Anionic Binding Site and 2,3-DPG Effect in Bovine Hemoglobin†

M. Marta,‡,§ M. Patamia,§ A. Colella,‡ S. Sacchi,‡ M. Pomponi,‡ K. M. Kovacs,|| C. Lydersen,⊥ and B. Giardina*,‡,§

Istituto di Chimica e Chimica Clinica e Centro CNR per la Chimica dei Recettori, UCSC, Facoltà di Medicina, Largo F. Vito 1, 00168 Roma, Italy, Department of Biology, University of Waterloo, Ontario, N2L3G1 Canada, and Department of Biology, Division of General Physiology, University of Oslo, N-0316 Oslo, Norway

Received May 18, 1998; Revised Manuscript Received July 31, 1998

ABSTRACT: It is generally believed that bovine hemoglobin (BvHb) interacts weakly with 2,3-diphosphoglycerate (2,3-DPG) in a chloride-free media and not at all in the presence of physiological concentrations of chloride (100 mM). This lack of interaction has raised several questions at both structural and evolutionary levels. Results obtained in this study via ^{31}P nuclear magnetic resonance (NMR) show that, even in the presence of 100 mM chloride ions, 2,3-DPG does, in fact, interact with bovine deoxy-Hb. This spectroscopic observation has been confirmed by oxygen binding experiments, which have also shown that, under certain conditions, chloride and 2,3-DPG may display a synergistic effect in modifying the oxygen affinity of bovine hemoglobin. It could be that this synergistic effect has its structural basis in a conformational modification induced by 2,3-DPG, possibly causing extra chloride anions to approach the positive charges which constitute the anion binding site. Another possibility, not necessarily an alternative, is the additional chloride binding site recently identified [Fronticelli, C., Sanna, M. T., Perez-Alvarado, G. C., Karavitis, M., Lu, A.-L., and Brinnigar, W. S. (1995) *J. Biol. Chem 270*, 30588−30592] involving lysine β76 that in bovine Hb substitutes for the alanine residue present in human hemoglobin. All of these findings are in agreement with the very low enthalpy of oxygenation that characterizes bovine Hb when both chloride and 2,3-DPG are present in concomitance. The results reported here clearly show that bovine hemoglobin does react with 2,3-DPG and is functionally affected by this organic phosphate. Hence, the very low intraerythrocytic concentration of 2,3-DPG (0.5 mM) in adult bovine red blood cells is the result of metabolic adaptation which cannot be explained solely by the different amino acid sequence at the level of the 2,3-DPG binding site.

Mammals display a wide range of whole blood oxygen affinity that mostly depends on three factors: the intrinsic oxygen affinity of hemoglobin, the concentration of intraerythrocytic 2,3-diphosphoglycerate (2,3-DPG),[1] and the response of Hb to DPG and chloride. It is well-known that, in human hemoglobin, binding of 2,3-DPG occurs at a specific site, which at neutral pH involves a cluster of eight positively charged amino acid residues (Val-NA1, His-NA2, Lys-EF6, and His-H21 of each β-chain), located on the dyad axis of the hemoglobin tetramer (*2*). A very interesting example of the effect of 2,3-DPG and chloride is the different functional modulation brought about by 2,3-DPG and chloride, respectively, in human adult Hb and in ruminant Hb. Thus, while 2,3-DPG is the major modulating factor for HbA in vivo, chloride appears to be the more relevant physiological effector for ruminant Hbs such as bovine Hb. In fact, ruminant hemoglobins, in the absence of 2,3-DPG but in the presence of physiological concentrations of chloride (100 mM), have an oxygen affinity similar to or even lower than that of human Hb fully saturated by 2,3-DPG. This observation is paralleled by the respective 2,3-DPG intracellular concentration of human and ruminant erythrocytes (≈5.0 vs 0.5 mM). The low intrinsic oxygen affinity of ruminant hemoglobins has been linked to the particular N-terminal region of the β-subunits, characterized by the deletion of the residue at position β1 and the presence of a hydrophobic residue (methionine or phenylalanine) which replaces the usual histidine residue at position β2. It has been suggested that this hydrophobic residue points into the interior of the protein, thereby mimicking 2,3-DPG in stabilizing the tertiary deoxy structure of the β-subunits (*2*).

On this basis, Perutz and collaborators (*3*) suggested that bovine Hb reacts weakly with 2,3-DPG in a chloride-free media and not at all in the presence of 100 mM chloride. However, Bunn (*4*), Fronticelli et al. (*5*, *6*), and Clementi et al. (*7*) have shown that both chloride and 2,3-DPG may modulate the oxygen affinity of bovine Hb in at least some experimental conditions.

This remains a point to be further investigated, not only because of the apparent discord between previous studies but also because, and in particular, of its evolutionary implications. In fact, from an evolutionary point of view the functional properties of bovine erythrocytes could be the

† This research was supported by the Italian CNR (Progetto Finalizzato Biotecnologie) and the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST).

‡ Istituto di Chimica e Chimica Clinica.

§ Centro CNR per la Chimica dei Recettori.

|| University of Waterloo.

⊥ University of Oslo.

[1] Abbreviations: Hb, hemoglobin; HbA, human adult hemoglobin; BvHb, bovine hemoglobin; 2,3-DPG, 2,3-diphosphoglycerate; Tris, tris-(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; HbCO, carboxy hemoglobin.

S0006-2960(98)01157-X CCC: $15.00
Published on Web 09/17/1998

result of (1) a molecular adaptation involving mainly the hemoglobin molecule that, by virtue of amino acid mutations at the level of the amino terminus of β-chains, is no longer able to bind the 2,3-DPG anion; (2) a metabolic adaptation by which the synthesis of 2,3-DPG is so heavily reduced that its physiological effect is no longer evident under in vivo conditions; and (3) the concomitant presence of both mechanisms reported above such that the specific function of bovine erythrocyte is the result of a complex network of evolutionary processes.

In an attempt to gain insight as to which scenario is correct, an investigation has been carried out through oxygen binding and NMR experiments on the interaction between bovine Hb and both chloride and 2,3-DPG. Moreover, to better understand the role played by the amino acid residues which constitute the anion binding site, and the possible existence of different modes of binding, a computer modeling study has been performed to explore the interaction of 2,3-DPG between both human and bovine Hbs.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals used during this study were of the highest quality commercially available from Aldrich Chemical Co. Bovine blood samples from the local slaughterhouse were collected in an isotonic NaCl solution containing 2 mM EDTA.

Protein Preparation. Hb was prepared and purified as previously described for human Hb (*8*). Chloride and any organic phosphates were removed by gel filtration on a Sephadex G-25 column (at 2−4 °C) and equilibrated with 10 mM Tris-HCl (pH 8.0), containing 0.1 M NaCl. Subsequently, the product was passed through a column of a mixed-bed ion-exchange resin (Bio-Rad AG 501-X8) (*9*).

Spectrophotometric Measurements. Each experiment was carried out with a CARY 3E (Varian) using thermostated cells (Haake L, Fisons). Concentrated stock solutions of 2,3-DPG were prepared by dissolving the sodium salt of 2,3-DPG acid (Sigma) in HEPES buffer.

Functional Characterization. The oxygen equilibrium curves of bovine Hb, in either the presence or absence of the specific heterotropic effector(s), were measured in 0.1 M HEPES buffer, within the pH range 6.7−7.8. Oxygen binding isotherms were determined by the tonometric method (*10*) at a protein concentration of 3−5 mg/mL between 20 and 37 °C. The overall oxygenation enthalpy, ΔH (kJ/mol), was calculated from the integrated van't Hoff equation $\Delta H = RT^2(\mathrm{d} \ln P_{50}/\mathrm{d}T)$ (*11*, *12*), where P_{50} is the partial pressure of the ligand at which 50% of the heme sites are oxygenated. An average standard deviation of ±8% for P_{50} values and of 10% for ΔH values was calculated.

Nuclear Magnetic Resonance Spectra. The NMR spectra were recorded with a Varian Gemini spectrometer operating at 121.48 MHz for ^{31}P. The ^{31}P spectrum was the result of averaging 31 000 transients at a repetition rate of 0.7 s^{-1}. All ^{31}P chemical shifts were referenced with respect to 85% H_3PO_4. In NMR experiments in the presence of 0.1 M NaCl, the 2,3-DPG concentration was about 1.4 mol/mol of Hb (1%). Hb samples at various pH values were obtained by adding 1.0 M MES (pH range 5.5−6.5) or 1.0 M HEPES (pH range 6.5−8.0). The final buffer concentration was 0.1 M. The temperature inside the probe was 20 °C. Chemical shifts (δ) were relative to H_3PO_4 ($\delta_{Cn-P} - \delta_{H_3PO_4}$). The δ scale was defined as positive in the low-field direction. The assignment of the resonances to the 3-phosphate (lower field) and to the 2-phosphate (higher field) was taken from Moon and Richards (*13*).

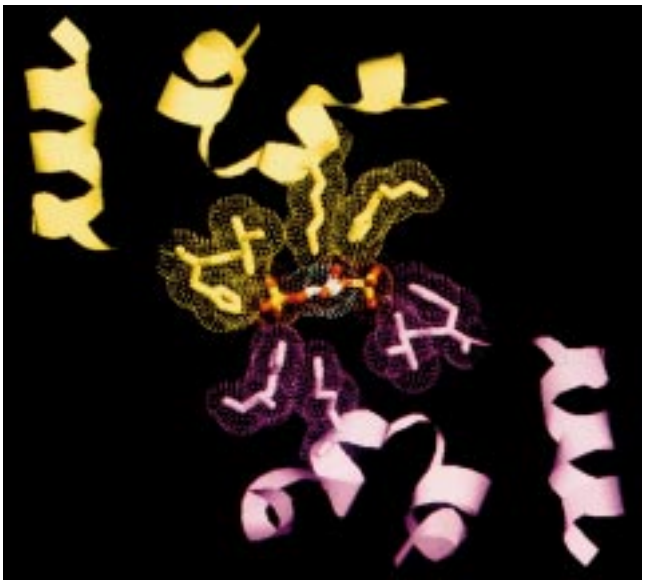

FIGURE 1: View of the human Hb−2,3-DPG complex. The van der Waals surface shows the narrow cavity upon binding with the effector.

Molecular Modeling. Computation results were obtained using software programs from TRYPOS Associates Inc. of St. Louis, MO, running on a Silicon graphics workstation.

The crystal structures of human deoxy-HbA (*14*) at 1.74 Å resolution (PDB, entry 2HHB) and bovine deoxy-Hb (*13*) at 2.2 Å resolution (PDB, entry 1HDA) were used as a basis for modeling procedures. 2,3-DPG was sketched and then subjected to energy minimization; the Powell method and the TRIPOS force field were used.

The most favorable approximation of intermolecular energy interaction between ligand and protein for human deoxy-HbA was obtained by a docking simulation of a 2,3-DPG molecule within the protein site. The complex was then subjected to energy minimization to improve the modeled dock; force field constraints were used to compel defined amino acids into a spatial relationship. In the complex, the asymmetric 2,3-DPG molecule interacts with the eight cationic groups present in the protein cavity in two opposite orientations related by a 180° rotation. For the 2,3-DPG carboxylate the following constraints were constructed to simulate the formation of salt bridges: NZ Lys82 β1 and C1 of 2,3-DPG (range distance either 2.8−3.2 or 5.8−6.2 Å); NZ Lys82 β2 and C1 (range distance either 5.8−6.2 or 2.8−3.2 Å). For both orientations (Lys82 β2 or Lys82 β1) of the 2,3-DPG carboxylate a constraint of 3.5 Å was settled between the following atoms: (a) N-terminal Val1 β1 and P−C3 of 2,3-DPG; (b) N-terminal Val1 β2 and P−C2 of 2,3-DPG; (c) CE1 His2 β2 and the same P−C2; (d) CE1 His2 β1 and P−C3 of 2,3-DPG; (e) CE1 His143 β1 and P−C2; (f) CE1 His143 β2 and P−C3 (Figure 1).

Two different situations were analyzed for bovine Hb, prompted by the NMR spectra (Table 1). The coordinates of the previously minimized HbA−2,3-DPG complex served as a starting reference to merge the 2,3-DPG molecule into

Table 1: p*K* Values for the Phosphate Groups in 0.1 M Buffer and 50 μL of D_2O in a 700 μL Final Volume[a]

	p*K*				
	2,3-DPG alone	2,3-DPG + Cl^-	2,3-DPG + Cl^- + deoxy-Hb	2,3-DPG + Cl^- + deoxy-Hb	2,3-DPG + Cl^- + HbCO
C3−P	7.27	7.36	7.20	7.11	7.19
C2−P	6.87	7.09	6.42	6.63	6.97

[a] 2,3-DPG concentration 4.3×10^{-4} M. Temperature 20 °C. For all of the ^{31}P NMR experiments, the molar ratio of 2,3-DPG to deoxyHb was between 0.9 and 1.1.

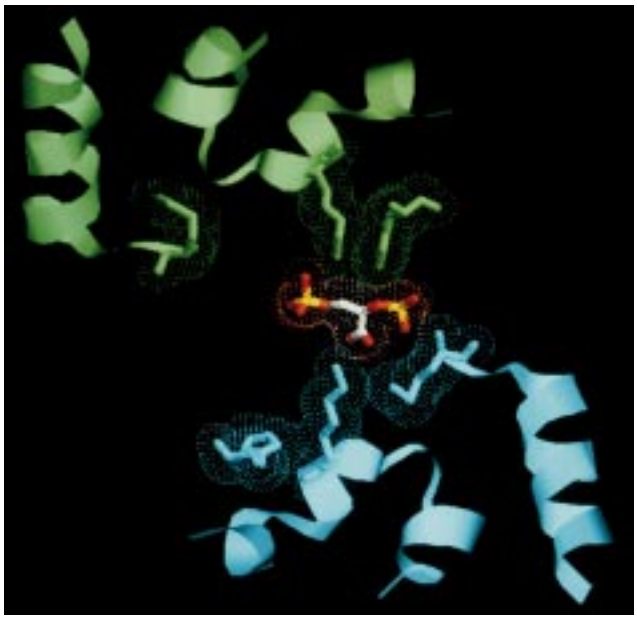

FIGURE 2: View of the bovine Hb−2,3-DPG complex. The van der Waals surface shows the enlargement of the cavity after complex formation.

the active site region of deoxy-Hb. The asymmetric 2,3-DPG molecule interacts with four cationic groups present in the protein cavity in two opposite orientations related by a 180° rotation. The following constraints were considered for one orientation (Figure 2): NZ Lys82 β1 (β1 chain is green) and C1 of 2,3-DPG (range distance 5.8−6.2 Å); NZ Lys82 β2 and the former C1 (range distance 2.8−3.2 Å); CE1 His143 β2 and P−C3 of 2,3-DPG and N-terminal Met2 β1 and the same P−C3 were both settled at 3.5 Å. The considered constraints for the other possible orientations were NZ Lys82 β2 and C1 (range distance 5.8−6.2 Å); NZ Lys82 β1 and the same C1 (range distance 2.8−3.2 Å); CE1 His143 β1 and P−C2 of 2,3-DPG and N-terminal Met2 β2 and the same P−C2 were both settled at 3.5 Å. Accuracy of docking was measured by the root-mean-squared deviation (RMS) between the initial crystallographic positions and those obtained by docking the 2,3-DPG molecule in the effector protein site.

RESULTS

^{31}P Nuclear Magnetic Resonance Experiments. High-resolution spectra were obtained with a Varian Gemini 300 instrument operating at 121.48 MHz for ^{31}P. ^{31}P chemical shifts (Figure 3), measured as a function of pH in the presence of chloride anions, showed a significant interaction between the phosphate groups of 2,3-DPG and the deoxygenated derivative of bovine Hb. However, from the p*K* values obtained by ^{31}P NMR (Table 1) it is clear that the interaction involves essentially the phosphate group on the C2 of 2,3-DPG and the deoxy state of the hemoglobin molecule. This interaction is almost abolished in the liganded derivative.

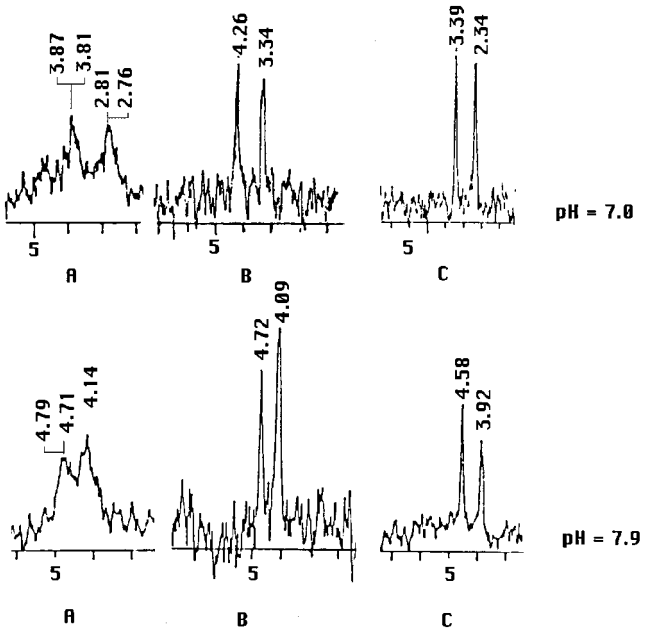

FIGURE 3: 121.48 MHz ^{31}P NMR spectra of the 2- and 3-phosphate groups of 2,3-DPG in 0.1 M MES buffer at different pH values and at 20 °C in the presence of (A) 0.1 M NaCl + deoxy-Hb (≈1.4:1 molar ratio to 2,3-DPG), (B) 0.1 M NaCl + HbCO (≈1.4:1 molar ratio to 2,3-DPG), and (C) 0.1 M NaCl. The assignment of the resonances to the 3-phosphate (lower field) and to the 2-phosphate (higher field) is taken from Moon and Richards (*13*). Absolute shifts are from external H_3PO_4.

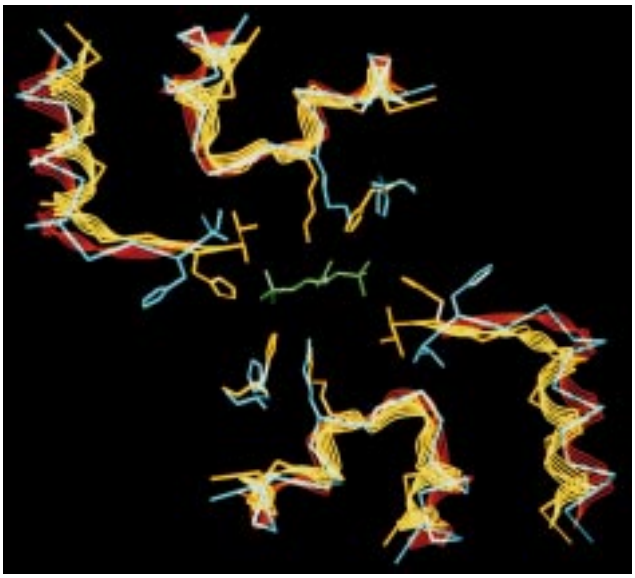

FIGURE 4: View showing the proposed relationship for human deoxy-Hb (cyan) and the human deoxy-Hb−2,3-DPG complex (orange).

Molecular Modeling. Minimization of the human HbA−2,3-DPG complex suggests a geometric structure substantially in agreement with that reported by Arnone (Figure 4) (*2*). In bovine deoxy-Hb, the replacement in the β chains of Val1−His2 by Met alone reduces the number of interactions (Figure 5) operative in the complex. The least energy

FIGURE 5: Drawing of the models of bovine deoxy-Hb (cyan bonds) and the deoxy-Hb−2,3-DPG complex (magenta) with Cα superimposed. Constraints are between C2−P (2,3-DPG) and Met2 β2 and between C2−P (2,3-DPG) and His143 β1.

conformations obtained show the following: (a) The perturbation induced by 2,3-DPG affected only one His143 β and both Lys82 β; all these residues moved toward the effector molecule in both human and bovine Hbs. (b) In human hemoglobin, as expected, binding of 2,3-DPG determined a narrowing of the cavity (Figure 4); in bovine Hb, due to the highly asymmetric 2,3-DPG binding, this restriction, although still present, seemed to concern only that part of the cavity where docking takes place (Figure 5). (c) In bovine Hb (Figure 5), the region of the cavity which is not directly involved in 2,3-DPG binding, appeared to be significantly wider (≈30%). The distance of NE2 (His143 β2) from SD (Met2 β1) has increased from 13.5 to 17.8 Å.

The structures obtained show that one of the two His143 β's is relatively distant, 6.4 Å, from the 2,3-DPG molecule while the other one, at 3.1 Å, established a hydrogen bond with one of the oxygen atoms of the phosphate group.

Moreover, the α-helical segments, generally indicated as "helices H", were superimposed in both structures (with and without 2,3-DPG), and only the ends of the β-chains lost their superimposition.

Bohr Effect. The pH dependence of the oxygen affinity of bovine Hb was measured in the presence of the various effectors in 100 mM Hepes buffer and at a protein concentration of 3−5 mg/mL. As shown in Figure 6, in the absence of both chloride and 2,3-DPG, BvHb is characterized by a low Bohr coefficient ($-\Delta \log P_{50}/\Delta\text{pH} = 0.29$), which increases progressively to 0.47 in the presence of chloride ions (100 mM) and to 0.68 in the presence of only 3 mM 2,3-DPG, reaching its maximum in the concomitant presence of chloride and 2,3-DPG ($\Delta \log P_{50}/\Delta p\text{H} = 0.74$). On the whole, oxygen binding experiments show that both chloride and 2,3-DPG are able to affect the functional behavior of bovine Hb, in full agreement with the data obtained by NMR and molecular modeling. This effect is not only limited to the absolute value of the oxygen affinity but also to the amplitude of the Bohr effect, which is significantly influenced by the presence of the effectors.

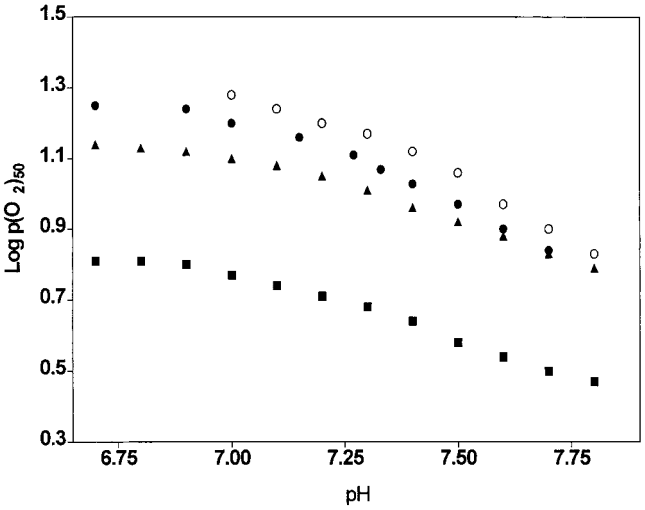

FIGURE 6: Effect of the pH on the oxygen affinity, at 20 °C and 0.1 M HEPES buffer, of stripped deoxy-Hb (squares), compared with its affinity in the presence of 3 mM 2,3-DPG plus 0.1 M NaCl (open circles), 0.1 M NaCl (triangles), and 3 mM 2,3-DPG (black circles).

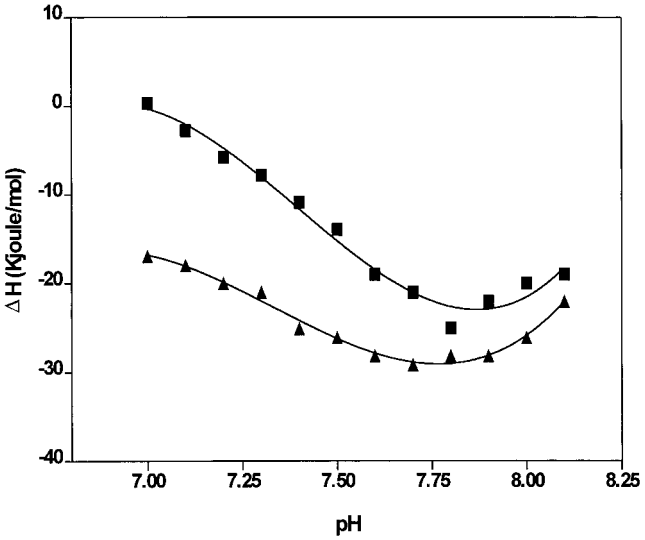

FIGURE 7: pH dependence, at 20 °C, of the oxygenation enthalpy (ΔH) for bovine Hb in the presence of 0.1 M NaCl (triangles) and 2,3-DPG plus 0.1 M NaCl (squares).

It may be worthwhile to mention that the oxygen affinity of BvHb decreases in a progressive way on going from stripped Hb (i.e., Hb free from both chloride and 2,3-DPG), to Hb + Cl^- (100 mM), to Hb + 2,3-DPG (3 mM), and finally to Hb + Cl^- (100 mM) + 2,3-DPG (3 mM). Hence, a small but still measurable sinergistic effect of chloride and 2,3-DPG is observable.

Effect of Temperature on Oxygen Affinity. Over the temperature range explored (20−37 °C), van't Hoff plots were linear within experimental error (data not shown). Figure 7 displays the pH dependence of the exothermic oxygenation enthalpy (ΔH). The smallest value (in absolute terms) is observed when both chloride and 2,3-DPG are present as functional modulators.

DISCUSSION

As outlined in the introduction, bovine erythrocytes are almost totally devoid of 2,3-DPG (*4*, *15*), and therefore their

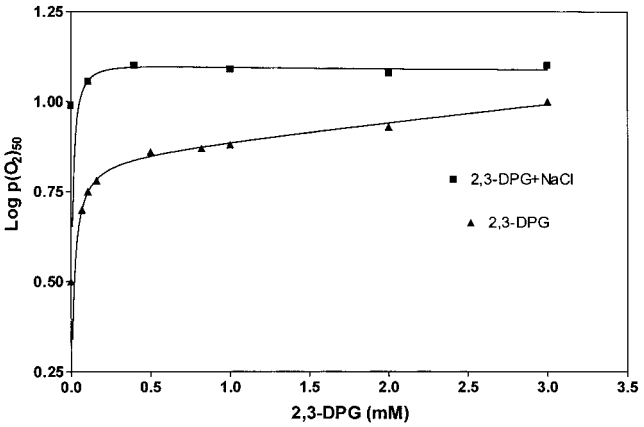

FIGURE 8: Effect of increasing concentrations (mM) of 2,3-DPG (triangles) and 2,3-DPG plus 0.1 M NaCl (squares) on the O_2 affinity of bovine Hb in 0.1 M HEPES buffer at 20 °C.

Hb is almost exclusively modulated by chloride ions. This fact, together with the deletion of the N-terminal amino acid residue of the β chains and the replacement of the adjacent His NA2 by a methionine, has led to the belief that ruminant Hbs do not bind 2,3-DPG, especially when in the presence of chloride.

The results presented here outline very clearly that 2,3-DPG does in fact bind to bovine Hb, not only in the absence but also in the presence of saturating concentrations of chloride ions. The confusion existing in the data found in the literature could be explained by the slight difference in the functional effect brought about by chloride and 2,3-DPG (Figure 6). In fact, the total effect of 2,3-DPG on the functional properties of ruminant hemoglobins and especially BvHb is certainly not comparable with that observable in other mammalian Hbs. In comparison to human HbA, the lower functional effect of 2,3-DPG on bovine Hb finds its structural counterpart in the different mode of binding, outlined here by the structural modeling approach. Therefore, compared to human Hb in which both p*K*s, for the phosphate groups on C2 and C3 of the 2,3-DPG molecule, are strongly shifted (*16*), binding of 2,3-DPG to bovine Hb appears highly asymmetrical since only the p*K* value of the phosphate group on C2 appears to be significantly shifted (from 6.87 to 6.42) (Table 1). On the basis of our structural modeling, this could be due to the interaction of the negatively charged groups of 2,3-DPG with Met2 β and His143 β of only one β-chain. The asymmetric binding of 2,3-DPG induces a widening of part of the central cavity (from 13.5 to 17.8 Å) that merits some comments. It has been recently proposed (*1*) that in bovine Hb there are no specific chloride binding sites and that the effect of chloride is a result of the widening of the central cavity on transition from the R (oxygenated derivative) to the T (deoxygenated derivative) structure. This would allow the diffusion of chloride to neutralize the excess of positive charges lining the central cavity, without being bound to any one of them. On the basis of this view, the low synergistic effect of chloride and 2,3-DPG could well be linked to the partial widening of the cavity imposed by the asymmetric binding of 2,3-DPG, which, in fact, may allow extra chloride ions to approximate the positive charges present in this site (Figures 6 and 8) and to further affect the oxygen affinity.

However, this view is complemented by the elegant results of site-directed mutagenesis obtained by Fronticelli et al. (*1*), who have clearly indicated the existence, in bovine Hb, of a particular, specific, and additional (with respect to human Hb) oxygen-linked chloride binding site involving a lysine (Ala in human hemoglobin) at position β76 and lysine β8. This site is disrupted upon oxygenation, allowing the bound chloride ion to be released. In interpreting the synergistic effect of chloride and 2,3-DPG, we cannot disregard the existence of this new oxygen-linked chloride binding site.

Finally, with reference to the thermodynamics of oxygen binding, the difference in the overall heat of oxygenation observable at pH 7.0 on going from 0.1 M chloride (≈ -18.5 kJ/mol) to 0.1 M chloride plus 3.0 mM 2,3-DPG (≈ -0.5 kJ/mol)) is worthy of note and strongly recalls a set of recent results obtained on fetal bovine hemoglobin (*7*). This was a very interesting case where the paradoxical situation of fetal erythrocytes characterized by an intracellular concentration of 2,3-DPG (5 mM) 10 times higher than that of adult cells has found a reasonable explanation in the lower overall heat of oxygenation displayed by fetal Hb when in the presence of 2,3-DPG and chloride at physiological concentration (*7*). Hence, the effect of 2,3-DPG on fetal bovine Hb, whose binding site for organic phosphate is identical to that of the adult protein, is also that of lowering the overall heat of oxygen binding so as to favor the heat exchange from fetus to mother. To explain the very low enthalpy of bovine Hb oxygenation when in the simultaneous presence of chloride and 2,3-DPG, we have to consider the additional chloride ions which are bound to this hemoglobin. The endothermic contribution made by the oxygen-induced release of the additional chloride ions would thus 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 in ruminant hemoglobin.

This entire body of data indicates that the physiological adaptation mechanisms of oxygen transport displayed by ruminant Hbs do not only involve the Hb molecule but also are the outcome of a complex network of metabolic interactions which result in a sophisticated control of 2,3-DPG synthesis. Considering the linkage that exists between maternal and fetal blood, 2,3-DPG synthesis plays an important role in determining the overall effect of temperature of the oxygenation process.

ACKNOWLEDGMENT

The authors thank Dr. E. Clementi for discussing these results and Mr. M. Di Iorio for technical assistance.

REFERENCES

1. Fronticelli, C., Sanna, M. T., Perez-Alvarado, G. C., Karavitis, M., Lu, A.-L., and Brinnigar, W. S. (1995) *J. Biol. Chem. 270*, 30588−30592.
2. Arnone, A. (1972) *Nature 237*, 146−149.
3. Perutz, M. F., Fermi, G., Poyart, C., Pagnier, J., and Kister, J. (1993) *J. Mol. Biol. 233*, 536−545.
4. Bunn, H. F. (1971) *Science 172*, 1049−1051.
5. Fronticelli, C. (1990) *Biophys. Chem. 37*, 141−146.
6. Fronticelli, C., Bucci, E., and Razynska, A. (1988) *J. Mol. Biol. 202*, 343−348.
7. Clementi, M. E., Scatena, R., Mordente, A., Condò, S. G., Castagnola, M., and Giardina, B. (1996) *J. Mol. Biol. 255*, 229−234.

8. Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1964) *Adv. Protein Chem. 19*, 73−222.
9. Marta, M., Patamia, M., Lupi, A., Antenucci, M., Di Iorio, M., Romeo, S., Petruzzelli, R., Pomponi, M., and Giardina, B. (1996) *J. Biol. Chem. 271*, 7473−7478.
10. Giardina, B., and Amiconi, G. (1981) *Methods Enzymol. 76*, 417−427.
11. Wyman, J. (1964) *Adv. Protein Chem. 19*, 223−286.
12. Antonini, E., and Brunori, M. (1971) *Hb and myoglobin in their reactions with ligands*, North-Holland Publishing Co., Amsterdam.
13. Moon, R. B., and Richards, J. H. (1973) *J. Biol. Chem. 248*, 7276−7278.
14. Fermi, G., Perutz, M. F., Shaanan, B., and Fourme, R. (1984) *J. Mol. Biol. 175*, 159−174.
15. Giardina, B., Arevalo, F., Clementi, M. E., Ferrara, L., Di Luccia, A., Lendaro, E., Bellelli, A., and Condò, S. (1992) *Eur. J. Biochem. 204*, 509−513.
16. Russu, I. M., Wu, S. S., Bupp, K. A., Ho, N. T., and Ho, C. (1990) *Biochemistry 29*, 3785−3792.

BI981157V