Tyr mutations, in the absence (empty circle) and presence (filled circle) of CO_2. The oxygen-binding curves of Hb Scuba lacking the Leu 100α→Phe, His 103α→Gln mutations were determined in the absence (empty square) and presence (filled square) of CO_2.

METHODS. Synthetic genes encoding the α- and β-globins of the Nile crocodile (*C. niloticus*) Hb were each constructed by assembling 14 oligodeoxynuclotides with the codons optimized for expression in *E. coli*[15]. All subsequent mutations were introduced by cassette mutagenesis[16]. Recombinant human Hbs, both wild-type and mutants, were produced essentially as described in ref. 17. Nile crocodile and all chimaeric Hbs were expressed under the control of either a tac or a lac–tac tandem promoter. Purification of these recombinant Hbs was as described[18]. Oxygen equilibrium curves were obtained using the automatic recording apparatus[19]. All the measurements were made at pH 7.4 in 50 mM bis-Tris with 0.1 M chloride ions at 25 °C, either in the presence or absence of 5% CO_2 which was in equilibrium with 21 mM bicarbonate ion in the buffer.

gen-binding properties. We synthesized genes encoding the α- and β-chains of Nile crocodile (*C. niloticus*) Hb and overproduced them in *Escherichia coli*. Figure 1*a* shows the oxygen-binding curves of recombinant human and crocodile Hb in the absence and presence of 5% CO_2. Bicarbonate ions markedly reduce the oxygen affinity of recombinant crocodile Hb, whereas human Hb shows only a small effect, confirming the results of ref. 4. The amino-acid sequences of human Hb and Nile crocodile Hb[5] are compared in Fig. 2. Thirty-two per cent of the residues in the α-subunit and 49% in the β-subunits have been substituted between these species. We first made hybrid Hbs, consisting of human α-chains and crocodile β-chains and vice versa, to see whether only one of the subunits is predominantly responsible for the bicarbonate effect. The α(crocodile)$_2\beta$(human)$_2$ showed no bicarbonate effect (Fig. 1*b*), but α(human)$_2\beta$(crocodile)$_2$ showed a small bicarbonate effect (data not shown). A cluster of amino-acid residues at the $\alpha_1\beta_2$-subunit interface, including Lys 38β, Arg 39β and Tyr 41β are conserved in all three sequenced crocodilians Hbs (*Caiman crocodylus*, *Alligator mississippiensis* and *C. niloticus*). We therefore synthesized a mutant human β-globin gene, hereafter called β(SC4), which includes the mutations Gly 29β → Ser, Leu 31β→Met, Thr 38β→Lys, Gln 39β→Arg and Phe 41β→Tyr at the $\alpha_1\beta_2$ subunit interface. Hb tetramers consisting of crocodile α-subunits and β(SC4)-subunits showed a full bicarbonate effect (Fig. 1*b*). However, introducing these five mutations alone into human Hb created only a small bicarbonate effect (data not shown), indicating that residues in the α-subunits are also necessary for the full bicarbonate effect.

To find the minimal number of crocodile residues required to create the bicarbonate effect in human Hb, we 'humanized' the α-globin scquence in chimaeric Hb (α(crocodile)$_2\beta$(SC4)$_2$) step by step. We could reduce the number of crocodile residues remaining in the α-subunit to only seven without losing the full bicarbonate effect. This new engineered Hb, named Hb Scuba, consists of human α-subunits with the Leu 34α→Cys, Ser 35α→Ala, Phe 36α→Tyr, Thr 38α→Gln, Thr 41α→Ile, Leu 100α→Phe, His 103α→Gln mutations and the β(SC4) subunits (Fig. 1*c*). Without the Leu 100α→Phe, His 103α→Gln mutations, this engineered Hb loses not only the bicarbonate effect but also cooperative oxygen binding. However, of these two residues, only the Leu 100α→Phe may be essential because two crocodilian species have a Gln residue at 103α. Although Hb Scuba retains the full bicarbonate effect, its oxygen affinity is 10 times higher than that of Nile crocodile Hb, both in the presence and absence of CO_2 (Fig. 1*a*, *c*). Additional mutations are therefore needed to lower the oxygen affinity. Figure 3 shows a schematic drawing of human deoxy-Hb in which the positions of the 12 residues mutated to create the bicarbonate effect are indicated. Most of these residues are clustered at the $\alpha_1\beta_2$-subunit interface, where the two subunits slide with respect to each other on oxygen binding. M. Perutz (personal communication) has been able to model a stereochemically plausible binding site for a bicarbonate ion in which the phenolate oxygen of Tyr 41β, the ε-amino group of Lys 38β, and the phenolate oxygen of a conserved Tyr 42α form hydrogen bonds to a bicarbonate ion. However, these two mutations alone in human Hb failed to create a bicarbonate ion effect. The residues involved in bicar-

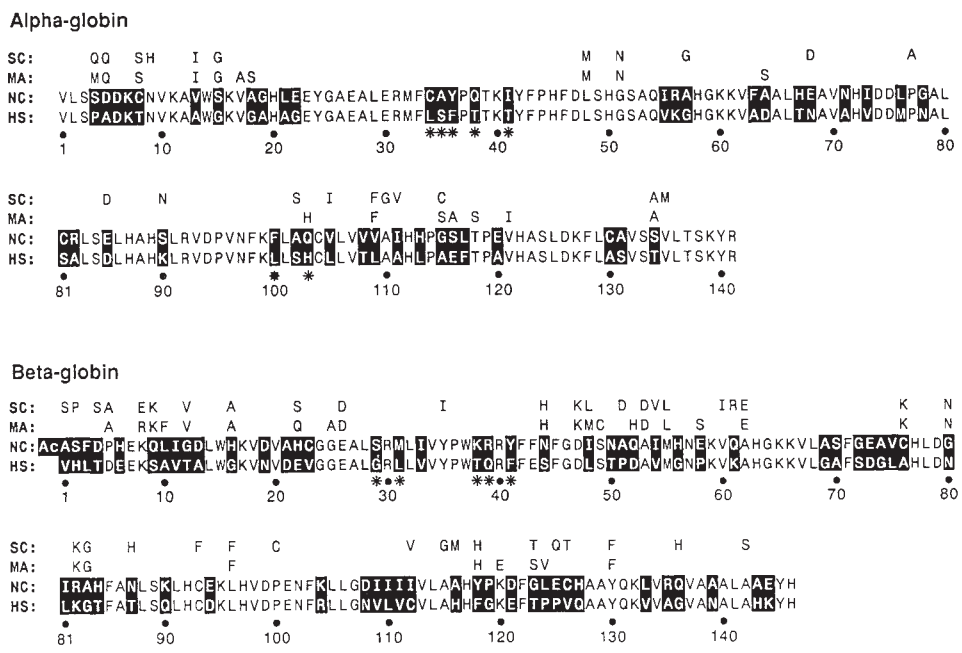

FIG. 2 Amino-acid sequences of human and three crocodilian Hbs (HS, human; SC, spectacled caiman, *C. crocodylus*; MA, Mississippi alligator, *A. mississippiensis*; NC, Nile crocodile *C. niloticus*)[5]. Residues that differ between human and Nile crocodile Hbs are highlighted. Amino-acid sequences of spectacled caiman and Mississippi alligator are shown only at positions where residues are not identical to those of Nile crocodile. Residues necessary for the transplantation of the bicarbonate effect into human Hb are indicated with an asterisk.

bonate ion binding are located at the $\alpha_1\beta_2$ contact between the β-chain C helix and the α-chain FG corner, which acts as a flexible joint during the allosteric transition. Therefore this is an ideal binding site for an allosteric effector, and bicarbonate ions act as a molecular clamp at the $\alpha_1\beta_2$ interface to stabilize the T state[11,12]. Crocodile Hb is the only known Hb to use this unique allosteric effector site.

Unlike other vertebrate Hbs, the oxygen affinity of crocodile Hb is not affected by organic phosphates such as 2,3-BPG and ATP because of the mutations in the central cavity between the two β-subunits[3,10]. However, the oxygen affinity of embryonic Hb of the saltwater crocodile (*Crocodylus porosus*) is significantly reduced by ATP[13]. A drop in the level of embryonic Hb is observed with the concomitant decrease in the ATP level toward hatching. The binding of organic phosphate and bicarbonate ions are not mutually exclusive and it is possible that an ancestral crocodile Hb may have had the organic phosphate effect when it acquired the bicarbonate ion effect. However, the selective advantage of having the bicarbonate effect would be sufficient in adult life, and crocodile adult Hb may have lost the selective pressure to maintain the organic phosphate effect.

There are 110 amino-acid replacements out of 287 residues (the α- and β-subunits contain 141 and 146 amino-acid residues, respectively) between human and Nile crocodile Hbs. We have shown that no more than 12 amino-acid replacements are needed to create the bicarbonate effect. These results indicate that an entirely new function which enables species to adapt to a new environment could evolve in a protein by a relatively small number of amino-acid substitutions in key positions, rather than by gradual accumulation of minor mutations. This observation is consistent with the neutral theory of molecular evolution[14] and Perutz's theory of protein speciation[6]. □

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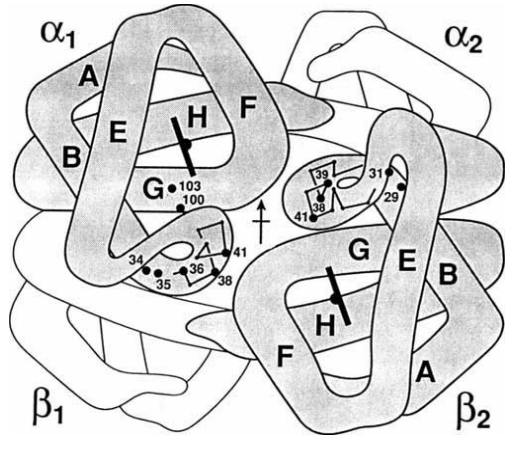

FIG. 3 A schematic drawing of human deoxyhaemoglobin. Filled circles indicate the positions of the 12 mutations introduced into human Hb to transplant the bicarbonate effect. Most of the residues are clustered at the $\alpha_1\beta_2$-subunit interface where the two subunits slide with respect to each other on binding oxygen. The spread in space of the positions of the required mutations indicates that not all can be directly involved in the formation of the bicarbonate-binding site; some must serve to perturb the structure to favour binding some distance away.