The chloride effect in the human embryonic haemoglobins

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The interactions of the three human embryonic haemoglobins with chloride ions have been investigated. Each of the three embryonic haemoglobins exhibits a unique pattern of oxygen-affinity-dependence on chloride ion concentration. Human embryonic haemoglobin Portland ($\zeta_2\gamma_2$) is found to be completely insensitive to chloride ion concentration. Haemoglobin Gower I ($\zeta_2\gamma_2$) shows a small concentration dependence, whilst haemoglobin Gower II ($\alpha_2\epsilon_2$) exhibits a dependence approaching that of the adult protein. The degree of co-operativity for each protein is essentially chloride concentration independent. The chloride-dependent and -independent components of the alkaline Bohr effects have been measured for each of the embryonic haemoglobins and compared with that of the adult protein. Both the chloride-binding data and the Bohr effect have been analysed in terms of the recently developed allosteric model proposed by Perutz [Perutz, Fermi, Poyart, Pagnier and Kister (1993) J. Mol. Biol. **233**, 536–545].

INTRODUCTION

Three different haemoglobins are synthesized during the earliest stages of human development [1]. From approximately week two to week twelve of gestation, activation of the necessary globin genes leads to the sequential synthesis of haemoglobins Portland ($\zeta_2\gamma_2$), Gower I ($\zeta_2\epsilon_2$) and Gower II ($\alpha_2\epsilon_2$) [2]. The impossibility of obtaining samples of these human embryonic proteins, from natural sources, has prevented any major investigation of their properties in the past. However by employing recent advances in molecular biology we have been able to produce relatively large quantities of each of these proteins by expression from plasmids within a yeast expression system [3,4]. Recently a series of investigations into the nature of the equilibrium process of oxygen binding to the three human embryonic haemoglobins has been undertaken and has clearly shown the likely role played by organic phosphates and protons in the control of the allosteric functioning of these proteins [4]. For many years the important role of chloride ions in the control of adult human haemoglobin function has been recognized [5]. However the origins of this effect, at the molecular level, have only recently been fully identified. In the adult haemoglobin molecule the effect of chloride ions appears to have two distinct origins. The central water-filled cavity in adult human haemoglobin is lined by specific amino acids which, under physiological conditions, contribute a net overall cationic excess to the cavity [6]. This cavity is wider in the deoxy form than in the oxy form of the protein and is capable of admitting chloride ions, which although randomly distributed within the cavity, neutralize the repulsion between subunits arising from the cationic excess [7–9]. Chloride ions thus play their prime role by altering the stability of the deoxy form relative to the oxy form by altering the internal electrostatic interactions. Using chemically modified and mutant haemoglobins it has been possible to identify a second role for chloride ions involving the α-amino group of Val-1α and Lys-82β which lie at opposite ends of the central water-filled cavity [10–12]. Although it was at first thought that chloride ions exert an analogous electrostatic effect by binding directly to these two residues [13] more recent X-ray diffraction analysis shows no evidence of such binding [6,14]. It is now believed that these two residues, which in fact lie at opposite edges of the central cavity [15], exert their effect primarily via an entropic contribution associated with a higher degree of mobility in the oxygenated structure [7].

MATERIALS AND METHODS

The three human embryonic haemoglobins were expressed in yeast expression systems, isolated and purified as previously described [4]. Oxygen-binding experiments were performed using a spectro-polarographic oxygen dissociation analyser designed and built in this laboratory. The automatic recording oxygen dissociation analyser consists of a 2.5 ml volume sample chamber constructed from a drilled Perspex block housed within the sample compartment of a DW2a dual-wavelength spectrophotometer (Aminco Corp., Silver Spring, MA, U.S.A.). The sample is stirred magnetically. The sample chamber is also fitted with a Clark-type oxygen electrode (Y.S.I., Yellow Springs, OH, U.S.A.) which is used to monitor the oxygen concentration in solution. The sample is maintained at 37 °C ($\pm$0.1) using a Julabo U3 circulating water bath (Julabo, Seelback, Germany). During an experiment, a sample of haemoglobin is progressively deoxygenated by bubbling through the sample a stream of O_2-free nitrogen which has been further scrubbed free of residual oxygen using an Oxy-trap chemical scrubber (Alltech, Deerfield, IL, U.S.A.) and equilibrated to 37 °C by passage through the circulating water bath. Dissolved oxygen concentration and haemoglobin saturation are simultaneously monitored via the Clark electrode and the dual-wavelength spectrophotometer. By measuring the difference in absorption at 450 and 439 nm the spectrophotometer automatically removes the optical noise arising from the passage of gas bubbles through the light beam. The absorbance output from the spectrophotometer is used either to drive the y axis of an x/y chart recorder or else is input directly into a dedicated 486 personal computer. Likewise the output

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from the Clark electrode is either used to drive the x axis of the x/y chart recorder or else is input directly into the computer. Input into the computer is via a programmable gain analogue-to-digital converter (Lab Master DMA, Scientific Solutions, Solon, OH, U.S.A.). Further analysis of the binding curves is performed using the non-linear curve-fitting facility provided by the Tablecurve computer program (Jandel Scientific, San Rafael, CA, U.S.A.).

In order to measure chloride-binding curves for the embryonic haemoglobins the protein was dissolved in 50 mM Hepes buffer, pH 7.4, in the presence of varying amounts of added sodium chloride. The effect of chloride ions on the alkaline Bohr effect was determined by comparison of the p_{50} values (the oxygen partial pressure necessary to achieve 50% saturation of the haemoglobin oxygen-binding sites) as a function of pH in the presence and absence of 100 mM sodium chloride in 50 mM Hepes buffer. All binding curves were determined using 60 μM haemoglobin containing 60 μM EDTA and 20 μg/ml catalase in order to limit oxidation of the sample. All curves were recorded on samples containing less than 5% methaemoglobin.

RESULTS

When oxygen equilibrium curves were measured for adult human haemoglobin and the three human embryonic haemoglobins at 37 °C in 50 mM HEPES buffer, pH 7.4, in the presence of various concentrations of chloride ions it was found that the co-operativity exhibited by each of the haemoglobins, expressed as the Hill coefficient, was essentially constant. The p_{50} values for each haemoglobin, however, showed a characteristic dependence on chloride ion concentration (Figure 1). In the complete absence of chloride ions the four haemoglobins showed remarkably similar p_{50} values (3.5 ± 0.4 mmHg). As the chloride ion concentration was increased the oxygen affinity of the adult protein and that of the human embryonic Gower II ($\alpha_2\epsilon_2$) and Gower I ($\zeta_2\epsilon_2$) proteins reached a maximal p_{50} value at around 1 M chloride concentration. The relative change in p_{50} for each of these proteins as a function of chloride concentration is however quite different. The relative sensitivity of each can best be expressed by the function $\Delta\log p_{50}/\Delta\log$ [Cl]. Using this measure to express the chloride sensitivity of the proteins we obtain values of 0.5, 0.31 and 0.04 respectively for the adult haemoglobin, haemoglobin Gower II and haemoglobin Gower I. Figure 1 also shows that haemoglobin Portland ($\zeta_2\gamma_2$) exhibits no sensitivity to the presence of chloride ions in solution.

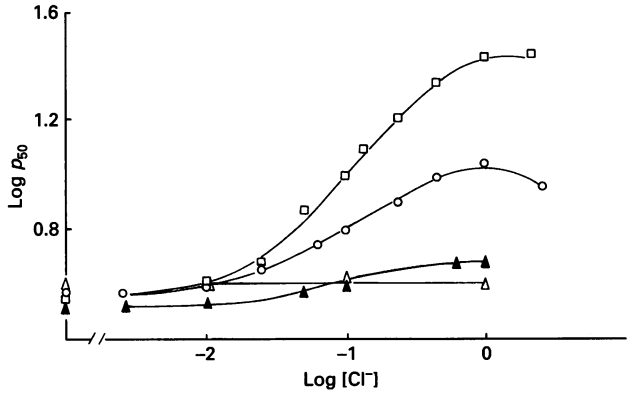

Figure 1 The effect of chloride ions on oxygen binding to human haemoglobins

Oxygen-binding curves were measured at 37 °C in 50 mM HEPES buffer, pH 7.4. Chloride ion concentration was altered by the addition of a concentrated solution of sodium chloride. A new protein sample was used for each data-point. The curves are shown for $\alpha_2\beta_2$ (□); $\alpha_2\epsilon_2$ (○); $\zeta_2\epsilon_2$ (△); and $\zeta_2\gamma_2$ (▲). The points on the y-axis correspond to the values obtained in the absence of chloride ions.

It has long been recognized that the Bohr effect and the chloride sensitivity in adult haemoglobin are at least in part linked functions [16,17], such that this protein exhibits what have come to be called 'chloride dependent' and 'chloride independent' components of the Bohr effect [7]. We have independently determined these components at 37 °C by measuring the Bohr effect for adult haemoglobin in 50 mM Bistris buffer supplemented with 100 mM sodium chloride and the Bohr effect in 50 mM Hepes buffer in the absence of chloride ions (Figure 2) In quantitative terms the Bohr effect can best be expressed using the function $\Delta\log p_{50}/\Delta$pH, usually referred to as the Bohr coefficient [18]. For the adult protein (Figure 2) we obtained a value of -0.45 in the presence of 100 mM sodium chloride and a value of -0.23 in the absence of chloride ions.

We next proceeded to determine the chloride-dependent and -independent Bohr components for each of the human embryonic haemoglobins, employing the same procedures as for the adult protein. Once again the embryonic proteins displayed distinct chloride-dependent Bohr effects. Haemoglobin Gower II presented results most similar to those of the adult protein (Figure 2b). In the presence of chloride ions the Bohr coefficient was -0.41 and in the absence of chloride ions -0.25. Embryonic haemoglobin Gower I ($\zeta_2\epsilon_2$) had a Bohr coefficient of -0.17 in the presence and -0.1 in the absence of chloride ions (Figure 2c). As would be predicted from Figure 1, embryonic haemoglobin Portland ($\zeta_2\gamma_2$) did not exhibit any chloride-dependent Bohr effect and displayed a Bohr coefficient of -0.26 both in the presence and absence of chloride ions (Figure 2d).

DISCUSSION

Although the allosteric role of chloride ions in the control of adult haemoglobin function has long been recognized, the mechanism whereby it achieves this control has only recently been rationalized [7]. The mechanism of action of chloride ions is envisaged as arising, principally, from the stabilization of the deoxy T state of the protein by means of the neutralization of excess cationic charges found within the central water-filled cavity of the molecule [7,9]. The chloride ions appear to perform this task by randomly associating with the excess positive charges provided by Val(NH_2)-1α, Lys-99α, His-103α, Val-1β, His-2β, Lys-82β, Arg-104β and His-143β of the adult protein [6]. This model fits well the experimental data obtained for the adult protein and a number of single-point mutants of the protein [6,9,19]. We have accepted this model as appropriate and extended its application to the embryonic haemoglobin data outlined above in the Results section. In the absence of detailed three-dimensional structures for the embryonic proteins we must make a number of assumptions. We have assumed that the overall structures of the embryonic proteins are the same as the adult protein and that the sequence-position-specific roles played by key amino acid residues are conserved between the proteins. Having made these assumptions we can now rationalize the observed chloride sensitivity of the embryonic proteins within the context of the model-specific roles played by certain amino acids as established by Perutz [7] and Bonaventura and co-workers [8,9].

Our measure of the chloride sensitivity for the adult protein (0.5) is very similar to that obtained previously for this protein when measured at 25 °C (0.45, [7]).

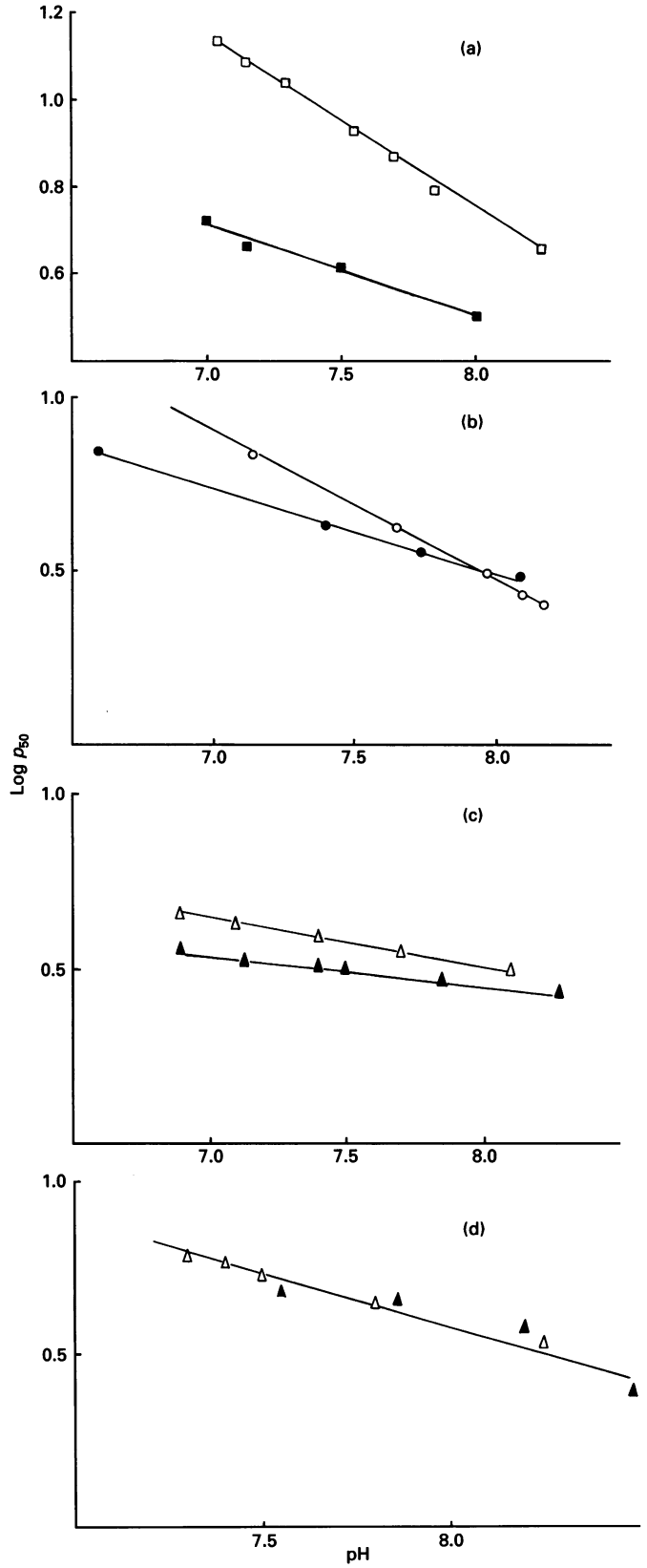

Figure 2 The chloride dependent Bohr effect of adult human and embryonic human haemoglobin

The Bohr effect is shown in the presence of 100 mM sodium chloride (open symbols) and the absence of added chloride ions (closed symbols) for the adult protein ($\alpha_2\beta_2$; **a**) and for human embryonic haemoglobins Gower II ($\alpha_2\epsilon_2$; **b**), Gower I ($\zeta_2\epsilon_2$; **c**) and Portland ($\zeta_2\gamma_2$; **d**).

Perutz et al., using mutant adult haemoglobin proteins, have shown that a net reduction by two positive charges (per dimer) within the central cavity is sufficient to reduce the chloride sensitivity of the adult protein from 0.45 to 0.035 (cf. haemoglobin Turrif Lys-99α → Glu [7]). A net loss of one positive charge from the central cavity is reported to reduce the chloride sensitivity from 0.45 to 0.22 (cf. haemoglobin Sherwood Forest Arg-104β → Thr [7]).

By inspection of the adult human deoxy haemoglobin structure (co-ordinates obtained from the Brookhaven Protein Data Base) and the amino acid sequences of the embryonic haemoglobins we can make the following proposals. The complete loss of chloride sensitivity from the embryonic haemoglobin Portland ($\zeta_2\gamma_2$) appears to arise from the loss of slightly more than two formal positive charges from within the central cavity. The N-terminal acetylation of the ζ-chain Val-1 residue found in this protein [4] is expected to reduce the chloride effect somewhat [6]. Although not formally within the central cavity, this residue expresses its action through an entropic effect associated with a higher degree of mobility of this residue in the oxygenated form [7]. The ζ-chain Ser-138 → Glu substitution on the other hand would clearly reduce the formal positive charge within the central cavity. Furthermore the γ-chain has a His-143 → Ser substitution at a site previously identified as a chloride-sensitive site [6]. A Pro-125 → Glu substitution might also contribute to a reduction of charge within the cavity. Thus, compared with the adult protein, the $\zeta_2\gamma_2$ protein is clearly expected to exhibit a significant reduction in the cationic excess and so no chloride dependence in its oxygen-binding properties.

In the case of the human embryonic haemoglobin Gower I ($\zeta_2\epsilon_2$), the model predicts the loss of two positive charges from the cavity, as the chloride sensitivity observed experimentally for this protein is 0.04. As outlined above, the ζ-chain carries a Ser-138 → Glu substitution which would reduce the cavity charge by one unit. Whilst the Val-1 ζ-chain N-terminal acetylation also makes a contribution, it would appear that the ϵ-chain also contributes one less positive charge than the β-chain. Inspection of the adult deoxy model does not conclusively identify any residue within the ϵ-chain which can be nominated with confidence in the absence of a detailed three-dimensional structure of the ϵ protein. Three residues appear possible candidates, namely His-116β → Thr; His-77β → Asn (M.F. Perutz, personal communication) or Pro-125β → Glu.

In the case of embryonic haemoglobin Gower II ($\alpha_2\epsilon_2$), as compared with the adult protein ($\alpha_2\beta_2$), it appears that the central cavity cationic excess is reduced by one positive charge. The α-chain is obviously common between the proteins and so the charge difference must be associated with the ϵ-chain, as outlined above.

It is thus satisfying that the model proposed to explain the chloride effect in the adult haemoglobin protein can reasonably be extended to the human embryonic proteins. A categorical statement on this effect must of course await detailed structure determinations of the oxy and deoxy forms of these proteins.

With regard to the physiological significance of these findings it is interesting to note that all of the embryonic haemoglobins exhibit almost identical intrinsic oxygen affinities with the adult protein, in the absence of chloride ions. The previously identified intrinsically high oxygen affinities of the embryonic haemoglobins, at high salt concentration [4], are thus an artefact and merely reflect their lower sensitivity towards chloride ions. Within the embryonic period of development, the progressive transition from the $\zeta_2\gamma_2$, $\zeta_2\epsilon_2$ to $\alpha_2\epsilon_2$ forms is matched by an increasing sensitivity towards chloride ions. Whether this increasing sensitivity is correlated with an increasingly dynamic

chloride environment for the embryonic erythrocytes is not at this stage known.

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