Diving behaviour and haemoglobin function: the primary structure of the α - and β -chains of the sea turtle (*Caretta caretta*) and its functional implications

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The amino acid sequence of the α - and β -chains of haemoglobin (Hb) from the loggerhead sea turtle (*Caretta caretta*) has been determined. Comparison with that of human Hb shows differences in several residues involved in both $\alpha_1\beta_1$ and $\alpha_1\beta_2$ packing contacts. On the whole, in spite of the mutations, the essential characteristics of both interfaces seem to be maintained. The functional properties of the sea turtle Hb have been investigated at different temperatures and as a function of proton, chloride and organic phosphate concentrations. In addition to overall similarities shared with most of the vertebrate Hbs previously described, this molecule shows significant differences which could

be related to the life behaviour of the turtle. In fact, while the shape of the Bohr-effect curve is well adapted for gas exchange during prolonged dives, the very small enthalpy change for O₂ binding ensures that O₂ delivery becomes essentially insensitive to the temperature changes of the environment. Moreover, and similarly to the case of emperor penguin Hb, the small alkaline Bohr effect appears to be only chloride-linked, since the pH dependence of the O₂ affinity is abolished in the absence of this ion. These functional characteristics are discussed on the basis of the primary structure of α - and β -chains.

INTRODUCTION

Among vertebrates, haemoglobins (Hbs) exhibit a great deal of variation in terms of absolute affinities for oxygen and in their susceptibility to control by metabolic effectors. These variations bring about a fine modulation of the O₂-binding properties of the molecule to fully meet the physiological requirements of a given species. This fine tuning, developed to a high degree in all vertebrate Hbs, is primarily based on the thermodynamic linkage between binding of allosteric effectors and the basic reaction of the haem-iron with oxygen.

A recent series of investigations on molecular adaptation to extreme environmental conditions [1–6] has provided insight into the role of temperature and of its interplay with heterotropic ligands in the modulation of Hb function. In line with these studies, and to widen the scope of the emerging scheme, we have recently undertaken an investigation of the functional properties of the Hb from the loggerhead sea turtle *Caretta caretta* [7]. This choice was made on the basis of the following main considerations: (a) turtles, being accomplished divers, show a remarkable adaptation to anoxic conditions, thereby pointing to the possibility of unusual functional and structural properties of their Hb; and (b) sea-turtle blood has to fulfil its O₂ transport function under a wide range of conditions, facing marked variations in pH levels and substantial temperature changes.

The previous characterization [7] has indicated that adult sea turtle Hb is characterized by (1) an intrinsic low oxygen affinity; (2) a strongly reduced amplitude of the Bohr effect for oxygen; and (3) a very low sensitivity to allosteric effectors and temperature. To elucidate the structure–function relationships in this Hb we have further characterized its functional properties and determined the amino acid sequence of its α - and β -chains. The

results, discussed at the structural level and in comparison with available data on other turtle and mammalian Hbs, highlight the existence of some peculiar features which may contribute to explaining the diving ability of sea turtles.

EXPERIMENTAL

Materials

Trypsin (EC 3.4.21.4) and 4-vinylpyridine, ATP, 2,3-diphosphoglycerate (DPG), inositol hexaphosphate (IHP) and Hepes were from Sigma; cyanogen bromide from Fluka; HPLC-grade acetonitrile from Aldrich; and sequence-grade reagents from Applied Biosystems. All reagents were of the highest purity commercially available.

Haemoglobin purification

Blood samples were collected with the official permission of the Italian Ministry of Agriculture and Forestry. Turtle Hb was obtained from haemolysate of erythrocytes washed three times with 1.3% (w/v) NaCl. Red cells were lysed with distilled water and removed by centrifugation. Turtle Hb was prepared as previously reported [7] and stripped by gel-filtration and ion-exchange chromatography [8].

Oxygen binding

Oxygen-binding isotherms were determined by the tonometric method [9] in the absence and presence of allosteric effectors. Spectrophotometric measurements were carried out with a Kontron 860 Wikon spectrophotometer.

Abbreviations used: DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; Hb, haemoglobin; HbA, human adult haemoglobin.

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The overall oxygenation enthalpy (ΔH in kcal/mol), corrected for the solubilization heat of O_2 (-3.0 kcal/mol), has been calculated from the integrated van't Hoff equation:

$$\Delta H = -4.574 [(T_1 T_2)/(T_1 - T_2)] \Delta \log P_{50}/1000$$

where P_{50} is the partial pressure of O_2 at which 50% of the haem is oxygenated and T_1 and T_2 are the initial and final temperatures respectively of the range considered. Over the temperature range explored (20–40 °C), van't Hoff plots were linear within the experimental error. Average S.D.s of $\pm 8\%$ for the values of P_{50} and of $\pm 15\%$ for ΔH values were calculated.

Amino acid sequence analysis

The α - and β -chains were purified by HPLC using a C_8 semipreparative reversed-phase column (Aquapore RP 300, 7×250 mm; Applied Biosystems), developed in 20 min with a linear gradient from 40 to 70% acetonitrile in 0.2% (v/v) trifluoroacetic acid that was generated in a Perkin-Elmer model 250 liquid chromatography instrument at a flow rate of 2.8 ml/min. The absorbance was monitored at 220 nm with a Perkin-Elmer model LC 290 spectrophotometer. Alkylation with 4-vinylpyridine and tryptic digestion were performed on 2 mg of α - and β -globins as previously described [4]. Another aliquot (2 mg) of the two globins was cleaved with cyanogen bromide in 70% trifluoroacetic acid overnight at room temperature. Cleavage at the single Asp-Pro bond [10] of the two globins was performed by incubation in 70% formic acid at 37 °C for 48 h.

Tryptic peptide mixtures and cyanogen bromide fragments were purified on a C_{18} reversed-phase analytical column (Aquapore RP 300, 0.6×250 mm; Applied Biosystems) and developed in 60 min with a linear gradient from 0 to 60% acetonitrile in 0.2% (v/v) trifluoroacetic acid at a flow rate of 0.6 ml/min. The absorbance of the effluent was monitored at 220 nm.

The N-terminal blocked β -globin was loaded onto Pro-Blott membranes and treated with anhydrous trifluoroacetic acid for 4 min at 45 °C. After removal of the acid the protein was incubated for 16 h at 65 °C [11]. The N-terminal tryptic blocked peptide was treated with anhydrous trifluoroacetic acid for 2 h at 55 °C and then subjected to Edman degradation. The fragments derived from acid cleavage were subjected to treatment with *o*-phthalaldehyde to block the non-Pro N-terminal residues [12]. Automated Edman degradation was carried out using an Applied Biosystems model 473 A pulsed-liquid sequencer with on-line detection of phenylthiohydantoin amino acids.

Amino acid analyses were carried out with an LKB 4151 Alpha Plus instrument using an LKB 2121 integrator after the hydrolysis of samples in 6 M HCl at 110 °C for 24 h.

RESULTS

Primary structure

The α - and β -chains of *C. caretta* Hb were purified by HPLC (Figure 1) and their purity was checked by PAGE in the presence of Triton X-100 [13].

Direct sequence analysis of the α -chain allowed collection of structural information for the first 48 residues. However, the β -chain was accessible to Edman degradation only after the application of the de-blocking procedure [11]; this gave structural information for the first 13 residues. Moreover we have applied the de-blocking procedure on the N-terminal peptide in solution. The Edman degradation of this peptide confirmed the presence of a threonine at the N-terminus of the β -chain. The complete amino acid sequence of the α - and β -chains of *C. caretta* Hb is

Figure 1 Reversed-phase HPLC separation of globin chains of *C. caretta* Hb

Details are given in the Experimental section.

presented in Figure 2 along with the corresponding sequences of human protein and Hb from the fresh water turtle *Chrysemys picta bellii* [14].

The sequence of the two globins was reconstructed following purification and analysis of the complete set of tryptic peptides. Overlaps were obtained by means of cyanogen bromide fragments and fragments formed through acid cleavage.

General aspects

The number of amino acid substitutions in the α - and β -globin chains of *C. caretta* compared with other reptiles is reported in Table 1. It is interesting to note that the *C. caretta* α -chain shows a smaller number of amino acid substitutions when compared with the α -globin of crocodiles and *Sphenodon punctatus*, than when compared with iguana, varanus and sea snake. On the other hand, the β -chain shows a reversed pattern of similarity, with a smaller number of substitutions when compared with the β -chain of the Hbs of *S. punctatus*, iguana and varanus than when compared with the crocodile β -chain. These data suggest that in *C. caretta* Hb the α - and β -chains have followed a divergent evolutionary pattern.

$\alpha_1\beta_1$ and $\alpha_1\beta_2$ interfaces

The differences which are found when comparing the sequences of α - and β -chains of *C. caretta* Hb with those of human Hb include several residues which have been shown by X-ray crystallography on human adult Hb (HbA) to be involved in $\alpha_1\beta_1$ packing contacts. In particular, we have found five substitutions in the α -chain and seven substitutions in the β -chain.

In normal human HbA the $\alpha_1\beta_1$ contact is very tight and known to remain mainly unchanged during the quaternary conformational change which accompanies ligand binding at the haem site. However several mutants whose substitution site is located at the $\alpha_1\beta_1$ interface do show a relevant perturbation of the O_2 affinity. Hence, amongst the substitutions found in the *C. caretta* β -chain, the $\beta 108(G10)$ Asn $\rightarrow$ Ser may be of interest because the two mutants found at the same position in human HbA, $\beta 108(G10)$ Asn $\rightarrow$ Asp (Hb Yoshizuka) [15] and $\beta 108(G10)$

Table 2 Oxygen-binding parameters for HbA, *C. caretta* and bovine Hbs

Experiments were performed in 0.01 M Hepes buffer (pH 7.2).

Condition	P_{50}		
	HbA (mmHg)	Turtle Hb (mmHg)	Bovine Hb (mmHg)
$[Cl^-] = 0$	2.0	13.0	5.1
$[Cl^-] = 0.1$ M	6.1	17.0	14.5
$[Cl^-] = 0.1$ M [DPG] = 1 mM	14.8	21.4	17.6
$[Cl^-] = 0.1$ M [IHP] = 1 mM	43	22	19.2
$[Cl^-] = 0.1$ M [ATP] = 1 mM	—	21.5	—

Table 3 Heterotropic effects in *C. caretta*, HbA and bovine Hbs at 25 °C

Experiments were performed in 0.01 M Hepes buffer (pH 7.2). P_{50} is expressed in mmHg.

Effect	P_{50}		
	Turtle	Bovine	HbA
$\Delta \log P_{50} \pm 0.1$ M Cl^-	0.15	0.45	0.48
$\Delta \log P_{50} \pm 1$ mM IHP	0.11	0.12	0.85
$\Delta \log P_{50} \pm 1$ mM DPG	0.1	0.09	0.85

to an important hydrogen-bond at the $\alpha_1\beta_2$ interface [20,21] are both conserved.

Recently [22], the bicarbonate-ion-binding site has been located at the $\alpha_1\beta_2$ subunit interface by making various human–crocodile chimaeric Hbs. Amongst the 12 mutations introduced at the level of the globins interface of the human Hb to create the bicarbonate effect, only one Leu31 β → Met is present at the $\alpha_1\beta_2$ interface of the *C. caretta* Hb, thus excluding allosteric control of the O₂ affinity based on the binding of bicarbonate ions.

Functional studies

In agreement with previous results [7], values of P_{50} for turtle Hb oxygenation, both in the absence and presence of allosteric effectors, are significantly higher than those of human Hb in the presence of its physiological cofactors. In particular, oxygen equilibria of turtle Hb at 25 °C in chloride-free medium show, under the same experimental conditions, an almost 6-fold decrease of intrinsic O₂ affinity relative to HbA and a 2-fold decrease with respect to bovine Hb, this latter taken as a prototype of a low O₂-affinity Hb (Table 2).

As far as the effect of DPG and IHP is concerned, turtle Hb appears to be very similar to the bovine protein. Thus turtle Hb is not able to discriminate between these two heterotropic ligands since the functional effect (Table 3) is almost the same, being 8–9 times lower than that observed for human HbA. However, the most striking difference between turtle Hb and both bovine and human Hbs is represented by the effect of chloride, which is almost three times lower (Tables 2 and 3) in turtle. The Bohr effect for O₂ binding to turtle Hb (Figure 3) appears to be 50 % smaller, both in the absence and presence of allosteric effectors, than that displayed by human Hb. Moreover, turtle Hb displays a Bohr effect characterized by a substantial shift of the mid-point of the transition towards acidic pH values (mid-point values for turtle and human Hb respectively are 7.0 and 7.7). Moreover, a set of O₂ equilibrium experiments in chloride-free Hepes buffer

Figure 3 Effect of pH on the oxygen affinity of penguin Hb (circles), *C. caretta* Hb (squares) and *Chelonia mydas* Hb (triangles)

Open symbols refer to data obtained in 0.1 M Hepes buffer in the absence of both chloride and organic phosphates. Closed symbols refer to experiments performed in 0.1 M Hepes buffer + 0.1 M NaCl. Data on penguin and *C. caretta* haemoglobins were obtained at $T = 20$ °C. Data on *C. mydas* haemoglobin were taken from [27] and were obtained at $T = 25.7$ °C.

(Figure 3) clearly shows that the Bohr effect of turtle Hb is completely abolished in the absence of chloride ions.

Another peculiar feature of turtle Hb is represented by the overall heat of oxygenation ΔH and its pH dependence (Figure 4). At pH 7.3, the overall ΔH is -1.8 kcal/mol, four times lower

Figure 4 pH dependence of the overall oxygenation enthalpy (ΔH in kcal/mol) for turtle Hb as obtained in 0.1 M Hepes buffer + 0.1 M Cl^-

Values of ΔH are characterized by an S.D. of $\pm 15\%$ and were obtained at between 20 and 40 °C.

than the value generally measured for vertebrate Hbs and even slightly lower than that observed in arctic mammals [5]. It should also be noted that the very small ΔH values are negative at neutral and alkaline pH values and positive at acid pH, being +1.2 kcal/mol at pH 6.5. Hence oxygenation is exothermic at the extreme alkaline limit of the Bohr effect and becomes endothermic at the other extreme, being zero at around pH 6.9.

DISCUSSION

Low intrinsic oxygen affinity

Looking at the primary structure reported above, turtle Hb differs from the human Hb by amino acid replacements in both the α - and β -chains. Most of the replacements occur in positions where they would not affect oxygen binding (Table 4). In the search for a structural explanation of the low intrinsic O_2 affinity of turtle Hb we have to consider one important amino acid alteration: the presence of a hydrophobic moiety, very likely an acetyl group, blocking the β -chain N-terminus threonine.

This replacement renders turtle Hb in some way similar to ruminant Hbs and in particular to the bovine protein. In this molecule the hydrophobic side-chain of the terminal Met in position NA2 β has in fact been suggested to adhere to the hydrophobic interior of the β -chains, thereby mimicking the effect of DPG on human deoxy Hb [23].

An elegant study [24] has shown that in bovine Hb (compared with HbA) the A helix and the N-terminal residues are shifted closer to the main body of the β -chains and the dyad axis of symmetry. As seen from the N-termini, these shifts are the consequence of the displacement of the N-terminus of helix A

toward the dyad by 2.1 Å and an anti-clockwise rotation of 12°. It is important to recall that the same type of shift is induced in the human protein by the binding of DPG [25]. Hence, this structural change has been suggested to be the cause of the intrinsic low O_2 -affinity of bovine Hb. Following the line of thought of these authors, the main amino acid replacements potentially involved in this structural rearrangement are: (1) Pro β 5(A2) → Ala which renders the first turn of helix A more flexible and may allow the two N-terminal residues to stretch and shift towards the dyad axis; (2) Tyr β 130(H8) → Phe which eliminates the hydrogen-bond found in human Hb between the hydroxyl group of Tyr130(H8) and the carbonyl oxygen of Leu14(A11) and allows helix A to move closer to helix E and the dyad axis.

The turtle Hb is characterized by both these amino acid substitutions, which, by analogy with the bovine Hb, could be the basis for the intrinsic low O_2 -affinity.

Moreover, in the R-structure of mammalian Hbs the terminal carboxyl of His β 146(HC3) forms an external salt bridge with the ϵ -amino group of the Lys β 144(HC1) of the same β -chain. In turtle Hb, the replacement of lysine by glutamine makes the existence of this salt-bridge impossible; the conformational rearrangement which brings His β 146 into the proximity of Glu β 94 during the R → T transition should therefore require less energy than in the HbA molecule. That the T-state (low-affinity conformation) of the Hb from turtle could be more populated compared with other Hbs, even in the liganded derivative, has indeed been indicated by EPR spectroscopy of the nitrosylated derivative [7] and is consistent with the Lys β 144 → Gln substitution.

Table 4 Important amino acid replacements distinguishing turtle from bovine and human Hbs

E, External; I, internal; $\alpha_1\beta_1$, $\alpha_1\beta_2$, subunit contacts; A, B, CH denote helices; NA, AB, BC etc. denote non-helical segments. The sequence numbers for the β -chain refer to bovine Hb. For human and turtle Hb add 1 to each number, except the first two.

α -Chain					β -Chain				
Sequence number	Turtle	Bovine	Human	Position	Sequence number	Turtle	Bovine	Human	Position
A2 4	Ser	Ala	Pro	E	NA1 1	X-Thr	Met	Val	Central cavity
A6 8	Ala	Gly	Thr	E	NA2 2	His	—	His	Central cavity
AB1 19	Gln	Gly	Ala	E	A2 4	Ala	Ala	Pro	E
B3 22	Glu	Ala	Gly	E	A6 8	His	Ala	Ser	E
E9 60	Lys	Ala	Lys	E	A11 13	Met	Phe	Leu	Surface crevice
E13 64	Leu	Ala	Asp	E	B1 18	Asn	Lys	Asn	E
E17 68	Asp	Lys	Asn	E	D2 50	Ser	Ala	Pro	$\alpha_1\beta_1$ E
E20 71	Asn	Glu	Ala	E	D7 55	His	Asn	Gly	E
EF2 73	Ile	Leu	Val	Surface crevice	E13 68	Asn	Asp	Gly	E
EF5 76	Ile	Leu	Met	I	E14 69	Ser	Ser	Ala	E
EF7 78	Thr	Gly	Asn	E	E17 72	Ser	Asn	Asp	E
F3 82	Ala	Glu	Ala	E	E19 74	Val	Met	Leu	I
G11 104	Cys	Ser	Cys	$\alpha_1\beta_1$	E20 75	Lys	Lys	Ala	E
G18 111	Arg	Ser	Ala	Surface crevice	EF4 79	Asp	Asp	Asp	E
GH3 115	Thr	Ser	Ala	E	F3 86	Asp	Ala	Thr	E
GH4 116	Leu	Asp	Glu	E	G6 103	Lys	Lys	Arg	Central cavity
					G14 111	Ile	Val	Cys	$\alpha_1\beta_1$
					G18 115	Met	Arg	His	$\alpha_1\beta_1$
					G19 116	His	Asn	His	E
					H3 124	Pro	Val	Pro	Surface crevice
									$\alpha_1\beta_1$
					H4 125	Trp	Leu	Val	Surface crevice
					H7 128	Ala	Asp	Ala	Surface crevice
					H8 129	Trp	Phe	Tyr	I
					HC1 143	Gln	Arg	Lys	E

It is interesting to note that the same substitution is found in those fish Hbs which display the Root effect [26], an enhanced version of the alkaline Bohr effect, and hence a drastic drop in the oxygen affinity at acid pH.

Effect of organic phosphates

As far as the organic phosphate effect is concerned, it should be recalled that multiple organic phosphates are found in the red cells of adult sea turtle. ATP predominates and is accompanied by smaller amounts of DPG [27]. In most mammalian Hbs, binding of these effectors occurs at a specific site, which at neutral pH involves a cluster of eight positively charged amino acid residues: His(NA2), Lys(EF6), His(H21) and the α -amino group of Val(NA1) of each β -chain located on the dyad axis of the Hb tetramer [25]. In the case of the Hb from *C. caretta*, the reduced influence of organic phosphates on the O₂ affinity may be explained on the basis of the substitutions observed at this binding site. The mutation His β 143(H21) $\rightarrow$ Leu and the presence of a blocking group at the β -chain N-terminus eliminate four of the eight positive charges contributing to the interaction with organic phosphates. The allosteric effect of these compounds is therefore greatly diminished.

Bohr effect

As far as the Bohr effect is concerned, its shape seems well adapted for gas exchange during prolonged dives. In fact the drastically reduced pH dependence of the O₂ affinity within the physiological pH range, preserves turtle Hb from a severe and uncontrolled stripping of oxygen in response to acidosis. The O₂ delivery during diving is thus modulated essentially by the partial pressure of the gas in the specific tissue. A molecular interpretation of this markedly reduced Bohr effect is not easy. The alkaline Bohr effect finds its molecular basis in the preferential binding of protons to deoxy Hb rather than to oxy Hb. His β 146 forms a strong ionic bond with Asp β 94 in the T-state but is highly solvated in the R-state and it is therefore a primary candidate as alkaline Bohr group [23,25]. It has been demonstrated that this residue contributes to the chloride-independent part of the Bohr effect of human Hb (approx. 40%). All the remaining chloride-dependent part has been clearly attributed to Val α 1 and Lys β 82 [29,30]. Surprisingly, and in spite of the presence of His β 146, the Bohr effect of turtle Hb, which is approx. 50% of that of HbA, is completely abolished in the absence of chloride ions. This peculiar behaviour is analogous to that of the Hb from the emperor penguin, which is also characterized by a strongly reduced Bohr effect that vanishes in the absence of Cl⁻ [31]. On the whole, this phenomenon seems to be general among diving vertebrates, as shown by Figure 3 where the Bohr effect of another turtle Hb is reported both in the presence and absence of chloride ions.

The complete absence of the Bohr effect in the absence of chloride implies that the salt-bridge between His β 146 and Glu β 94 is either not closed in deoxy Hb or fails to open on oxygenation of the *C. caretta* Hb molecule. Although the latter case seems more likely, only a chrysallographic analysis of the molecule will give a definitive answer.

Temperature effect

The overall ΔH of O₂ binding represents the sum of the 'intrinsic' heat for the binding of the ligand and of all the other contributions

which derive from ligand-linked processes. In human Hb, more exothermic values are observed at very alkaline pH values where the Bohr effect is negligible. On going towards acid pH values the apparent ΔH of O₂ binding becomes less and less exothermic due to the increasing endothermic contribution of the Bohr protons, which cancels some of the heat released upon O₂ binding. Although with different absolute values (in the case of turtle Hb the ΔH of O₂ binding is strongly reduced compared with that of human HbA), the same behaviour applies to turtle Hb. The apparent heat of oxygenation is exothermic, even if small, for pH values higher than 6.9, and becomes endothermic at the other extreme of the Bohr curve due to the contribution of the Bohr protons. On the whole, the very small enthalpy change for O₂ binding could be regarded as a case of molecular adaptation through which O₂ delivery becomes essentially independent of the water temperature.

Moreover, it strongly suggests that the overall ΔH is positive at just those pH values (lower than 6.9) which may be reached by turtle tissues during prolonged dives due to the decrease in pH brought about by lactic acid production. Under these conditions, Hb reaching the tissues will find a more acid pH which lowers its oxygen affinity and renders positive its ΔH of oxygenation. This will favour the deoxygenation process, especially when the animal faces colder water during its diving activity.

In conclusion, the determination of the amino acid sequence of the Hb from the sea turtle *C. caretta* allowed the detection of specific amino acid substitutions which could be responsible for some of the peculiar functional properties displayed by this Hb.

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REFERENCES

- 1 Brix, O., Bardgard, A., Mathisen, S., El-Sherbini, S., Condo', S. G. and Giardina, B. (1989) *Comp. Biochem. Physiol.* **94B**, 135–138
- 2 Giardina, B., Brix, O., Nuutinen, M., El-Sherbini, S., Bardgard, A., Lazzarino, G. and Condo', S. G. (1989) *FEBS Lett.* **247**, 135–138
- 3 Giardina, B., Condo', S. G., Petruzzelli, R., Bardgard, A. and Brix, O. (1990) *Biophys. Chem.* **37**, 281–286
- 4 Petruzzelli, R., Barra, D., Bossa, F., Condo', S. G., Brix, O., Nuutinen, M. and Giardina, B. (1991) *Biochim. Biophys. Acta.* **1076**, 221–224
- 5 di Prisco, G., Condo', S. G., Tamburrini, M. and Giardina, B. (1991) *Trends Biochem. Sci.* **16**, 471–474
- 6 Conti, E., Casale, E., Ascenzi, P., Coletta, M., Condo', S. G., Merli, A., Giardina, B., Bordo, D. and Bolognesi, M. (1992) *Biochem. Biophys. Res. Commun.* **187**, 1063–1070
- 7 Giardina, B., Galtieri, A., Lania, A., Ascenzi, P., Desideri, A., Cerroni, L. and Condo', S. G. (1992) *Biochim. Biophys. Acta.* **1159**, 129–133
- 8 Ascenzi, P., Coletta, M., Desideri, A., Petruzzelli, R., Polizio, F., Bolognesi, M., Condo', S. G. and Giardina, B. (1992) *J. Inorg. Biochem.* **45**, 31–27
- 9 Giardina, B. and Amiconi, G. (1981) *Methods Enzymol.* **76**, 417–427
- 10 Landon, M. (1977) *Methods Enzymol.* **47**, 145–149
- 11 Wellner, D., Panneerselvam, C. and Horecker, B. L. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1947–1949
- 12 Brauer, A. W., Oman, C. L. and Margolies, M. N. (1984) *Anal Biochem.* **137**, 134–142
- 13 Alter, B. P., Goff, S. C., Efremov, G. D., Gravely, M. E. and Huisman, T. H. J. (1980) *Br. J. Haematol.* **44**, 527–534
- 14 Kleinschmidt, T. and Sgouras, J. (1987) *Biol. Chem. Hoppe-Seyler* **368**, 579–615
- 15 Imamura, T., Fujita, S., Ohta, Y., Hanada, M. and Yanese, T. (1969) *J. Clin. Invest.* **48**, 2341–2348
- 16 Moo-Penn, W. F., Wolff, J. A., Simon, G., Vacek, M., Jue, D. L. and Johnson, M. H. (1978) *FEBS Lett.* **92**, 53–56
- 17 Kohne, E., Behnken, L. J., Leupold, D., Rogge, H., Martin, H. and Kleihauer, E. (1979) *Haemoglobin* **3**, 365–370

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- 18 Baldwin, J. M. (1980) *J. Mol. Biol.* **136**, 103–128
- 19 Hashimoto, M., Ishimori, K., Imai, K., Miyazaki, G., Morimoto, H., Wada, Y. and Morishima, I. (1993) *Biochemistry* **32**, 13688–13695
- 20 Ishimori, K., Morishima, I., Imai, K., Fushitani, K., Shih, D. T., Tame, J., Pagnier, J. and Nagai, K. (1989) *J. Biol. Chem.* **264**, 14624–14626
- 21 Imai, K., Fushitani, K., Miyazaki, G., Ishimori, K., Kitagawa, T., Wada, Y., Morimoto, H., Morishima, I., Shih, D. T. and Tame, J. (1991) *J. Mol. Biol.* **218**, 769–778
- 22 Komiyama, N. H., Miyazaki, G., Tame, J. and Nagai, K. (1995) *Nature (London)* **373**, 244–246
- 23 Perutz, M. F., Kilmartin, J. V., Nishikura, K., Fogg, J., Butler, P. G. J. and Rollem, H. S. (1980) *J. Mol. Biol.* **138**, 649–670
- 24 Perutz, M. F., Fermi, G., Poyart, C., Pagnier, J. and Kister, J. (1993) *J. Mol. Biol.* **233**, 536–545
- 25 Arnone, A. (1972) *Nature (London)* **237**, 146–149
- 26 Root, R. W. (1931) *Biol. Bull. Woods Hole Mass.* **61**, 427–456
- 27 Lutz, P. L. and Lapennas, G. N. (1982) *Resp. Physiol.* **48**, 75–87
- 28 Perutz, M. F. (1970) *Nature (London)* **228**, 726–739
- 29 Shih, D. T. and Perutz, M. F. (1987) *J. Mol. Biol.* **195**, 419–422
- 30 Shih, D. T., Luisi, B. F., Miyazaki, G., Perutz, M. F. and Nagai, K. (1993) *J. Mol. Biol.* **230**, 1291–1296
- 31 Tamburrini, M., Condo, S. G., di Prisco, G. and Giardina, B. (1994) *J. Mol. Biol.* **237**, 615–621

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