The measurement of the intrinsic alkaline Bohr effect of various human haemoglobins by isoelectric focusing

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We have used isoelectric focusing to measure the differences between the pI values of various normal and mutant human haemoglobins when completely deoxygenated and when fully liganded with CO. It was assumed that the $\Delta pI(\text{deox.} - \text{ox.})$ values might correspond quantitatively to the intrinsic alkaline Bohr effect, as most of the anionic cofactors of the haemoglobin molecule are 'stripped' off during the electrophoretic process. In haemoglobins known to exhibit a normal Bohr coefficient ($\Delta \log P_{50}/\Delta pH$) in solutions, the $\Delta pI(\text{deox.} - \text{ox.})$ values are lower the higher their respective pI(ox.) values. This indicates that for any particular haemoglobin the $\Delta pI(\text{deox.} - \text{ox.})$ value accounts for the difference in surface charges at the pH of its pI value. This was confirmed by measuring, by the direct-titration technique, the difference in pH of deoxy and fully liganded haemoglobin A₀ ($\alpha_2\beta_2$) solutions in conditions approximating those of the isoelectric focusing, i.e. at 5°C and very low concentration of KCl. The variation of the $\Delta pH(\text{deox.} - \text{ox.})$ curve as a function of pH (ox.) was similar to the isoelectric-focusing curve relating the variation of $\Delta pI(\text{deox.} - \text{ox.})$ versus pI(ox.) in various haemoglobins with Bohr factor identical with that of haemoglobin A₀. In haemoglobin A₀ the $\Delta pI(\text{deox.} - \text{ox.})$ value is 0.17 pH unit, which corresponds to a difference of 1.20 positive charges between the oxy and deoxy states of the tetrameric haemoglobin. This value compares favourably with the values of the intrinsic Bohr effect estimated in back-titration experiments. The $\Delta pI(\text{deox.} - \text{ox.})$ values of mutant or chemically modified haemoglobins carrying an abnormality at the N- or C-terminus of the α -chains are decreased by 30% compared with the ΔpI value measured in haemoglobin A₀. When the C-terminus of the β -chains is altered, as in Hb Nancy ($\alpha_2\beta_2^{\text{TYR-145} \rightarrow \text{ASP}}$), we observed a 70% decrease in the ΔpI value compared with that measured in haemoglobin A₀. These values are in close agreement with the estimated respective roles of the two major Bohr groups, Val-1 α and His-146 β , at the origin of the intrinsic alkaline Bohr effect [Kilmartin, Fogg, Luzzana & Rossi-Bernardi (1973) *J. Biol. Chem.* **248**, 7039–7043; Perutz, Kilmartin, Nishikura, Fogg, Butler & Rollema (1980) *J. Mol. Biol.* **138**, 649–670]. In other mutant haemoglobins it is demonstrated also that the $\Delta pI(\text{deox.} - \text{ox.})$ value may be decreased or even suppressed when the substitution affects residues involved in the stability of the tetramer. These results support the interpretation proposed by Perutz, Kilmartin, Nishikura, Fogg, Butler & Rollema [(1980), *J. Mol. Biol.* **138**, 649–670] for the mechanism of the alkaline Bohr effect, and also indicate that the transition between the two quaternary configurations is a prerequisite for the full expression of the alkaline Bohr effect.

Among the different functional properties of mammalian haemoglobins the alkaline Bohr effect is of particular interest, as it concerns both O₂ delivery

Abbreviations used: Hb, haemoglobin; P_{50} , partial pressure of O₂ at half-saturation of haemoglobin; Bistris, 2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

to the peripheral tissues and the homoeostatic regulation of pH of extracellular fluids. However, despite its extensive study for more than 30 years, its molecular mechanism is still controversial (Kilmartin *et al.*, 1973; Matthew *et al.*, 1979). One of the more recent questions about this mechanism is the following: does the alkaline Bohr effect originate

exclusively from pK changes of specific residues and mainly from His-146 β , Val-1 α and His-122 α (Perutz *et al.*, 1969, 1980; Kilmartin *et al.*, 1973, 1980; Nishikura, 1978), or does it result from a widespread change in ionization of titratable groups within the molecule (Matthew *et al.*, 1979)? An example of this controversy appeared recently when it was reported that 'in the absence of phosphate His-146 β does not contribute significantly to the alkaline Bohr effect' (Russu *et al.*, 1980). During recent years we have used isoelectric focusing for determining the isoelectric pH (pI) of various human haemoglobins in solutions that were completely deoxygenated or were fully liganded with CO, following a technique first described by Park (1973) and later used more extensively by Bunn (1977). Our aim in using this technique was to screen for abnormal mutant haemoglobins that may be 'silent' in routine isoelectric focusing, i.e. that may have the same pI as HbA₀ when oxygenated but a lower one in the deoxygenated form. The present paper deals with results of the difference between pI values (ΔpI) of deoxygenated [pI(deox.)] and fully liganded [pI(ox.)] human haemoglobins known to exhibit, in solutions, either a normal or a reduced Bohr coefficient ($\Delta \log P_{50}/\Delta pH$).

The ΔpI of a haemoglobin that represents the difference in the surface charges between the deoxy and oxy forms of the protein may be considered as a direct measurement of the intrinsic Bohr effect, as most of the anionic cofactors are 'stripped' during the electrophoretic process. It has the main advantage of being determined at a high concentration of the protein present in the small volume where it focuses. This is a convenient way to avoid the major problem encountered when the measurements are made in diluted salt-free solution, where, particularly with mutant haemoglobins, the dimer-tetramer equilibrium constant may be shifted in favour of the dimers and thus lead to erroneous values of the Bohr effect. One major pitfall of this technique is that it gives, for any haemoglobin molecule, only one point of its Bohr curve, that corresponding to the pH of its pI value. We have obviated this problem by measuring the ΔpI of various human haemoglobins with $\Delta \log P_{50}/\Delta pH$ values equal or close to the normal value of -0.50 when measured in physiological conditions of pH, temperature and ionic strength but with widely different pI(ox.) values, such as HbF_{II}, HbS, HbC, HbE and HbA₂. This was performed with the idea that the change in ΔpI with pI(ox.) will give at least one segment of a normal isoelectric-focusing Bohr curve with which those of abnormal haemoglobins could be compared. These results were also compared with the ΔpH (deox.-ox.) curve obtained through direct titration of deoxygenated and fully liganded HbA₀ solutions in conditions similar to those of isoelectric

focusing, i.e. at 5°C and very low chloride concentration. A very good agreement between the two methods was obtained.

With the view to bringing new information to bear on the role played by specific groups within the haemoglobin molecule at the origin of the alkaline Bohr effect, we report specifically on pI values of human haemoglobins carrying an abnormality at the C-terminal region of either the β - or the α -chains. These are important, since 80–90% of the Bohr protons released on ligation of tetrameric HbA₀ have been attributed to the disruption of salt-bridges present between the N-terminal and the C-terminal residues in the deoxyhaemoglobin molecule (Perutz, 1970; Perutz *et al.*, 1980). In addition, the pI values of haemoglobins carrying an abnormal residue at the α_1 - β_2 contact are reported also, since this region is of crucial importance for both the quaternary changes occurring on ligation of the haem group and for the full expression of the alkaline Bohr effect (Perutz *et al.*, 1980).

Materials and methods

Haemoglobins

Isolated haemoglobins were prepared from fresh blood haemolysates by DEAE-Sephadex column chromatography as already described (Poyart *et al.*, 1980a). Haemoglobin solutions were then stripped of organic and inorganic anions by ion-exchange chromatography (Dowex 70-AG 50-1 X8 resin) and concentrated under pressure with the Aminco system (PM 10 membrane). They were stored until use in liquid N₂. In certain instances mutant haemoglobins from heterozygous carriers were not separated from the normal HbA₀ component, and the stripped haemolysates were used as such for the isoelectric-focusing experiments. Mixed-valency hybrids of HbA₀ were a gift from Dr. H. S. Rollemma (University of Nijmegen, Nijmegen, The Netherlands). The preparation and purity of Hb $\alpha_2^{des-Arg-141}\beta_2$ (des-Arg-Hb) and of derivatives of HbA₀ specifically carbamoylated at the α -amino group of the α -chain or β -chain terminal valine residue ($\alpha_2^C\beta_2$, $\alpha_2\beta_2^C$) have been described previously (Poyart *et al.*, 1980a,b).

Isoelectric focusing

Isoelectric focusing was performed in cylinders of polyacrylamide gels (12 cm $\times$ 0.5 cm diam.) in the Bio-Rad apparatus (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The gels were 4% (w/v) acrylamide/bisacrylamide plus NNN'-tetramethylethylenediamine and sodium persulphate (from Bio-Rad Laboratories); 2% (w/v) ampholytes (pH gradient 6–8 or 7–9) (LKB Produkter, Bromma, Sweden) were added to the acrylamide solutions. The electrolytes were 10 mM-H₃PO₄ and

10mM-NaOH. Each electrolyte solution was first evacuated of dissolved gases under vacuum and next equilibrated with pure N_2 in a closed glass vessel. The acid was admitted in the lower compartment of the apparatus under a pressure of N_2 , and a constant stream of N_2 was allowed to bubble in the acid solution until the beginning of the experiment. The deoxygenated NaOH solution was transferred similarly in the upper compartment but under a 2–3 cm-thick layer of paraffin oil. Much care was taken during this manoeuvre to avoid any contact with room air. The whole system was thermostatically controlled at $5 \pm 1^\circ\text{C}$. A pre-focusing period of 20–30 min was allowed for stabilizing the pH gradient in the gels (450 V, 1 mA per cylinder). At the beginning of this period 50 μl of 0.1% $Na_2S_2O_4$ (prepared anaerobically) was layered at the top of each gel, under the oil, with a tuberculin syringe. After the pre-focusing period, samples of the haemoglobin solutions were layered on the gels in a similar way. $Na_2S_2O_4$ was not used in experiments with the mixed-valency hybrids.

Preparation of the haemoglobin solutions

Samples of the purified haemoglobin solutions or of the haemolysates were diluted to approx. 0.6 g of tetramer/dl in 50mM-BisTris/HCl buffer, pH 6.8, at 25°C containing 10mM-NaCl and 0.5 g of sucrose/dl in a glass tonometer sealed on to an optical cuvette (2 mm light-path). The solutions were deoxygenated under pure argon until the ratio of absorbances $A_{554.5}/A_{540}$ was at least equal to 1.25 (Cary 219 spectrophotometer). The deoxygenated haemoglobin solution was withdrawn from the tonometer with a Hamilton syringe that had been flushed carefully beforehand with N_2 , and 40–50 μl (200–250 μg of tetrameric haemoglobin) was layered at the top of the gels. The rest of the solution remaining in the syringe was converted into its liganded form by addition of a small amount of CO and deposited on a separate gel. Because of the small variations in the pH gradient or in the length of the different gels, a marker haemoglobin (the pI of which was known) was deposited in each cylinder 20–30 min before or after the deoxyhaemoglobin solution. This was either fully liganded HbA_2 or HbA_0 , depending on the migration of the haemoglobin under investigation. The duration of the focusing period was usually 4–5 h at 5°C .

Determination of the pI of deoxy- and oxy-haemoglobins

At the end of the experiments the glass cylinders were removed and the distances of the different bands from the marker haemoglobin were recorded on graph paper. These distances were also calculated from the densitometric records of the gels (Quick Scan Junior; Laboratoires Helena, Paris,

France). This procedure for the assessment of the pI of the different bands assumed linearity of the pH gradient in the gels. This was checked several times by removing the gels from the cylinders and slicing them into 0.5 cm-thick pieces, which were eluted in 0.5 ml of 10mM-KCl for 18 h. The pH of each sample was then measured at 5°C (PHM 62 pH-meter; Radiometer, Copenhagen, Denmark). Fig. 1 shows that good linearity was indeed found in the pH range where most of the haemoglobin tested focused. Fig. 2 gives a further check of this linearity. This represents the densitometric records of isoelectric-focusing gels of a mixture of HbS and HbA_0 mixed in their oxy forms, before deoxygenation. The asymmetric hybrid $\alpha_2\beta_A\beta_S$ focused exactly midway from the two mother haemoglobins. The results given in the Tables are the means of at least four or five determinations and agreed within 0.02 pH unit. Further, the pI values of the fully liganded haemoglobins determined by the cylinder method were simultaneously compared with those obtained by the thin-slab isoelectric-focusing technique (Multiphor; LKB Produkter) (Krishnamoorthy *et al.*, 1976) and agreed very closely in the same range of experimental error.

Titration of deoxy and liganded HbA_0 at 5°C and low chloride concentration

To establish whether the difference between the pI values of deoxy- and fully liganded haemoglobin

Fig. 1. pH gradient of cylinder polyacrylamide gels containing 4% (w/v) ampholytes (pH 6–8) used for the measurement of the pI of various human haemoglobins. pH was measured at 5°C in eluates of 0.5 cm-thick slices in 10mM-KCl. The broken lines indicate the pH range where most of the human haemoglobins tested migrate. The open symbols represent the mean pH values corresponding to at least four gels in six different experiments. Arrows indicate the pI values of five human haemoglobins in the oxy form that, in solution, exhibit a normal Bohr coefficient ($\Delta\log P_{50}/\Delta\text{pH}$). O-R refers to Hb Ouled-Rabah (see Table 1). Experimental details are indicated in the text.

Fig. 2. Densitometric record of a polyacrylamide gel on which a mixture of HbA₀ and HbS (3:2, w/w) were mixed in their oxy forms and then rapidly deoxygenated before focusing. HbA₂, completely liganded with CO, was layered 20 min after the mixture of deoxy-HbA₀ and deoxy-HbS. The peak between HbA₀ and HbS indicates the presence of the asymmetrical hybrid $\alpha_2\beta_A\beta_S$, which focused exactly midway between the two mother haemoglobins. Experimental details are indicated in the text.

bears a quantitative relationship with the alkaline Bohr effect, it was necessary to obtain information on the direct-titration curves of deoxygenated and oxygenated haemoglobin solutions in conditions similar to those used for isoelectric focusing, i.e. at 5°C and at very low concentration of chloride. This was done with aqueous solutions of purified HbA₀ (0.7–0.9 mM in haem) containing 2 mM-KCl. The stock oxyhaemoglobin solutions were firstly extensively dialysed against double-distilled water at 4°C and adjusted to the final concentration. They were next deoxygenated under pure argon in glass tonometers until the spectrophotometric criteria were obtained ($A_{554.5}/A_{540} \geq 1.25$). The concentration of the protein was calculated from the absorption coefficients at these wavelengths. A 2.5 ml Hamilton syringe was used to transfer 1.5 ml of the deoxygenated solutions into the plastic Radiometer vessel, which had been carefully flushed with humidified argon. The vessel contained the circular magnetic stirrer and was closed with a rubber bung with one hole for the pH electrode (Radiometer GK 2321 C) connected to a PHM 64 Radiometer pH-meter. A second hole was used for the capillary of an Aglar micrometer syringe. A third hole was used for a precision mercury thermometer. Admission of gases, either argon or CO, was allowed through two different circuits with stainless-steel needles connected through stainless-steel tubes to the

humidifiers and gas tanks. The whole system was thermostatically controlled. The pH of the deoxygenated haemoglobin solution was adjusted with deoxygenated 10 mM-KOH or 10 mM-HCl (Titrasol; Merck, Darmstadt, Germany), and the corresponding amount of deoxygenated 10 mM-KCl (Suprapur; Merck) was added up to a final concentration of 2–3 mM with a Hamilton syringe. Once the pH readings and temperature of the deoxyhaemoglobin solution had stabilized, humidified CO was admitted for a short period of time (20–30 s), and the pH of the fully liganded haemoglobin solution was recorded. Then the solution was removed from the vessel and transferred into a 2 mm-light-path optical cuvette. The full combination of ligand with the haemoglobin in the solution was checked by measuring the absorbance ratio $A_{568.5}/A_{538.3}$: this was at least equal to 1.014. The absorbance was also used to calculate the final concentration of haemoglobin in solution ($\epsilon_{568.5} = 14.21 \text{ mM-haem}^{-1} \cdot \text{cm}^{-1}$). The titration curve was constructed from eight to ten points in each experiment and by plotting the pH against the amount of HCl added per mol of haem present. In other experiments a known amount of 10 mM-KOH or 10 mM-HCl was added to the carbonmonoxyhaemoglobin solution to titrate back to the pH of the initial deoxyhaemoglobin solution. This allowed to calculate directly the amount of oxygen-linked Bohr protons.

Results and discussion

It is obvious from Table 1 that the ΔpI values of various human haemoglobins that exhibit a normal alkaline Bohr effect in diluted solutions (2 oxygen-linked protons per tetramer in the presence of 0.1 M-NaCl at 25°C) vary considerably and are the lower the higher their respective pI values. One should note also that the present values are approx. 0.4 pH unit higher than usually reported pI values. This is due to the fact that these were measured from the pH gradient at 5°C, the difference being 0.4–0.45 pH unit from those measured at room temperature. Fig. 3(a) shows the variation of the difference in pH between deoxy- and liganded HbA₀ solutions as a function of the oxy-form pH measured at 5°C and at very low concentration of KCl. As expected, this difference is lowered with increasing pH. In Fig. 3(b) the ΔpI values of haemoglobins listed in Table 1 are compared with the same curve. All the ΔpI values of these haemoglobins fall very close to the curve, but are not randomly distributed; most are slightly below the expected curve. This may be due either to experimental errors or to the presence of the small amount of chloride in the titration experiments. [It was checked that the addition of 4–8% (w/v) ampholytes to a solution of

Table 1. Values of isoelectric points (pI) of various human haemoglobins in the oxy [pI(ox.)] and deoxy [pI(deox.)] configurations

These haemoglobins are known to exhibit in diluted solutions a Bohr coefficient ($\Delta\log P_{50}/\Delta\text{pH}$) similar to that measured in HbA₀, in physiological conditions of pH (7.0–7.2), temperature and ionic strength (0.1 M). The functional properties of Hb Ouled-Rabah have been reported (Elion *et al.*, 1973). $\alpha_2\beta_2^C$ is the carbamoylated derivative of HbA₀ at Val-1 β . Experimental details are indicated in the text.

Haemoglobin	Structure	pI(ox.)	pI(deox.)	$\Delta\text{pI}(\text{deox.} - \text{ox.})$
A ₀	$\alpha_2\beta_2$	7.355	7.525	0.170
F _{II}	$\alpha_2\gamma_2$	7.465	7.63	0.175
A ₂	$\alpha_2\delta_2$	7.84	7.92	0.08
S	$\alpha_2\beta_2^{\text{Glu-6} \rightarrow \text{Val}}$	7.635	7.765	0.13
Ouled-Rabah	$\alpha_2\beta_2^{\text{Asn-19} \rightarrow \text{Lys}}$	7.68	7.815	0.135
C	$\alpha_2\beta_2^{\text{Glu-6} \rightarrow \text{Lys}}$	7.88	8.0	0.12
E	$\alpha_2\beta_2^{\text{Glu-26} \rightarrow \text{Lys}}$	7.86	7.98	0.12
$\alpha_2\beta_2^C$	$\alpha_2\beta_2^{\text{Val-1-CNO}}$	7.32	7.52	0.20

Fig. 3. (a) Variation of the difference of pH between deoxygenated and fully liganded HbA₀ solutions with the pH of the liganded HbA₀ solution [pH(ox.)] and (b) values of $\Delta\text{pI}(\text{deox.} - \text{ox.})$ of haemoglobins listed in Table 1 compared with the same curve (-----)

(a) These measurements were made in solutions of HbA₀ 0.7–0.9 mM in haem, at 2–3 mM-KCl and at 5°C. The broken line was fitted by inspection. (b) Apart from HbA₂ all these haemoglobins are considered to have a normal ΔpI value. Experimental details are indicated in the text.

HbA₀ in 50 mM-Bistris/HCl buffer, pH 6.8, containing 0.1 M-NaCl has no effect on the P_{50} . HbA₂ shows a small ΔpI compared with HbA₀ at the same oxy-form pH.

de Bruin & Janssen (1973) have demonstrated the identity of the alkaline Bohr effect between the two proteins in the presence of 0.1 M-KCl and at 25°C. We did not find, from the structural differences between the β - and δ -chains, any clear reason for this discrepancy. The difference between the pI values of oxy-HbA₀ and oxy-HbS is 0.28 pH unit, which corresponds to the difference of 2 positive surface charges per tetramer between these two haemoglobins. Therefore one may calculate that a change of 1 pH unit corresponds to a variation of 7 charges per tetramer. This is close to the value of 8 charges reported by others in similar conditions (Bunn & Riggs, 1979). Conversely, one may calculate that the ΔpI of HbA₀ (0.17 pH unit) accounts for a difference on 1.20 charges per tetramer between deoxy- and oxy-HbA₀. This value is in good agreement with the results obtained by titration by Rollemans *et al.* (1975) for the alkaline Bohr effect of HbA₀ at 5 mM-KCl ($\Delta z = 1.4$), by Perutz *et al.* (1980) of 1 oxygen-linked proton per tetramer in the absence of chloride or that calculated by Van Beek & de Bruin (1980). A maximum value of 0.98 oxygen-linked proton at pH 6.85 has been measured in a back-titration experiment during the present study (results not shown). One may conclude therefore that the ΔpI determined in the present experiment is in close quantitative agreement with the estimated values of the intrinsic alkaline Bohr effect.

Table 2 gives the results obtained in human haemoglobins where low values for the alkaline Bohr effect have been reported when measured in diluted solutions. The first three haemoglobins (Hb Suresnes, $\alpha_2\text{Val-1, carbamoylated } \beta_2$ and des-Arg-Hb) have oxy-form pI values lower than that of oxy-HbA₀, but still have low ΔpI values between their respective deoxy and oxy configurations. This indicates a diminished intrinsic alkaline Bohr effect that may be attributed to the disruption of the salt-bridges formed in the deoxy configuration, between the N- and C-termini of the α -chains

Table 2. Values of isoelectric points (pI) of various human haemoglobins either completely deoxygenated or fully liganded with CO before isoelectric focusing

These haemoglobins have substitutions of residues or chemical modifications at one of the two major sites responsible for the alkaline Bohr effect. The functional properties of the mutant haemoglobins have been described: Hb Suresnes (Poyart *et al.*, 1980a); Hb Nancy (Gacon *et al.*, 1975); Hb Necker Enfants Malades (Wajcman *et al.*, 1980). The functional properties of the carbamoylated derivative of HbA₀ at Val-1 α ($\alpha_2^S\beta_2$) have been reported by O'Donnell *et al.* (1979) and those of des-Arg-Hb by Kilmartin *et al.* (1975) and Van Beek *et al.* (1978). Experimental details are indicated in the text.

Haemoglobin	Structure	pI (ox.)	pI (deox.)	Δ pI (deox. — ox.)
Hb Suresnes	$\alpha_2^{\text{Arg-141} \rightarrow \text{His}}\beta_2$	7.31	7.43	0.12
$\alpha_2^S\beta_2$	$\alpha_2^{\text{Val-1-CNO}}\beta_2$	7.32	7.44	0.12
Des-Arg-Hb	$\alpha_2^{\text{des-Arg-141}}\beta_2$	7.33	7.44	0.11
Hb Nancy	$\alpha_2\beta_2^{\text{Tyr-145} \rightarrow \text{Asp}}$	7.25	7.295	0.045
Hb Necker-Enfants Malades	$\alpha_2^{\text{His-20} \rightarrow \text{Tyr}}\beta_2$	7.29	7.45	0.16

(O'Donnell *et al.*, 1979; Poyart *et al.*, 1980b) and/or to the very low L-allosteric constant of these haemoglobins at this low ionic strength. Further, at least for Hb Suresnes and des-Arg-Hb, no dependence of $\log P_{50}$ with pH has been observed when it was measured at low chloride concentration (5 mM-NaCl) in 1 g/dl haemoglobin solutions and at 25°C (Poyart *et al.*, 1980a). As we measured a distinct Δ pI by the isoelectric-focusing method, we conclude that these deoxyhaemoglobins should be mostly dimeric in solutions. This was effectively observed from kinetic experiments performed with Hb Suresnes (Poyart *et al.*, 1980a). The fourth haemoglobin in Table 1 (Hb Nancy) (Gacon *et al.*, 1975) has an aspartic acid residue substituted for the tyrosine-145 residue in the β -chains. This lowers its pI (ox.) by 0.105 unit compared with that of HbA₀. This mutant haemoglobin has an increased affinity for O₂, very low co-operativity in O₂ binding and low alkaline Bohr effect, probably because 'the absence of the hydrogen bond between the OH group of Tyr-145 and the CO group of Val-93 destabilizes the T structure' (Perutz, 1970). Hence in Hb Nancy His-146 β would remain 'floating' in the deoxy state (Perutz, 1970; Kilmartin *et al.*, 1973) and keep its low pK_a (about 7.0), estimated in the oxy form, thus diminishing the alkaline Bohr effect. The very low Δ pI observed in Hb Nancy supports this interpretation. It is also worth mentioning that the decreases in the Δ pI values of the haemoglobins with a disrupted interaction between the N- and C-termini of their α -chains account for approx. 30% of the Δ pI measured in HbA₀. Similarly, the decrease in the Δ pI value for Hb Nancy accounts for as much as 70% of the Δ pI of HbA₀. Within experimental errors, including differences in the technical approaches, these values are very close to those established by Kilmartin *et al.* (1973) for the respective participation of the α -chains and β -chain Bohr groups to the overall alkaline Bohr effect. In Table 2 we have also reported the results obtained with Hb Necker-

Enfants Malades (Wajcman *et al.*, 1980), where the histidine-20 α is replaced by a tyrosine residue. It has been reported by Ohe & Kajita (1980) that histidine-20 α evidences a pK shift on ligation of deoxy-HbA₀ and could be consequently considered as an alkaline Bohr group. The value of the Δ pI measured in Hb Necker Enfants Malades, similar to that of HbA₀, demonstrates that in the conditions of isoelectric focusing this is not so.

To further exemplify the observations that could be made from the measurement of the Δ pI in mutant haemoglobins, the results obtained in several well-characterized abnormal haemoglobins are shown in Tables 3 and 4. We were not able to measure any significant difference between the pI of deoxy and oxy forms of mixed-valency hybrids, which indicates that in the present conditions these haemoglobins do not show any quaternary or tertiary changes, whether or not the remaining ferrous haem groups are combined with ligands.

Also in Table 3 are shown the results for three haemoglobins that bear a substitution involving a residue at the α_1 - β_2 contact. All three haemoglobins have low Δ pI values, and it is even completely abolished in Hb Rothschild. Table 4 shows results obtained with some other mutant haemoglobins carrying various substitutions and whose functional properties are well characterized. Apart from Hb Ty Gard (Bursaux *et al.*, 1978), all the haemoglobins listed have diminished Δ pI values when compared with HbA₀. However, most of them have been reported to exhibit a normal or slightly diminished alkaline Bohr effect in physiological conditions of pH, temperature and ionic strength. This would indicate that O₂-linked anion binding, and particularly chloride binding, may compensate for the alteration of the intrinsic alkaline Bohr effect.

In addition, these various examples may help us to understand more precisely why the structural alterations of the haemoglobins lead to the functional abnormality. It has been stressed above that Δ pI is

Table 3. Values of isoelectric points (pI) of various human haemoglobins after complete deoxygenation and after full combination with CO

These haemoglobins are known to exhibit in solutions a low Bohr coefficient. The three mutant haemoglobins bear substitution of a residue normally involved in the α_1 - β_2 contact. All these haemoglobins show high O₂ affinity and diminished haem-haem interaction, probably due to a low L-allosteric constant in the conditions of the isoelectric focusing. Hb Kempsey was studied by Bunn *et al.* (1974), Hb Hôtel Dieu by Blouquit *et al.* (1980), Hb Rothschild by Gacon *et al.* (1977) and the mixed-valency hybrids by Rollema *et al.* (1976). Experimental details are indicated in the text.

Haemoglobin	Structure	pI (ox.)	pI (deox.)	ΔpI (deox. - ox.)
Hb Kempsey	$\alpha_2\beta_2^{Asp-99 \rightarrow Asn}$	7.435	7.48	0.045
Hb Hôtel Dieu	$\alpha_2\beta_2^{Asp-99 \rightarrow Gly}$	7.43	7.52	0.090
Hb Rothschild	$\alpha_2\beta_2^{Tyr-37 \rightarrow Arg}$	7.72	7.72	0.00
$\alpha_2^+ \beta_2$	$\alpha_2^{Fe^{3+}} \beta_2^{Fe^{2+}}$	7.50	7.51	0.01
$\alpha_2 \beta_2^+$	$\alpha_2^{Fe^{2+}} \beta_2^{Fe^{3+}}$	7.455	7.46	0.00

Table 4. Values of pI (ox.) and pI (deox.) and functional characteristics of various human mutant haemoglobins
The functional properties of these haemoglobins have been reported: Hb Hope (Thillet *et al.*, 1974; Steinberg *et al.*, 1976), Hb Malmö (Lorkin *et al.*, 1970; Perutz *et al.*, 1980), Hb Creteil (Thillet *et al.*, 1976), Hb Ty Gard (Bursaux *et al.*, 1978) and Hb Broussais (de Traverse *et al.*, 1966). Experimental details are indicated in the text. Key: $\nearrow$, increased, $\searrow$, decreased, and N, normal compared with the values measured in HbA₀ solutions.

Haemoglobin	Structure	P_{50}	n_{50}	$\Delta \log P_{50}$			
				ΔpH	pI (ox.)	pI (deox.)	ΔpI (deox. - ox.)
Hb Hope	$\alpha_2\beta_2^{Gly-136 \rightarrow Asp}$	$\nearrow$	$\searrow$	N	7.30	7.42	0.12
Hb Malmö	$\alpha_2\beta_2^{His-97 \rightarrow Gln}$	$\searrow$	$\searrow$	N or $\searrow$	7.26	7.39	0.13
Hb Creteil	$\alpha_2\beta_2^{Ser-89 \rightarrow Asn}$	$\searrow$	$\searrow$	$\searrow$	7.32	7.42	0.10
Hb Ty Gard	$\alpha_2\beta_2^{Pro-124 \rightarrow Gln}$	$\searrow$	N	N	7.325	7.475	0.15
Hb Broussais	$\alpha_2^{Lys-90 \rightarrow Asn} \beta_2$	N	N	N	7.19	7.306	0.12

strongly affected if the substituted residue lies at one of the two distal extremities in either the α - or the β -chain. The scheme in Fig. 4 indicates that in both des-Arg-Hb and Hb-Nancy, for example, it is the pI values of the deoxy forms that appear to be mostly altered; this is as expected if the pK of either Val-1 α or His-146 β remains unchanged or low in the ligand-free haemoglobins.

The question is now to explain why or how can the ΔpI be altered when the substitution of a residue lies far away from the Bohr-group salt-bridges? One simple hypothesis is that this could happen if the substitution of the residue involves a change in T $\rightleftharpoons$ R quaternary-structure equilibrium, in either the deoxy or the oxy form. Such interpretation is supported by Hb Kempsey (Table 3). It may be seen from Fig. 4 that the pI(ox.) of Hb Kempsey is higher than that of HbA₀, as one could expect from the substitution of aspartic acid for arginine in a region of the protein freely accessible to the water molecules. Strikingly, the pI(deox.) of Hb Kempsey is lower than that of HbA₀. This may indicate that deoxy-Hb Kempsey, in the salt-free conditions of the isoelectric-focusing technique, is largely in the R quaternary structure, hence its low pI(deox.) value. This interpretation confirms the functional studies performed with this mutant haemoglobin (Bunn

Fig. 4. Schematic representation of ΔpI (deox. - ox.) of various human haemoglobins as measured by isoelectric focusing

Compared with HbA₀ (left-hand block) all these haemoglobins have lowered ΔpI and also low Bohr coefficients in solution. The broken lines indicate the missing part of the ΔpI if HbA₀ is taken as the reference. The arrows indicate which of the two pI values, either that of the deoxy or the oxy form, appears to be preferentially altered according to the hypothesis discussed in the text.

et al., 1974). Conversely it has been shown that solutions of Hb Hope, whether in the presence or absence of anions, show a low affinity for O₂, a normal alkaline Bohr effect and a low Hill coefficient (Thillet *et al.*, 1974; Steinberg *et al.*, 1976). It has been postulated (Perutz & Lehmann, 1968) that the low affinity for O₂ of this mutant haemoglobin may be explained by a newly formed salt-bridge between the substituted Asp-136 β (H 14) and Val-1 β of the same β -chain, possibly leading to a stabilization of the T structure. It was also suggested that the presence of Asp-136 β in Hb Hope 'raises the pK of Val-1 β more in oxy than in deoxy' compared with the pK of Val-1 β in HbA₀ (Perutz *et al.*, 1969). It may be calculated from the pI values of Hb Hope (Table 4) and those of HbA₀ (Table 1) that the difference in pI(deox.) between the two haemoglobins is 0.105 pH unit and that the difference is 0.05 pH unit between their pI(ox.) values. In other terms, the change in pI(ox.) due to the presence of the aspartic acid residue in Hb Hope is less than the change observed in the pI(deox.), so supporting the interpretation mentioned above.

Finally, we want to comment on the mutant Hb Rothschild, which carried a substitution at the α_1 - β_2 contact and exhibits no Δ pI between the oxy and deoxy forms (Table 3). Functional studies in diluted isolated Hb Rothschild solutions have revealed that this mutant haemoglobin has a lower affinity for O₂ than has HbA₀, small co-operativity in O₂ binding and a diminished alkaline Bohr effect (Gacon *et al.*, 1977). At very low concentration of chloride Hb Rothschild keeps its low O₂ affinity and low co-operativity, but with a complete inhibition of the alkaline Bohr effect (H. Wajcman & G. Gacon, personal communication). The high pI value observed in the present study for Hb Rothschild, together with the absence of change on combination with ligand, indicates that, in the conditions of isoelectric focusing, Hb Rothschild does not undergo any quaternary change between the deoxy and oxy configurations. Because of its low O₂ affinity, one may postulate that the oxy-form band is mostly populated with liganded tetramers in the T quaternary structure.

In conclusion, the determination of the Δ pI(deox.—ox.) of human haemoglobins through isoelectric focusing represents a convenient way to estimate the intrinsic alkaline Bohr effect, i.e. the anion-independent part of the Bohr effect. In various haemoglobins exhibiting a normal $\Delta\log P_{50}/\Delta$ pH value in solution, the Δ pI decreases with increasing pI(ox.), indicating the pH-dependence of the intrinsic Bohr effect. Analyses of Δ pI values of mutant haemoglobins carrying substitutions at residues normally involved in a Bohr-group salt-bridge support the interpretation made by Perutz *et al.* (1980) that the bulk of the protons released on

combination of the ligand with the tetramer originate from His-146 β and from Val-1 α . In other mutant haemoglobins it is demonstrated also that the Δ pI may be decreased or abolished when the substitution leads to a destabilization of one of the two quaternary structures of the tetramer and/or to a low value of the L-allosteric constant. This supports the view that the quaternary transition from the deoxy to the oxy configuration is a prerequisite for the full expression of the alkaline Bohr effect (Perutz *et al.*, 1980).

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