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Adaptation to Extreme Environments: Structure–Function Relationships in Emperor Penguin Haemoglobin

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The functional properties of the single haemoglobin (Hb) of Emperor penguin (*Aptenodytes forsteri*) have been investigated at different temperatures as a function of proton and organic phosphate concentration. The complete amino acid sequence has been established. Comparison with that of human HbA shows 12 substitutions in the contact regions of $\alpha\beta$ dimers. In addition to overall similarities shared with most of the avian Hbs previously described, this Hb shows significant differences, which could be related to the peculiar behaviour of this penguin. In particular we may consider that: (1) the shape of the Bohr effect curve seems well adapted for gas exchange during very prolonged dives, preserving penguin Hb from a sudden and not controlled stripping of oxygen; (2) the very minor enthalpy change observed at lower pH could be an example of molecular adaptation, through which oxygen delivery becomes essentially insensitive to exposure to the extremely low temperatures of the environment. Moreover, the small alkaline Bohr effect has been found to be only chloride-linked, since the pH dependence of the oxygen affinity is totally abolished in the absence of this ion. These functional characteristics are discussed on the basis of the primary structure of α and β-chains.

Keywords: haemoglobin; penguin; amino acid sequence; temperature; oxygen affinity

1. Introduction

The present report is part of a series of investigations on the molecular adaptation to extreme environmental conditions, which in Arctic mammals (Brix *et al.*, 1989, 1990; Giardina *et al.*, 1989, 1990; Petruzzelli *et al.*, 1991; di Prisco *et al.*, 1991; Conti *et al.*, 1992) and diving animals (Brix *et al.*, 1990; Giardina *et al.*, 1992*a*,*b*) have provided insight into the role of temperature and of its interplay with heterotropic ligands in the modulation of Hb‡ function.

Along this line we have undertaken an investigation on the functional properties of the Hb of Emperor penguin (*Aptenodytes forsteri*). In fact penguins, of all birds, are the most fully adapted to water and to extreme cold, possessing a body morphology particularly suited to a cold liquid medium. On the whole, *A. forsteri* was chosen for the following main reasons: (1) the extremely low temperature that penguins have to face throughout the year, and (2) the fact that the respiratory system of penguins has to satisfy the oxygen demands arising from the extreme conditions of the Antarctic habitat and the characteristic diving behaviour.

The peculiar functional properties shown by Emperor penguin Hb are discussed in light of the amino acid sequence of α and β-chains and in comparison with other available sequences of mammalian and avian Hbs.

2. Experimental

(a) *Materials*

DEAE-cellulose (DE52) was from Whatman; trypsin (EC 3.4.21. 4), treated with L-1-tosylamide-2-phenylethylchloromethylketone, from Cooper Biomedical; Tris and

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‡ Abbreviations used: Hb, haemoglobin; P_6-inositol, inositol hexakisphosphate; P_{50}, partial pressure of oxygen required to saturate 50% of the haems; HbA, human adult haemoglobin; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; OPA, *o*-phthalaldehyde.

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bis-Tris from Sigma Chemical Co.; all sequanal-grade reagents from Applied Biosystems; HPLC grade acetonitrile from Carlo Erba. All other reagents were of the highest purity commercially available.

(b) *Hb purification*

Blood samples, kindly supplied by G. L. Kooyman, were drawn from adult Emperor penguins in the rookery located at Cape Washington (Antarctica).

Red cells, washed 3 times with isotonic NaCl solution, were lysed with 2 vol. of 1 mM Tris·HCl (pH 8.0); stromas and nuclei were eliminated by centrifugation. Hb was purified by ion-exchange chromatography of the haemolysate on a DE52 column (2·2 cm × 20 cm), equilibrated with 10 mM Tris·HCl (pH 7·6). Cellulose acetate electrophoresis of the haemolysate and purified Hb was performed as described (D'Avino & di Prisco, 1989). Hb was converted to the stable carbonmonoxy derivative and stored at −80°C or, alternatively, stabilized in the cyanmet form (Di Iorio, 1981) and precipitated in 60% saturated ammonium sulphate; the wet sediment was stored at 4°C. The former derivative was oxygenated in air under light exposure; after reduction with sodium dithionite, the latter derivative was spontaneously converted to the oxy form during gel filtration on a Sephadex G-25 column (1 cm × 20 cm), equilibrated with 10 mM Tris·HCl (pH 8·0).

(c) *Oxygen binding*

Oxygen-binding isotherms were determined by the tonometric method (Giardina & Amiconi, 1981), in the absence and presence of allosteric effectors, between 20°C and 40°C. The overall oxygenation enthalpy (ΔH, kcal/mol; 1 kcal = 4·184 kJ), corrected for the heat of oxygen solubilization (−3 kcal/mol), was calculated from the integrated van't Hoff equation:

$$\Delta H = -4{\cdot}574 \cdot [(T_1 \cdot T_2)/(T_1 - T_2)] \cdot \Delta \log P_{50}/1000.$$

Over the temperature range 20 to 40°C, van't Hoff plots were linear within experimental error. An average standard deviation of ±8% for values of P_{50} and of ±15% for ΔH values was calculated.

Spectrophotometric measurements were carried out with a Kontron 860 Uvikon spectrophotometer. Concentrated stock solutions of P_6-inositol (0·1 M) were prepared by dissolving the sodium salt in water and adjusting the pH to neutrality with concentrated phosphoric acid.

(d) *Amino acid sequence analysis*

The globin mixture was prepared by the acid-acetone method at −20°C (Rossi-Fanelli *et al.*, 1958). The α and β-chains were purified by reverse-phase FPLC on a ProRPC HR 5/10 column (Pharmacia). Solvents were 0·1% (v/v) trifluoroacetic acid (A) and acetonitrile (B). The purity of the globin chains was checked by SDS/PAGE, in a 9% to 15% (w/v) polyacrylamide linear gradient containing 8 M urea. Alkylation of cysteyl residues by reaction with 4-vinylpyridine and tryptic digestion of *S*-pyridylethylated chains were carried out as described (Tamburrini *et al.*, 1992).

After enzymatic cleavage of the globin chains, the peptide mixture was dissolved in 0·1% trifluoroacetic acid, centrifuged to remove the insoluble core and analysed by reverse-phase HPLC on a μ-Bondapak C_{18} column (Waters, 0·39 cm × 30 cm), equilibrated with solvent A (0·1% trifluoroacetic acid). Elution was carried out by an increasing gradient of solvent B (0·08% trifluoroacetic acid in acetonitrile).

The single, acid-sensitive Asp–Pro bond was cleaved in intact globin chains (Landon, 1977). The sample was dissolved in 70% (v/v) formic acid, spotted on a polybrene-coated filter and incubated in a heat-sealed plastic envelope at 42°C for 24 h. After incubation, the filter was used directly for sequencing.

Gas-phase hydrolysis of intact globins or tryptic peptides was carried out under nitrogen in 6 M HCl containing 1% (w/v) phenol, at 160°C for 1 h. Hydrolysates were analysed on a model 420A automatic amino acid derivatizer from Applied Biosystems, connected on line with a model 130A phenylthiocarbamyl amino acid analyser. Alternatively, samples were hydrolysed by the automatic hydrolysis station of the model 420A, and analysed as described above.

Tryptophan was determined after liquid-phase hydrolysis in 3 M mercaptoethanesulphonic acid at 110°C for 20 h (Penke *et al.*, 1974); cysteine was oxidized with performic acid for 2 h at 0°C (Hirs, 1967) and determined as cysteic acid after liquid-phase hydrolysis in 6 M HCl at 110°C for 20 h. Analyses were carried out on a Carlo Erba model 3A29 automatic amino acid analyser.

Amino acid sequence analysis was performed with a model 477A automatic sequencer from Applied Biosystems, equipped with a 120A analyser for the on-line detection of phenylthiohydantoin amino acids. After cleavage of the Asp–Pro bond, sequencing of the internal region of the globin was carried out after blocking the non-Pro *N*-terminal residue with OPA (Brauer *et al.*, 1984).

3. Results

(a) *Primary structure*

The electrophoretic analysis of the haemolysate showed a single component. Following ion-exchange chromatography, the α and β-chains were separated by reverse-phase FPLC (Figure 1). The purity was checked by SDS/PAGE.

The N termini of α and β-chains were accessible to Edman degradation, and sequencing procedeed for 24 and 35 residues, respectively. Following Asp-Pro cleavage, the sequences from Pro95 to Phe128 in the α-chains, and from Pro100 to Leu133 in the β-chains, were elucidated. After tryptic digestion of *S*-pyridylethylated globins, the peptide mixtures were fractionated by reverse-phase HPLC. Figure 2A shows the elution pattern of the peptides of the α-chain; all peptides were separated, except T6 and T13 (separated on the same column by a slightly modified gradient, not shown). The elution pattern of the β-chain peptides is shown in Figure 2B; poor separation of T7B and T10 did not impair amino acid and sequence analyses. T3 and T7A were not resolved; however, due to their different yields, sequencing was carried out directly in the mixture. T11 was extracted with 0·1% (w/v) SDS from the insoluble core, and purified by reverse-phase HPLC (not shown) under different conditions. The peptides were aligned with the sequences of the N-terminal portion, of internal

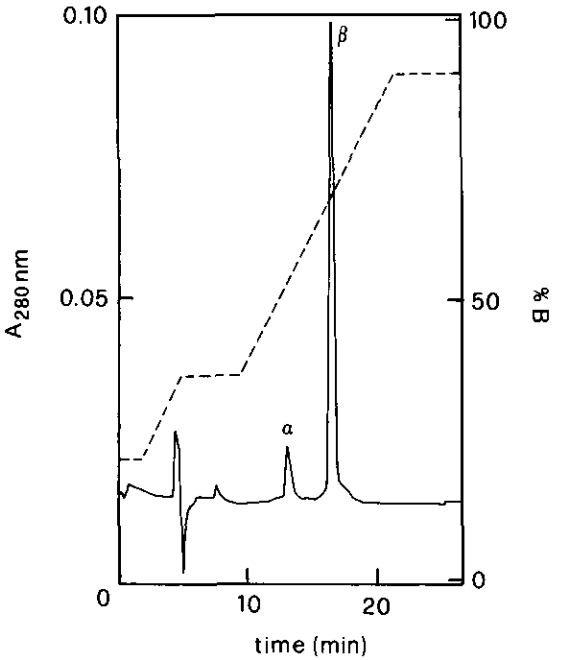

Figure 1. Reverse-phase FPLC of the globin chains of Emperor penguin Hb. Details are given in Experimental.

Table 1
Amino acid composition of α and β-chains of Emperor penguin Hb

	α-Chain	β-Chain
Asx	12·2 (12)	14·1 (14)
Thr	5·6 (7)	4·5 (5)
Ser	11·6 (12)	7·1 (7)
Glx	10·1 (10)	11·6 (11)
Pro	4·9 (5)	5·1 (5)
Gly	6·1 (6)	7·2 (7)
Ala	17·1 (17)	20·3 (20)
Cys	2·1 (2)	2·5 (3)
Val	10·5 (11)	12·8 (13)
Met	1·7 (2)	1·9 (2)
Ile	6·5 (7)	5·4 (6)
Leu	14·1 (14)	16·9 (17)
Tyr	3·6 (4)	2·1 (2)
Phe	7·1 (7)	8·1 (8)
His	9·5 (10)	6·6 (7)
Lys	12·3 (12)	10·1 (10)
Arg	3·1 (3)	5·8 (6)
Trp	0 (0)	3·3 (3)
No. of Residues	141	146

Relative molar amounts of amino acid residues are given. The number of residues from the sequences are indicated in parentheses.

regions after Asp-Pro cleavage, and on the basis of homologies with other known sequences.

The primary structure of Emperor penguin Hb is reported in Figure 3. The amino acid composition of the α and β-chains are summarized in Table 1; the molecular mass, calculated from the sequence, is 15,470 and 16,166 Da, respectively, in good agreement with the values calculated using SDS/PAGE (15,200 and 16,000 Da).

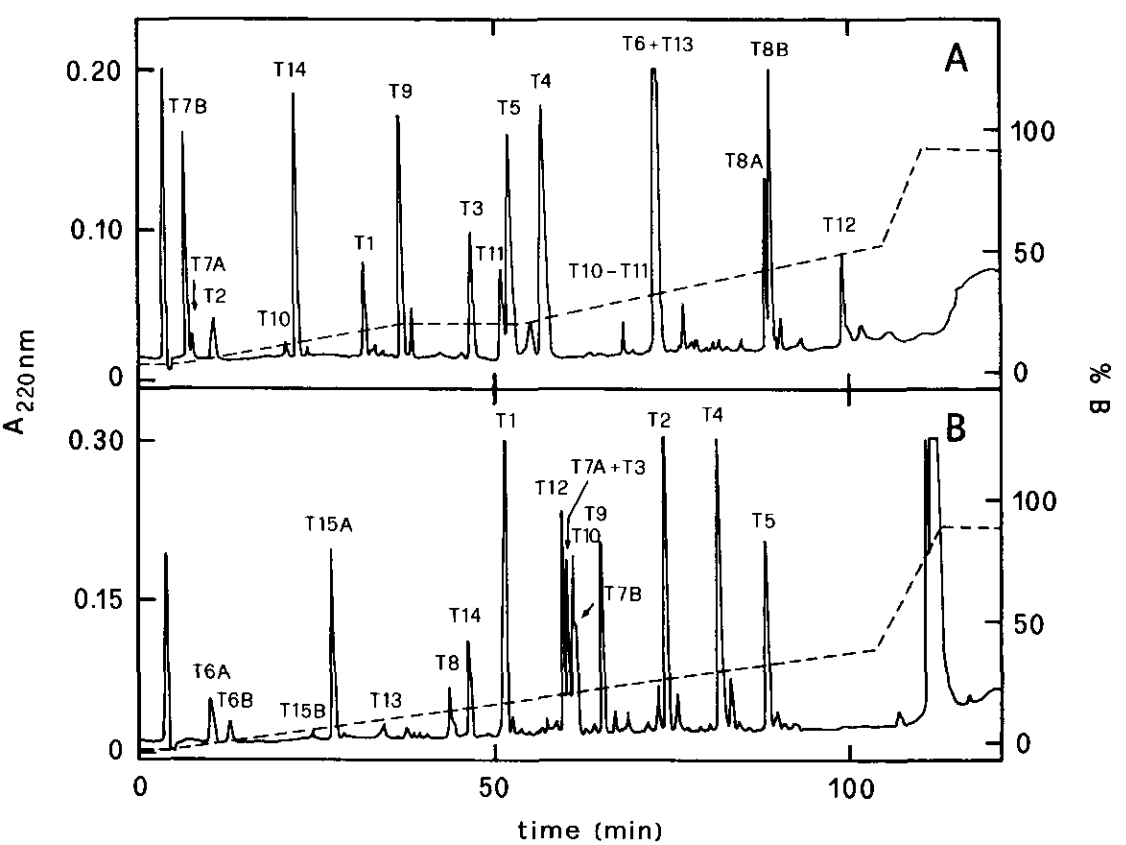

Figure 2. Reverse-phase HPLC of the tryptic peptides of α (A) and β (B)-chains. Details are given in Experimental.

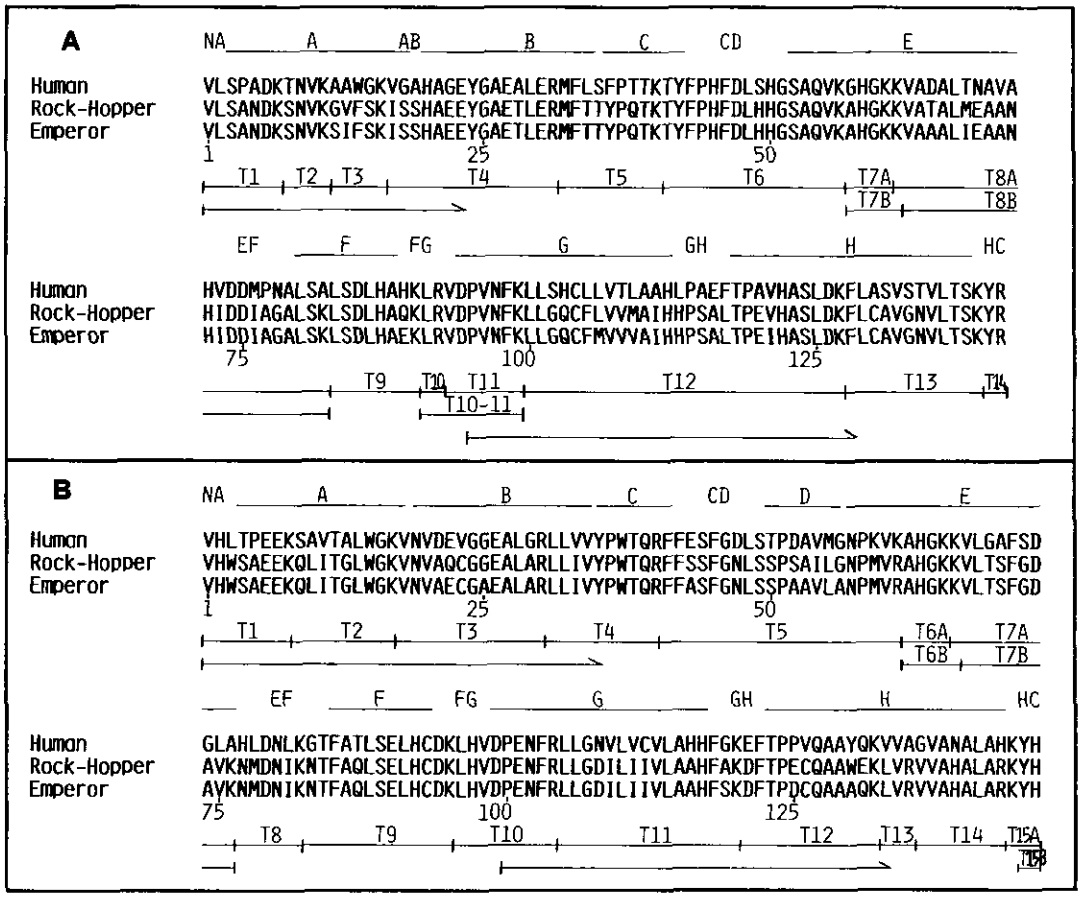

Figure 3. Amino acid sequence of Emperor penguin α (A) and β (B)-chains. The sequences of Rock-Hopper penguin and adult human Hbs are aligned for comparison. T1 to T15B represent the tryptic peptides; the arrows denote sequence portions established by automatic Edman degradation of the intact chain and after Asp-Pro cleavage.

(b) *Functional characterization*

Although the physiological cofactor of bird Hbs is P_5-inositol, the effect of organic phosphates on the oxygen affinity of Emperor penguin Hb was investigated using P_6-inositol, since most of the available data on Hb from other species concern the interaction with this effector. It has been shown (Brygier & Paul, 1976; Vandecasserie *et al.*, 1976; Lutz, 1980; Giardina *et al.*, 1985) that the effect of P_5-inositol is only slightly lower than that of P_6-inositol. This observation was fully confirmed by the data reported in Figure 4, which illustrates the oxygen affinity, expressed as $\log P_{50}$, of red blood cells and of purified Hb in the absence and presence of P_6-inositol. At 37°C, the Bohr effect of red blood cells was identical to that displayed by purified Hb in the presence of 3 mM P_6-inositol; the value of the Hill coefficient (not shown) did not change significantly under the different conditions and was close to 2.5. Hence the data reported herewith are physiologically meaningful.

In the absence and presence of P_6-inositol, the Bohr effect appeared greatly reduced (the Bohr coefficient, $\Delta\log P_{50}/\Delta$pH being $-0{\cdot}25$) in comparison to Hbs of most vertebrates (di Prisco *et al.*, 1991) at all temperatures tested. Addition of 3 mM P_6-inositol induced a marked decrease of the oxygen affinity, without changing the shape of the Bohr effect curve: at pH 7·5 and 37°C, P_{50} was increased from 4·1 mmHg to 35·5 mmHg in the presence of the effector. However, the Bohr effect appeared to be totally dependent on the presence of chloride ions, since in experiments carried out in chloride-free Hepes buffer, both at 20°C and at 37°C, the pH dependence of the oxygen affinity was completely abolished (see Figure 4).

Another peculiar feature of *A. forsteri* Hb is the influence of pH on the overall heat of oxygenation (ΔH) (Figure 5). In the absence of P_6-inositol, ΔH was close to $-9{\cdot}5$ kcal/mol, and was virtually pH-independent, even in the pH region in which the Bohr effect is operative. In contrast, in the presence of the effector, oxygenation became less and less exothermic at decreasing pH. At pH 6·5 the overall ΔH value was $-3{\cdot}4$ kcal/mol, a twofold lower value than that generally measured in vertebrate Hbs and comparable to that observed in Arctic mammals (Brix *et al.*, 1989,1990; Giardina *et al.*, 1989, 1990; Petruzzelli *et al.*, 1991; di Prisco *et al.*, 1991). However, on the basis of what is known on the interaction of P_6-inositol with Hb, dissociation of this effector should be strongly endothermic, and this would imply a large reduction of the overall ΔH

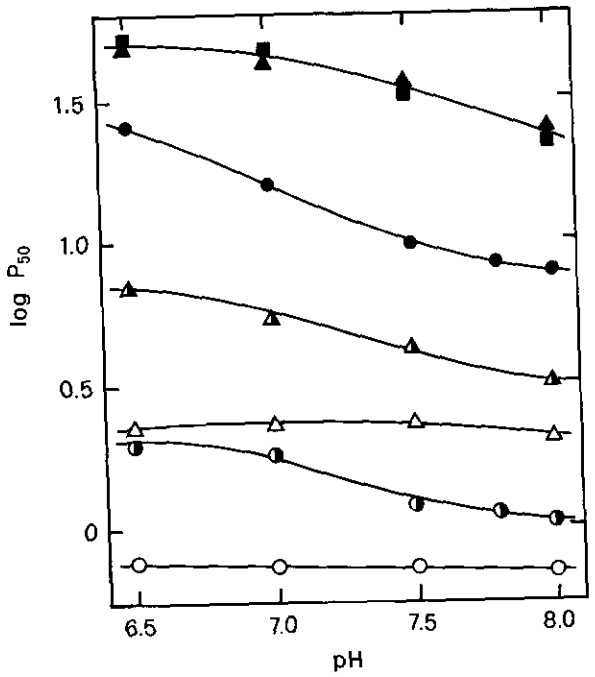

Figure 4. Effect of pH on the oxygen affinity at 20°C (circles) and 37°C (triangles), in 0·1 M Hepes buffer (open symbols), and in 0·1 M bis-Tris·HCl or Tris·HCl buffers containing 0·1 M NaCl, in the absence (half-filled symbols) and presence (filled symbols) of 3 mM P_6-inositol. Squares refer to experiments at 37°C on erythrocytes in iso-osmotic buffer.

of oxygenation. Hence, in order to ascertain the supposed peculiarity of the very low ΔH of oxygen binding observed at acid pH for penguin Hb, a

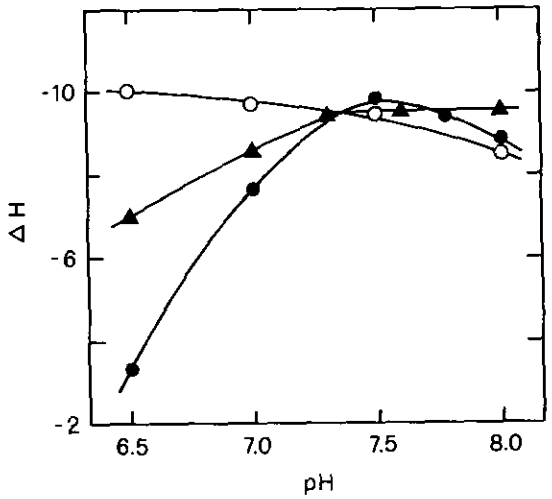

Figure 5. Overall oxygenation enthalpy (kcal/mol of oxygen) as a function of pH of penguin (circles) and pigeon (triangles) Hbs, in 0·1 M bis-Tris·HCl or Tris·HCl buffers containing 0·1 M NaCl, in the absence (open symbols) and presence (filled symbols) of 3 mM P_6-inositol. The values were calculated from the van't Hoff equation, using data from oxygen equilibrium experiments at different temperatures, and are corrected for the heat of oxygen solubilisation (−3 kcal/mol).

series of experiments was performed, under the same experimental conditions, on Hbs from other birds, e.g. pigeon (*Columba livia*) and flamingo (*Phoenicopterus ruber roseus*). The results reported in Figure 5 indicate that, at pH 6·5, pigeon Hb is characterized by an overall ΔH of oxygen binding which is almost twice as high as that observed in penguin Hb. Flamingo Hb shows the same behaviour (not shown).

4. Discussion

Previous studies have dealt with penguin blood metabolism, mainly focussed on haematological and physiological aspects (Lenfant *et al.*, 1969; Nicol *et al.*, 1988). The only complete amino acid sequence of a penguin Hb published so far is that of the Rock-Hopper penguin (*Eudyptes crestatus*: Huber *et al.*, 1988), one of the smaller-sized sub-Antarctic penguins.

This paper reports the complete amino acid sequence of the α and β-chains of the single Hb from the adult Emperor penguin. Previous papers (Monier *et al.*, 1973; Schnek *et al.*, 1978) reported the incomplete sequence of the α-chain of this Hb. The sequence reported here differs considerably from that published in the above-mentioned papers.

Emperor penguin Hb has a very high degree of sequence identity with Rock-Hopper Hb: 94% and 93% for α and β-chains, respectively. The invariant residues in the chains of all vertebrates, as well as those participating in the haem contacts in human Hb (Dickerson & Geis, 1983), are conserved. Among the sliding contact residues, Gln replaces Thr in αC3(38), and Ala replaces Glu in βCD2(43). Further replacements were found among the packing contact residues. In fact, in comparison with human Hb, the α-chain shows changes at six $\alpha_1\beta_1$ and one $\alpha_1\beta_2$ contact positions. The β-chain only has five substitutions at $\alpha_1\beta_1$ contact positions. The residues involved in the organic phosphate binding site (Arnone, 1972) are identical to those of other avian Hbs (Table 2); hence in comparison with human

Table 2

*Amino acid residues for the binding of organic phosphates in Hbs from Emperor penguin (*Aptenodytes forsteri*), chicken (*Gallus gallus*) and pigeon (*Columba livia*)*

Residue	Species			
	Human	Penguin	Chicken	Pigeon
NA1 (β1)	Val	Val	Val	Val
NA2 (β2)	His	His	His	His
EF6 (β82)	Lys	Lys	Lys	His
H13 (β135)	Ala	Arg	Arg	Arg
H17 (β139)	Asn	His	His	His
H21 (β143)	His	Arg	Arg	Arg

The residues involved in the DPG binding site of human HbA are reported for comparison. All the other amino acid sequences have been obtained from Kleinschmidt & Sgouros (1987).

HbA, at position β143 there is Arg and not His, and two other basic residues (Argβ135 and Hisβ139) are found in the internal cavity. Therefore, the much lower heat liberated on oxygenation of penguin Hb in comparison with that observed in the case of other bird haemoglobins cannot be explained on the basis of the heat absorbed on P_6-inositol dissociation from its binding pocket, and should be linked to other still unknown structural features.

Although the interactions with protons and organic phosphates are qualitatively similar to those found in other vertebrate Hbs, some peculiar features could be linked to the life style of this bird.

Firstly, the shape of the Bohr effect curve at 37°C seems well adapted for gas exchange during prolonged dives. In fact, similar to another diving organism, i.e. the sea turtle *Caretta caretta* (Giardina *et al.*, 1992*a*), the pH dependence of the oxygen affinity, within the physiological pH range, is drastically reduced, preserving this Hb from a severe and uncontrolled stripping of oxygen in response to acidosis. Hence, during diving, the oxygen delivery should be modulated essentially by the partial pressure of the gas at the specific tissue.

A molecular interpretation of the marked reduction of the amplitude of the Bohr effect in penguin Hb is not easy. In fact, the alkaline Bohr effect finds its molecular basis in the preferential binding of protons to deoxyHb with respect to oxyHb, and its amplitude is related to the number of protons bound per deoxyHb tetramer minus the number of protons bound to oxyHb. Hisβ146 is a primary candidate for the "alkaline Bohr group" because it forms a strong ionic bond with Aspβ94 in the T state, whereas it is highly solvated in the R state (Perutz, 1970; Perutz *et al.*, 1980). It has been shown (Shih & Perutz, 1987; Shih *et al.*, 1993) that Hisβ146 contributes to the chloride-independent part of the Bohr effect of human HbA (approx. 40%), and that Valα1 and Lysβ82 contribute to all of the remaining chloride-dependent part. Despite the presence of Hisβ146, the Bohr effect of penguin Hb (which is approx. 60% with respect to that of HbA) is abolished in the absence of chloride ions. This observation implies that the salt bridge between Hisβ146 and Aspβ94 is either not closed in deoxy or fails to open on oxygenation of the haemoglobin molecule. Although the latter case seems more likely, only a crystallographic analysis of the molecule will give a definite answer.

Secondly, during prolonged dives, the peripheral tissues of penguins are characterized by acid pH (Kooyman, 1975) and by a lower temperature than that of the rest of the body; due to the low ΔH value observed at acid pH, oxygen transport is not impaired at flippers and feet (which experience a lower temperature and great muscular activity), allowing the bird to endure more prolonged periods of anaerobiosis. Moreover, penguins hibernate and reproduce on the fast ice, and the skin temperature of the feet (in permanent contact with ice) falls near 0°C. Following egg laying, the male incubates the egg, keeping a stand-up position for about 64 days, holding it on the feet against the body to prevent it from freezing, and living on stored fat reserves. This would produce a significant metabolic acidosis, which in turn may benefit tissue respiration at the feet, once again because of the low ΔH value of oxygenation observed at acid pH.

Further investigations at the structural level are required for a more refined interpretation of the molecular mechanisms at the basis of the functional modulation of this haemoglobin.

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