From the Arctic to fetal life: physiological importance and structural basis of an 'additional' chloride-binding site in haemoglobin[1]

M. Cristina DE ROSA*, Massimo CASTAGNOLA*, Claudia BERTONATI*, Antonio GALTIERI† and Bruno GIARDINA*[2]

*Institute of Biochemistry and Clinical Biochemistry and CNR Institute of Chemistry of Molecular Recognition, Catholic University of Rome, L.go F. Vito 1, 00168 Rome, Italy, and †Department of Organic and Biological Chemistry, University of Messina, Salita Sperone 31, Villaggio S. Agata, 98166 Messina, Italy

Haemoglobins from mammals of sub-Arctic and Arctic species, as well as fetal human Hb, are all characterized by a significantly lower ΔH of oxygenation compared with the majority of mammalian haemoglobins from temperate species (exceptions are represented by some cold-resistant species, such as cow, horse and pig). This has been interpreted as an adaptive mechanism of great importance from a physiological point of view. To date, the molecular basis of this thermodynamic characteristic is still not known. In the present study, we show that binding of extra chloride (with respect to adult human Hb) ions to Hb would significantly contribute to lowering the overall heat of oxygenation, thus providing a molecular basis for the low effect of temperature on the oxygenation–deoxygenation cycle. To this aim, the oxygen binding properties of bovine Hb, bear (*Ursus arctos*) Hb and horse Hb, which are representative of this series of haemoglobins, have been studied with special regard to the effect of heterotropic ligands, such as organic phosphates (namely 2,3-diphosphoglycerate) and chloride. Functional results are consistent with a mechanism for ligand binding that involves an additional binding site for chloride ion. Analysis of computational chemistry results, obtained by the GRID program, further confirm the hypothesis that the reason for the lower ΔH of oxygenation is mainly due to an increase in the number of the oxygen-linked chloride-binding sites.

Key words: chloride-binding site, haemoglobin, oxygen affinity, oxygenation enthalpy, molecular modelling.

INTRODUCTION

The work done over the years on haemoglobins from different species has pointed out the physiological importance of the overall enthalpy change associated with the reaction of Hb with oxygen [1–4]. In fact, binding of oxygen to Hb is generally an exothermic reaction and a decrease in temperature induces an increase in oxygen affinity. This phenomenon could be very dangerous for mammals which are confronted to very low environmental temperatures (in the Arctic the environmental temperature can reach values down to − 40 °C), so their haemoglobins are characterized by much lower enthalpy values. Figure 1 shows the binding curves of human and bovine (a well-known cold-resistant species) Hb at 30 °C and 10 °C and the percentage of deoxygenation which could be obtained on going from an oxygen pressure of 100 mmHg (where 1 mmHg = 0.133 kPa), about that existing at the level of the lungs, to an oxygen pressure of 40 mmHg, i.e. about that of the tissues. As evident from the Figure, on passing from a temperature of 30 °C (about the temperature at the level of the lungs) to a temperature of 10 °C (even lower temperatures can be observed at the level of peripheral tissues in conditions of very low environmental temperature) in the case of human adult Hb, almost no oxygen can be delivered to the tissues due to the great increase of oxygen affinity caused by the decrease in temperature linked to the specific overall enthalpy change of the oxygenation reaction.

In other words, with the external temperature as low as − 40 °C, it is vital to reduce the temperature dependence of oxygen binding as much as possible. In this way, in fact, deoxygenation of Hb will require much less heat, and oxygen can still be released from the blood and transferred to the peripheral and colder tissues [5–9], as indicated by the case of bovine Hb (represented in Figure 1 by the broken lines). Thus, contrary to human adult Hb, bovine Hb, being characterized by a significantly lower enthalpy change of oxygenation and therefore by a significantly lower temperature dependence of the oxygenation–deoxygenation reaction, is still able to release a significant amount of oxygen even at 10 °C (about 12 % desaturation, Figure 1). This allows the organism to maintain a given metabolic rate, even if this is obviously lower than that which is observable at higher temperatures.

This is also the case of reindeer (*Rangifer tarandus*) [10,11] and musk ox (*Ovibos muschatos*) [12], whose haemoglobins are almost insensitive to temperature changes, and oxygen delivery is not drastically impaired at the level of skin, legs, etc., which may be up to 10 °C cooler than the lungs. The same has been shown to apply to whale (*Balaenoptera acutorostrata*) and penguin (*Aptenodytes forsteri*): the first has to sustain the metabolic activity of tail and fins muscles by an adequate release of oxygen, notwithstanding the unfavourable temperature gradient [7,8], and the second has to overcome the difficulties related to the cold Antarctic water and the lack of oxygen during extended diving [9].

This series of adaptive mechanisms is enriched by the case of fetal human Hb that, although representative of a different situation, adds useful information for the emerging overall scheme and for the physiological relevance of the overall enthalpy change associated with oxygen transport. Fetal human Hb is known to display at 20 °C a lower affinity for oxygen than HbA when both proteins are in the absence of DPG (2,3-diphosphoglycerate). Upon addition of DPG, the oxygen affinities of the two proteins become identical due to the lower effect of this organic phosphate on fetal human Hb, because of the well-known amino acid substitutions present in γ chains. It is only as the temperature rises to 37 °C that a higher oxygen affinity of fetal human Hb with respect

Abbreviations used: DPG, 2,3-diphosphoglycerate; MD, molecular dynamics; RMSD, root mean square deviation.

[1] Bruno Giardina dedicates this work to the memory of Eraldo Antonini who inspired his work on the comparative aspects of haemoglobin.

[2] To whom correspondence should be addressed (email bgiardina@rm.unicatt.it).

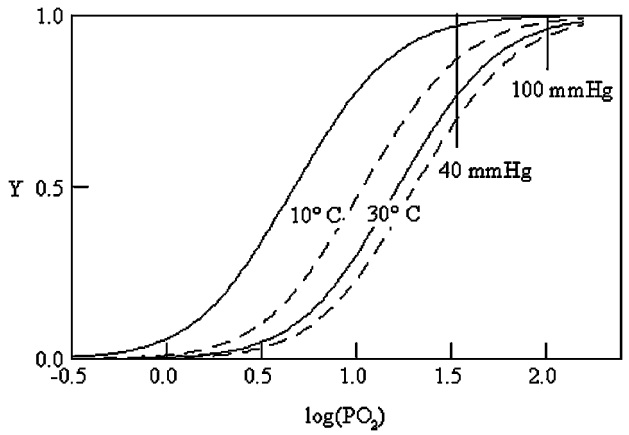

Figure 1 Saturation curves of human and bovine Hb

Theoretical curves of saturation of human (continuous line) and bovine (broken line) haemoglobins at 10 and 30 °C were obtained using the known functional parameters [13–15]. Tissues and alveolar pressures are represented by 40 and 100 mmHg respectively.

to adult human Hb is observed. It is clear from this observation that fetal human Hb has a decreased ΔH of oxygenation when compared with maternal Hb [13].

As the structural reasons of this mechanism are unknown, much of the experimental effort in the last number of years has been devoted to the identification of the regions of the molecule which may be able, through specific mutations, to modulate the thermodynamics of oxygen binding.

In this respect, it could be useful to recall the elegant site-directed mutagenesis experiments performed on adult human Hb by Fronticelli et al. [14,15] in order to clarify the molecular basis of the peculiar functional characteristics of bovine Hb, which is characterized by an intrinsically low oxygen affinity and is known not to be modulated by DPG *in vivo*. These experiments have clearly indicated the existence in bovine Hb of a particular, specific and additional (with respect to human Hb) oxygen-linked chloride-binding site that involves a lysine residue (replaced by alanine in human Hb) at position β76. This residue together with Lys-β8 and His-β77 (present in both human and bovine Hb) constitutes a new chloride-binding site. Also, as bovine Hb is characterized by a low ΔH of oxygen binding [3], we thought that the endothermic contribution made by the oxygen-induced release of the 'additional' chloride ions (one for each β chain) may contribute significantly to lowering the overall heat of oxygenation, providing us with a molecular basis to explain the particular thermodynamics of the oxygenation–deoxygenation cycle that characterize the Hb from mammals living in extreme environments.

In this perspective, we have inspected the amino acid sequences of a number of haemoglobins which are known to be characterized by a small value of the oxygenation enthalpy, from reindeer to fetal human Hb, and we observed that all of them present a cluster of basic residues in β8, β76 and β77 position. Then, in order to verify that this cluster of amino acids represents indeed an oxygen-linked binding site for chloride ion (i.e. the T state of Hb is characterized by a higher affinity for chloride with respect to the R state), a detailed functional characterization and a computational chemistry study have been carried out on a number of examples of haemoglobins characterized by a low effect of temperature. Our findings suggest that the cluster of basic residues in β8, β76 and β77 positions, indeed, represents an 'additional' chloride-binding site and that it provides the structural basis for an adaptive mechanism of significant physiological importance.

EXPERIMENTAL

Brown bear (*Ursus arctos*) blood samples

Blood samples were drawn, under anaesthesia, from the cephalic vein of a brown bear, aged 22 months, in the Zoological Garden of the University of Oulu (Finland). Blood was collected into an iso-osmotic NaCl solution containing 2 mM EDTA.

Horse and bovine blood samples

Blood samples were collected from the jugular vein without anaesthesia into an iso-osmotic NaCl solution containing 2 mM EDTA.

Methods

In all the three cases, red blood cells were washed three times with isotonic NaCl solution by centrifugation at 800 ***g***; at the end of the washing procedure, the packed cells were lysed by adding 3 vol. of cold water. Stroma were removed by centrifugation at 9000 ***g*** for 30 min.

Removal of organic phosphates and inorganic ions was obtained by passing the haemolysate firstly through a Sephadex G-25 column, equilibrated with 0.01 M Tris/HCl (pH 8.0), 0.1 M NaCl, and then by recycling the protein solution through a column of mixed-bed ion-exchange resin (Bio-Rad, AG 501 × 8).

The solution of DPG were prepared by dissolving the sodium salt of DPG (Sigma) in water or in the appropriate buffer.

Oxygen-binding isotherms, in the absence and in the presence of the effectors, have been determined by the tonometric method and/or by Hemox-Analyser [16] at a protein concentration of 3–5 mg/ml. The overall oxygenation enthalpy (ΔH, kcal/mol; where 1 cal = 4.184 J) has been calculated from the integrated van't Hoff equation:

$$\Delta H = -4.574[(T_1 \times T_2)/(T_1 - T_2)]\Delta \log P_m/1000$$

where P_m is the median pressure of the ligand. Due to the highly symmetric shape of the binding curves, P_m is almost equal to P_{50}, which is the partial pressure at which 50% of haem molecules are oxygenated.

Curve-fitting, as a function of effector concentration, was carried out by using least-squares fitting procedure according to the following equation:

$$\log P^{obs}{}_m = \log P^{o}{}_m + R \log\{(1 + K_d[M])/(1 + K_o[M])\}$$

where $P^{obs}{}_m$ refers to the oxygen affinity observed at a given concentration [M] of the effector under investigation, $P^{o}{}_m$ corresponds to the oxygen affinity displayed in the absence of the effector, K_d and K_o are the association equilibrium constants for the effector to unliganded and liganded Hb respectively, and R corresponds to the number of effector binding sites for haem (i.e. $R = 0.25$ for DPG).

Confidence limits of the data are $\pm 8\%$ for P_{50} and $\pm 15\%$ for ΔH values.

Grid searches

Investigation of the primary structure of haemoglobins displaying a low ΔH of oxygenation (Table 1) was performed using SWISS-PROT database, and the presence in the sequences of three basic residues in β8, β76 and β77 position are displayed (Table 2). Identification of oxygen-linked binding sites for Cl^- was carried out on human adult Hb and extended to all haemoglobins, which are characterized by a low ΔH of oxygenation, whose three-dimensional structure is available in the Protein DataBank: bovine

Table 1 ΔH of oxygenation of adult human Hb in comparison with ΔH of oxygenation of haemoglobins from other species in the absence and in the presence of DPG

Conditions are 0.1 M Hepes buffer plus 0.1 M NaCl, pH 7.4. Confidence limit of ΔH values is $\pm$ 15 %.

	ΔH (kJ/mol)	
Hb	− DPG	+ DPG
Adult human	− 41.0	− 36.0
Fetal human	− 35.5	− 27.2
Reindeer	− 14.0	− 14.0
Musk ox	− 15.0	− 15.0
Pig	− 34.0	− 17.0
Bovine	− 27.2	− 18.1
Horse	− 28.4	− 20.0
Bear	− 34.5*	− 29.3*

* Determined at pH 7.3.

Table 2 Amino acid residues at β8, β76, β77 position in the various haemoglobins displaying a low ΔH of oxygenation

		Amino acid		
Hb	Position ...	β8	β76	β77
Adult human		Lys	Ala	His
Fetal human		Lys	Lys	His
Reindeer		Lys	Lys	His
Pig		Lys	Lys	His
Bovine		Lys	Lys	His
Horse		Lys	His	His
Bear		Lys	Lys	Asn

Hb, horse Hb and fetal human Hb. The co-ordinates of T and R conformations for human adult Hb (PDB codes 1hhb and 1hho respectively), bovine Hb (PDB codes 1hda and 1g09 respectively), bovine Hb (PDB codes 2dhb and 1g0b respectively) and of T conformation (the only available) for fetal human Hb (PDB code 1fdh) were used for this analysis.

Favourable binding sites for Cl^- were calculated using the program GRID [17], which determines the interaction energy of a 'probe', representing a chemical fragment, at each point of an orthogonal grid which encloses the structure of a target macromolecule. The grid spacing was set at 0.5 Å. Oxygen-linked binding sites for chloride ion were calculated using a grid which surrounds each protein structure, exceeding it by 5 Å in each dimension. The standard GRID energy function and parameters were used to calculate the interaction energy.

In the calculations the positions of all the core atoms were fixed, but in GRID it was possible to take into account the flexibility of side chain atoms of the protein which can move towards the probe (when there is attraction) or away from it (when there is repulsion) [18].

Results can be displayed as contour maps, which indicate favourable locations for the Cl^- ion (the 'probe'), using InsightII (Accelrys, San Diego, CA, U.S.A.).

MD (molecular dynamics) simulations of the predicted Hb–Cl^- complexes

MD simulations were carried out using AMBER 1994 force field [19], implemented in the Discover program (Accelrys). The starting positions of the Cl^- ions were introduced in the crystallographic structures of bovine Hb, horse Hb and fetal human Hb exactly in correspondence with the minimum energy positions calculated by GRID program within the cluster β8, β76 and β77. No distance constraints were introduced to prevent a bias in the modelling, i.e. the chloride ions are free to leave the protein. In order to mimic aqueous solvent conditions each complex was soaked with a 4 Å layer of water molecules resulting in the introduction of about 1800 water molecules and a distance-dependent di-electric constant was also used [20]. Sodium ions were introduced as free unbound counterions, ensuring that each simulation was thus electrically neutral. These ions did not co-ordinate with any of the protein residues at any time during the simulation [21]. The solvent molecules were equilibrated initially by energy minimization and subsequently by performing 12 ps of MD. After energy minimization of the whole system (protein + chloride + water + counterions) by 2000 steps of steepest descent followed by approx. 3000 steps of conjugate gradient until the maximum derivative values were lower than 0.1 kcal/mol per Å, MD trajectories were calculated. A non-bonding cut-off of 12 Å was used. Temperature was slowly increased from 0 to 300 K in 20 ps and then maintained constant for the whole simulation time. The Verlet leapfrog algorithm [22] was applied using a time-step of 1.0 fs. The simulations were performed for 200 ps and co-ordinates and energy values were collected every 1000 steps for further analysis. The average structures over the last 50 ps of MD were analysed by the program PROCHECK (version 3.3.2) [23] to ascertain the stereochemical quality of the model structures.

RESULTS

Functional studies

Several years ago [24] it was shown, for the first time, that in bear Hb chloride ions and DPG can modulate the oxygen affinity in a synergistic way, such that their individual effect is enhanced whenever they are both present in saturating amount. The thermodynamic analysis of such a feature has clearly indicated that in bear Hb there are two classes of chloride-binding sites, one acting synergistically with DPG and another one which overlaps with the organic phosphate interaction cleft and is therefore only fully operative in the absence of DPG. Almost in the same period, Fronticelli et al. [14,15] have indicated the existence, in bovine Hb, of the specific and additional (with respect to human Hb) oxygen-linked chloride-binding site, as described in the Introduction. In order to shed more light on this phenomenon, especially in relation to its potential physiological relevance, we have analysed in detail the oxygen-binding properties of bear, horse and bovine haemoglobins, all of which display a basic residue at position 76 of their β chains.

Figures 2–4 show the dependence of the oxygen affinity for bovine, bear and horse haemoglobins respectively on the concentration of either chloride and DPG alone, or when the other effector is already present in saturating amounts.

In all the three cases the experiments, performed within the physiological pH range, have shown that chloride and DPG act in a synergistic way. As evident from the Figures, the synergistic effect, indicated by the arrow, corresponds to about 0.12–0.20 $\Delta\log P_m$ (depending on the specific protein) and therefore could well be of physiological significance.

Since human Hb does not display a basic residue at position 76 of its β chain and is not characterized by a synergistic effect of the two effectors (see Figure 5, reported for comparison), one could well attribute this peculiar functional behaviour to the presence of the additional chloride-binding site proposed by Fronticelli et al. [14,15].

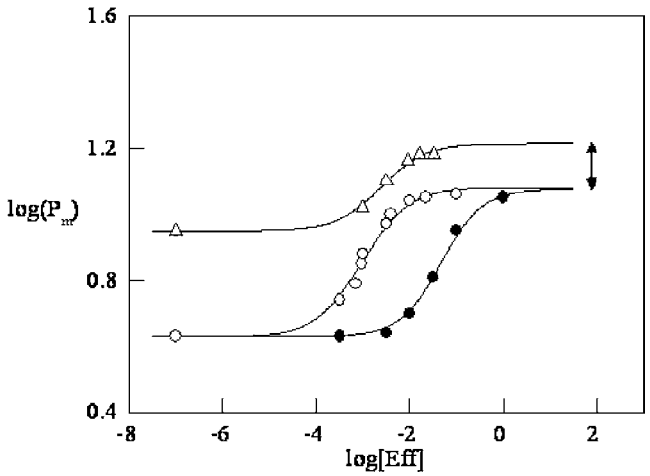

Figure 2 Chloride and DPG effect on the oxygen affinity of bovine Hb

Effect of chloride ions (●) and DPG concentration in the absence (○) and in the presence (△) of 0.1 M chloride on the oxygen affinity of bovine Hb. $\Delta\log P_m = 0.12$. Conditions were 0.1 Hepes buffer at pH 7.4 and 20 °C. Confidence limit of P_{50} values is 8 %.

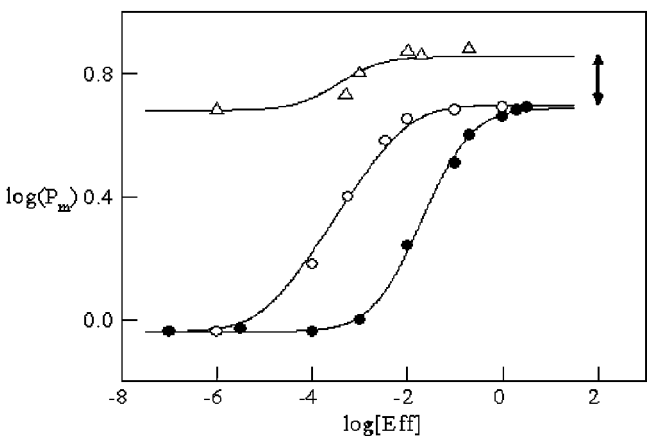

Figure 3 Chloride and DPG effect on the oxygen affinity of bear Hb

Effect of chloride ions (●) and DPG concentration in the absence (○) and in the presence (△) of 0.1 M chloride on the oxygen affinity of bear Hb. $\Delta\log P_m = 0.20$. Conditions were 0.1 Hepes buffer at pH 7.3 and 20 °C. Confidence limit of P_{50} values is 8 %.

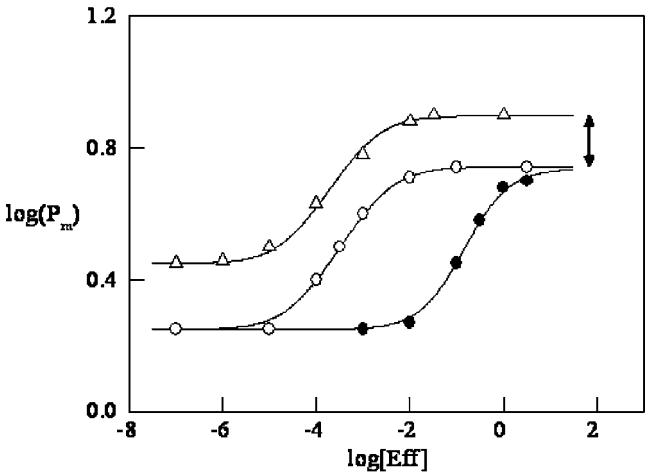

Figure 4 Chloride and DPG effect on the oxygen affinity of horse Hb

Effect of chloride ions (●) and DPG concentration in the absence (○) and in the presence (△) of 0.1 M chloride on the oxygen affinity of horse Hb. $\Delta\log P_m = 0.16$. Conditions are 0.1 Hepes buffer at pH 7.4 and 20 °C. Confidence limit of P_{50} values is 8 %.

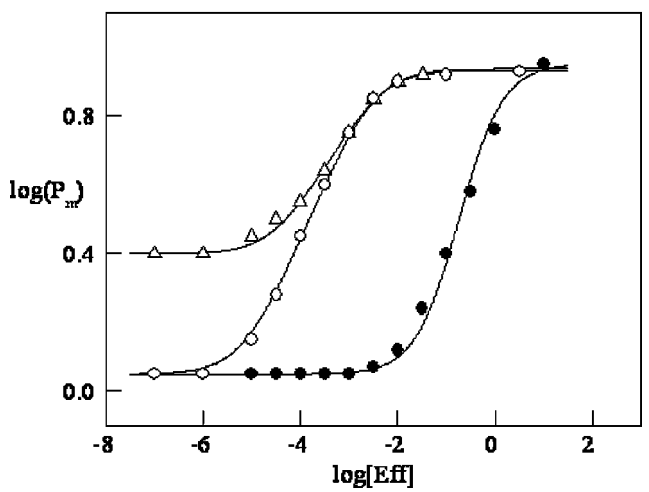

Figure 5 Chloride and DPG effect on the oxygen affinity of adult human Hb

Effect of chloride ions (●) and DPG concentration in the absence (○) and in the presence (△) of 0.1 M chloride on the oxygen affinity of adult human Hb. Conditions were 0.1 Hepes buffer at pH 7.4 and 20 °C. Confidence limit of P_{50} values is 8 %.

The experimental data on the effect of chloride on the oxygen affinity has allowed to determine the number of oxygen-linked chloride ions, using the Wyman's equation described in the Experimental section. The number of chloride ions is four for adult human Hb and six for bovine Hb. A particular case is represented by bear Hb that can be taken as a typical example of those haemoglobins which are characterized by a low effect of temperature only in the contemporaneous presence of both DPG and chloride. In this case, fitting of the titration curve (obtained under complete saturation with DPG) reported at the top of Figure 3 indicates the involvement of two additional chloride binding sites. This finding is in agreement with the results obtained by the computational approach (see below).

Potential oxygen-linked binding sites for Cl^-

The oxygen-linked sites (i.e. those that interact more in deoxy-Hb than in oxy-Hb) for chloride anion predicted by GRID program in HbA, bovine Hb, horse Hb and fetal human Hb are listed in Table 3.

Computational studies have shown that the major oxygen-linked sites in adult human Hb are the regions around Val-α1 and Val-β1, Lys-β82 in the DPG-binding cleft, as identified by different experimental investigations [25,26].

Energetically favourable binding sites for Cl^- were then predicted within bovine Hb. Calculations predict an additional oxygen-linked binding site for bovine Hb site with respect to those identified in HbA (Table 3) due to the presence of a lysine residue in both β8 and β76 position. The presence of this site is enhanced by the application of the flexibility option of GRID program when the amino acid side chains of lysine residues are free to move [18].

Figure 6 shows GRID contour maps for Cl^- ion with bovine Hb in the absence and in the presence of the flexibility option. GRID

Table 3 Oxygen-linked binding sites for chloride anion computed by GRID program

Hb	α-Chains site	DPG site	Additional site
Adult human	Val-α11 Ser-α1131 Arg-α2141	Val-β1 Lys-β82	–
Bovine	Val-α11 Asn-α1131 Arg-α2141	Met-β2 Lys-β82	Lys-β8 Lys-β76 His-β77
Horse + DPG	Val-α11 Ser-α1131 Arg-α2141	Va-lβ1 Lys-β82	Lys-β8 His-β76 His-β77
Fetal human + DPG	Va-lα11 Ser-α1131 Arg-α2141	Gly-γ1Lys-γ82	Lys-γ8 Lys-γ76 His-γ77

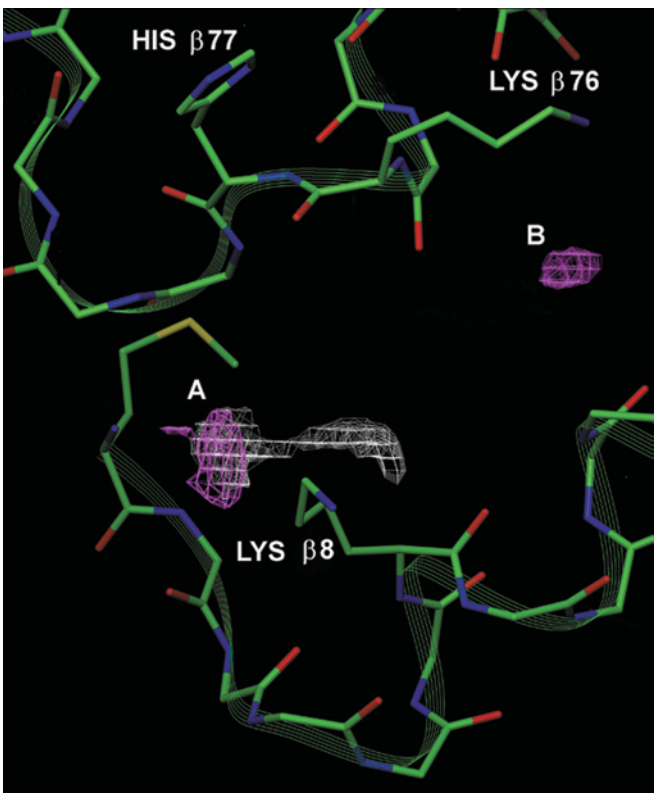

Figure 6 Predicted interaction between Cl^- ion and bovine Hb

Bovine Hb structure (PDB code: 1hda) is shown colour-coded by atom type (C, green; O, red; N, blue). In the absence of flexibility in the amino acid side chains a GRID contour map (violet coloured) was calculated at −8 kcal/mol, showing that the interaction area for Cl^- is localized near Lys-β8 (A) and Lys-β76 (B). In the presence of flexibility, a GRID contour map (white coloured) was calculated at −9.0 kcal/mol, showing one main area of interaction located between Lys-β8 and Lys-β76. The contour maps are displayed using InsightII (Accelrys).

results are displayed as contour maps using InsightII (Accelrys) showing regions of Hb where Cl^- would make favourable interactions. Without flexibility of the side chains there are two minima (whose corresponding maps, A and B, are violet coloured) which are mainly due to the interaction with Lys-β8 and Lys-β76. On the contrary, in the presence of flexibility it appears a single deeper minimum (whose corresponding map is white coloured) located between Lys-β8 and Lys-β76, since lysine side chains can move simultaneously.

The same theoretical method has been applied to the study of the interaction of chloride ion with horse Hb, taking into account the presence of DPG in the central cavity between the β chains [27,28]. An additional chloride-binding site (Table 3) was predicted for horse Hb, in the presence of DPG, in the same region previously identified for bovine Hb. The minimum energy area calculated by GRID program within β chain of horse Hb is due to the interaction of chloride ion with Lys-β8, His-β76 and His-β77.

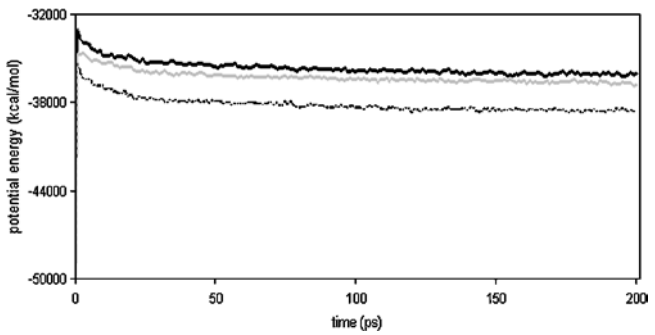

Figure 7 Time evolution of the potential energy

Plots of potential energy versus time over the MD trajectory for bovine Hb (dark line), horse Hb (grey line) and fetal human Hb (broken line) chloride complexes.

A reduced (compared with bovine Hb and horse Hb), but still evident, interaction of chloride ion within the cluster Lys-γ8, Lys-γ76, His-γ77 of fetal human Hb, in the presence of DPG, was calculated by GRID. The reduced interaction of chloride within the additional binding site of fetal Hb can be explained by the presence of a glutamate residue in γ5 position of fetal human Hb, which is occupied by an alanine residue in bovine Hb and by a glycine residue in horse Hb. The introduction of a negative charge in the region surrounding the site would therefore partially destabilize the binding of an additional chloride anion. This hypothesis has been confirmed by the construction of a mutant fetal Hb, γ(Glu-5 → Ala), in which the replacement entirely recovers the interaction area previously calculated for bovine Hb.

GRID results were then used for positioning Cl^- ion within the cluster β8, β76 and β77 of bovine Hb, horse Hb and fetal human Hb and consequently for building the corresponding Cl^- complexes.

Analysis of MD trajectories

Plots of potential energy versus time for the MD simulations of bovine Hb, horse Hb and fetal human Hb in the presence and absence of Cl^- ion are shown in Figure 7. Profiles show a plateau after only 50 ps, indicating that equilibrium has been attained in all simulations. The stability of the complexes was analysed by inspecting the RMSD (root mean square deviation) of each protein from the corresponding crystallographic structure. Figure 8 shows that all the structures reached an averaged constant RMSD, with respect to the starting structure, after the initial 60 ps.

In Figure 9 dynamic structures obtained every 20 ps during the whole course of the MD simulations are superimposed. In these images, only the amino acids surrounding Cl^- ion in the additional binding site are displayed. Note that the amino acid residues, as well Cl^- ions, are well superimposed and tightly clustered in bovine Hb (Figure 9A). Chloride ions are a bit less clustered in horse Hb (Figure 9B), and this effect is significantly enhanced in fetal Hb (Figure 9C).

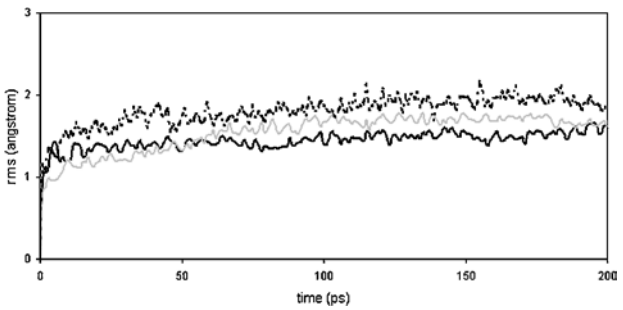

Figure 8 Time evolution of the backbone atoms rms deviation

RMSDs of the backbone atoms with respect to the crystallographic structures as a function of simulation time for bovine Hb (continuous line), horse Hb (grey line) and fetal human Hb (broken line) chloride complexes.

DISCUSSION

In Table 1 the ΔH of oxygenation for human Hb is reported in comparison with that of other species which, apart from fetal Hb, representative of a very peculiar physiological situation, are able to survive under extreme temperature conditions. In all these cases, from reindeer to horse Hb and to fetal human Hb, the haemoglobins are characterized by a small value of the oxygenation enthalpy that is essential to ensure an adequate release of oxygen to the colder peripheral tissues.

As the structural reasons for this feature are unknown, much of the experimental effort has been devoted to the identification of the regions of the molecule which may be able to modulate the thermodynamics of oxygen binding.

A potentially interesting and intriguing result of the inspection of the amino acid sequences is that all the selected haemoglobins present a cluster of basic residues in β8, β76 and β77 positions (Table 2). In this respect it is informative to correlate the different contributions to the thermal effects measured when oxygen binds to Hb [29]. These may be summarized as: (a) intrinsic heat of oxygenation, namely the heat involved in the binding of oxygen to the haem iron; (b) heat of ionization of oxygen-linked ionizable groups (Bohr groups); (c) heat associated with the T $\rightarrow$ R allosteric transition; and (d) heat of binding of other ions, such as organic phosphates and chloride.

Hence, Hb can alter its overall thermodynamics either by changing the structural characteristics of the T $\rightarrow$ R allosteric transition and/or by linking the basic reaction with the binding of different ions and effectors whose thermodynamics contribute to the overall effect of temperature. In the latter case, the structural basis of the thermodynamic modulation outlined above has to be searched at the level of heterotropic ligands. The functional data shown above indicate, in all the haemoglobins examined apart from the case of human HbA, the existence of different binding sites for chloride and DPG allowing simultaneous interaction with the protein without negative interference. Moreover, the synergistic effect suggests that in these haemoglobins the two classes of heterotropic interacting sites have direct and separate communication pathways with the haem and the observed phenomenon is not a pseudo-linkage [24]. In particular, the functional data point to the existence of an additional binding site for chloride which may also have an important physiological meaning related to the effect of temperature on oxygen transport. Thus, two more chloride ions per tetrameric molecule should be released upon oxygenation of Hb and, since this is an endothermic process, we should observe a lowering of the exothermic character of the oxygenation reaction and therefore a lower influence of temperature (Table 1). We have done a set of calculations showing that bovine Hb binds two additional chloride ions with respect to human Hb and that this corresponds to a higher (in absolute value, since its sign depends on the way we look at the reaction, i.e. oxygenation or deoxygenation) free energy change of about 100 kJ/mol for Hb tetramer. However, this value contains both the enthalpic and the entropic contributions. In any case, since binding of chloride ions to a given protein is based on electrostatic interactions, it is extremely difficult that the free energy change could have only an entropic contribution and therefore binding of two additional oxygen-linked chloride ions may well contribute to decrease the overall enthalpy change of the oxygenation–deoxygenation cycle, thereby lowering significantly the temperature dependence of oxygen release at the level of the tissues.

The interesting finding is the substitution of Ala-76β(E20) by a lysine residue, which has been suggested to result in the formation of a cluster of three positively charged amino acid residues [together with His-77β(EF1) and Lys-β8(A5)] which make up a chloride-binding site. The rational for this chloride-binding site, located at the external surface of the molecule, is linked to the movement of the N-terminal residues, occurring upon oxygen dissociation, toward the interior of the protein. This movement, in fact, produces a distortion of the A helix which, in turn, results in the displacement of Lys-β8(A5) toward the E helix, thereby forming the cluster of positive amino acid residues responsible for chloride binding [14,15]. The distortion of the A helix upon ligand binding plays a crucial role, since it is due to this distortion that the chloride-binding site becomes oxygen-linked, acquiring its modulation function.

These considerations have found support by crystallographic data of the deoxy human mutant β(V1M + H2del), one of the pseudo-bovine haemoglobins developed by Fronticelli et al. [14,15]; thus they show that the presence of a methionine at position β_1 and the deletion of histidine at position β_2 places the N-terminus in close proximity to Asp-β79, thereby weakening the interaction present between Asp-β79 and Lys-β8. In this way, Lys-β8 acquires a higher conformational flexibility and can establish with Lys-β76 and His-β77 a new chloride-binding site. This site, specific and additional with respect to human Hb, is 'oxygen-linked', since it disappears upon oxygenation. In fact, upon oxygenation, the interaction between Met-β1 and Asp-β79 would be disrupted and that between Lys-β8 and Asp-β79 would be re-established: the consequence is the release of the bound chloride.

Fetal human, horse and bear haemoglobins, recall the case of pig Hb [30], being representative of those haemoglobins which are characterized by a small value of the oxygenation enthalpy only in the contemporaneous presence of both chloride and DPG. The N-terminal region of the β chains in these haemoglobins is different from that of ruminant Hb and therefore may not favour (in the absence of organic phosphates) the disruption of the interaction between Lys β8 and Asp β79, which is an essential requisite for the binding site to show up. This is supplied by DPG whose interaction, at the level of its binding pocket, does induce a distortion in the A helix similar to that discussed above [31], thereby influencing the protein structure in such a way to put Lys-β8 in the right conditions for switching 'on' the additional binding site. In the absence of DPG, distortion of the A helix could not occur, and the chloride-binding site, even if potentially present, would not be able to detect the presence of oxygen bound to haem. In other words, it is through the protein-mediated interaction between chloride and DPG that the Hb molecule displays all its functional properties observed under physiological conditions.

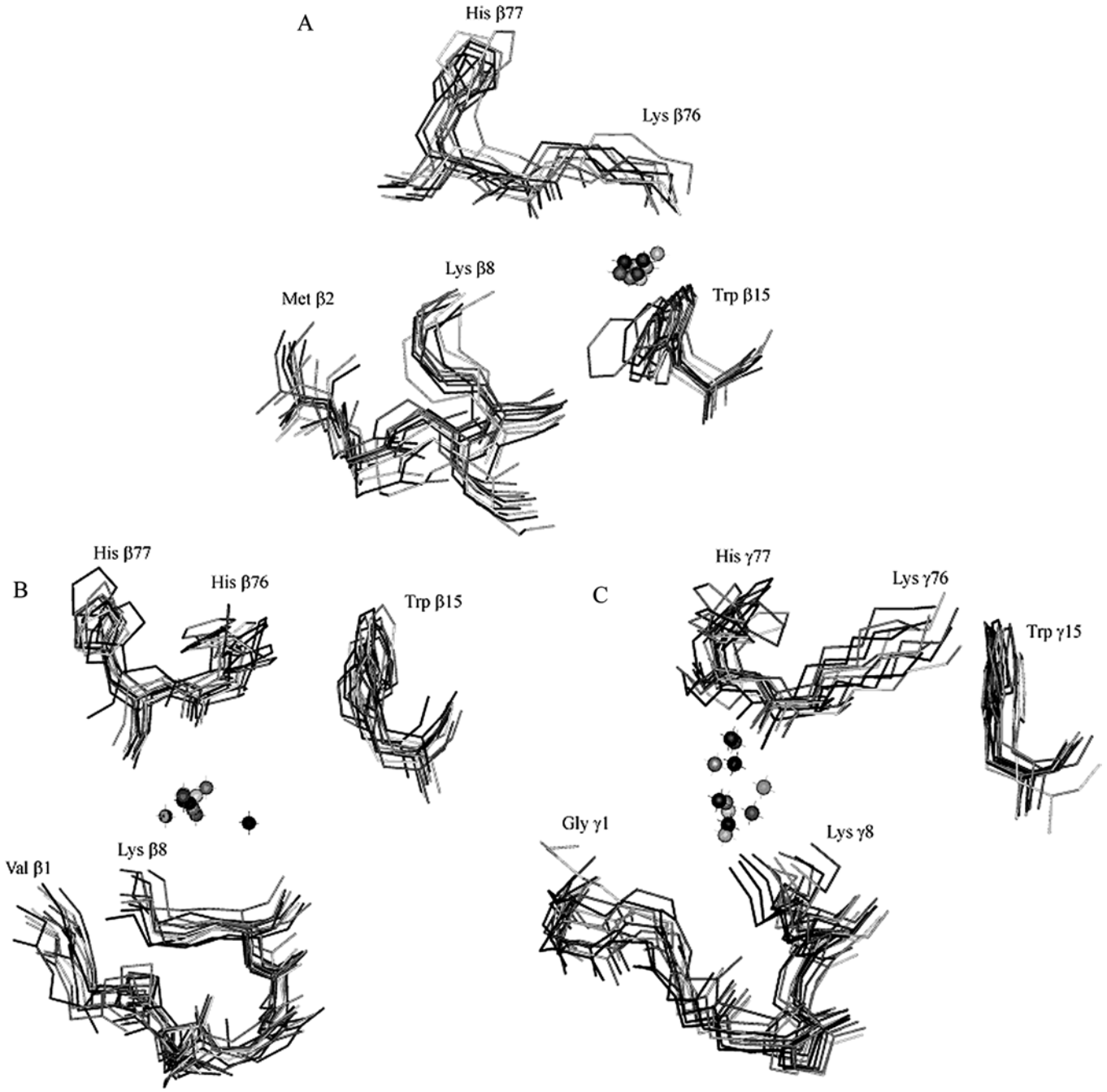

Figure 9 Dynamic structures obtained every 20 ps during the course of the MD simulations

Superimposition of archive structures obtained during the MD simulations of bovine Hb–Cl (**A**), horse Hb–Cl (**B**) and fetal human Hb–Cl (**C**) complexes.

The computational studies herein reported on haemoglobins characterized by a low ΔH of oxygenation and consequently, as showed in Table 2, by a basic (lysine or histidine) residue in $\beta 76$ position, suggest that the reason for the lower oxygen affinity of bovine Hb in the presence of chloride, compared with that for human Hb, is mainly due to an increase in the number of chloride-binding sites, rather than an increase in the affinity of the same binding sites of HbA.

The plausible binding sites of chloride ion in bovine Hb, horse Hb and fetal human Hb have been investigated using the crystallographic structures of the proteins and computational methods. Specifically, using GRID, a minimum energy region for chloride ion was identified in each of the three systems within the β chains, due to the presence of basic residues in $\beta 8$, $\beta 76$ and $\beta 77$ position. The complexes generated from GRID were submitted to MD simulations. Observing the mobility of chloride ion during MD simulations, the predicted binding site was found to be significant in all the three systems (Figures 9A, 9B and 9C) and their differences in agreement with their different experimental properties (see Table 1).

Computational studies performed on horse and fetal Hb, in both the presence and in the absence of organic phosphates, would also

confirm our hypothesis that the effector might help to 'switch on' the additional site for chloride (Table 3).

In conclusion, the whole body of the data suggests that the region of the molecule comprising Lys-β8, Lys-β76 and His-β77 is one of the keys (if not the only one) to be used to modulate the thermodynamics of ligand binding in Hb. This represents the molecular basis of an adaptive mechanism of great importance from a physiological point of view.

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