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Identification of Histidine-122α in Human Haemoglobin as One of the Unknown Alkaline Bohr Groups by Hydrogen–Tritium Exchange

By KAZUKO NISHIKURA*
Medical Research Council Laboratory of Molecular Biology, University Postgraduate Medical School, Hills Road, Cambridge CB2 2QH, U.K.

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Human carbonmonoxy- and deoxy-haemoglobins were incubated at 37°C in ^3H_2O at various pH values to measure the pH-dependent hydrogen–tritium exchange at the C-2 position of the imidazole ring of histidine-122α. To obtain the pseudo-first-order rate constants for the exchange, k, the two peptides containing histidine-122α were isolated and the amounts of tritium incorporated were determined. The rate constants gave pK values for the histidine of 6.1 in carbonmonoxyhaemoglobin and 6.6 in deoxyhaemoglobin, showing that it contributes about 20% to the total alkaline Bohr effect and about 10% at pH 7.4.

The interaction between the binding of oxygen and hydrogen ions to the haemoglobin molecule is known as the Bohr effect (Bohr *et al.*, 1904). Below pH 6.0, oxyhaemoglobin binds more protons than does deoxyhaemoglobin, and above pH 6.0, in the physiological range of pH, deoxyhaemoglobin binds more protons than oxyhaemoglobin. These phenomena are called the acid and alkaline Bohr effects respectively. Wyman (1939) showed that the differential titration curve for oxygenation could be accounted for by a model in which each subunit has two oxygen-linked ionizing groups that change their pK values on oxygenation, one from 5.25 to 5.75 (the acid Bohr groups) and the other from 7.81 to 6.80 (the alkaline Bohr groups).

Up to now two residues, histidine-146β and valine-1α, have been identified as the alkaline Bohr groups. In deoxyhaemoglobin the imidazole moiety of histidine-146β forms a hydrogen bond with the carboxy group of aspartate-94β, and the α-amino group of valine-1α extends a hydrogen bond to an inorganic anion, which is also linked to the guanidinium group of arginine-141 of the opposite α-chain (Arnone *et al.*, 1976). When these hydrogen bonds break on oxygenation (Perutz *et al.*, 1968), the pK values of the imidazole and α-amino groups are lowered and protons are released (Perutz *et al.*, 1969). Valine-1α is responsible for about 20% and histidine-146β for about 40% of the alkaline Bohr effect, leaving another 40% still to be accounted for (Kilmartin & Rossi-Bernardi, 1973). Perutz (1970) has proposed that a further contribution may come from histidine-122α. However, the evidence relating to this proposal is conflicting (Tuchinda *et al.*, 1975; Brunori, 1975; M. F. Perutz, J. V. Kilmartin, K. Nishikura, J. H. Fogg & P. J. G. Butler, unpublished work). I have therefore tried to measure the pK change of histidine-122α on oxygenation directly, and to estimate its exact contribution to the alkaline Bohr effect.

Ohe *et al.* (1974) have developed a hydrogen–tritium exchange method for the determination of the pK values of individual histidine residues in intact proteins, which has been applied successfully to pancreatic ribonuclease (Ohe *et al.*, 1974) and bovine trypsin (Krieger *et al.*, 1976). The pH-dependence of the exchange reaction has been explained by a mechanism that involves a rate-determining abstraction of the C-2 proton of the imidazole ring by OH^- or by H_2O, followed by a fast protonation (Vaughan *et al.*, 1970). By this method, I have found pK values for histidine-122α of 6.1 in carbonmonoxyhaemoglobin and 6.6 in deoxyhaemoglobin at 37°C, which shows that this histidine residue is one of the alkaline Bohr groups not previously identified. Its contribution is estimated as about 20% of the total alkaline Bohr effect and 10% at pH 7.4. The remaining Bohr effect, about 20%, may come from a basic residue near the 2,3-diphosphoglycerate-binding site in the central cavity (M. F. Perutz, J. V. Kilmartin, K. Nishikura, J. F. Fogg & P. J. G. Butler, unpublished work).

Experimental

Materials

^3H_2O (1 Ci/ml) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Bio-Solv Formula BBS-3 was obtained from Beckman

* Present address: Division of Biochemistry, Tsukuba University School of Medicine, Sakura, Niihari, Ibaraki 300-31, Japan

Instruments, Fullerton, CA, U.S.A. Trypsin treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one and α-chymotrypsin were from Worthington Biochemical Co., Freehold, NJ, U.S.A. Nitrogen (oxygen-free) was obtained from British Oxygen Co., Ipswich, U.K., and carbon monoxide was obtained from BDH (Laboratory Gas Service) Ltd., Poole, Dorset, U.K. Human blood was obtained as partially filled bottles less than 1 week old from the National Blood Transfusion Centre, Cambridge, U.K.

Methods

Hydrogen–tritium exchange of histidine residues in haemoglobin. Human haemoglobin was prepared (Kilmartin & Rossi-Bernardi, 1971) and stripped of all diphosphoglycerate by passage through a Dintzis column (Nozaki & Tanford, 1967).

The tritium exchange procedure was based on the method of Krieger *et al.* (1976). Desalted haemoglobin solution, buffer and water were deoxygenated separately in tonometers by evacuation and flushing with nitrogen and injected anaerobically into a sealed incubation tube which had been evacuated and filled with either nitrogen or carbon monoxide. The buffers used were 0.1M-sodium cacodylate/HCl in 0.1M-NaCl (pH4.9–5.5), 0.05M-Bistris {2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol}/HCl in 0.1M-NaCl (pH5.5–7.5), and 0.1M-Tris/HCl in 0.1M-NaCl (pH7.5–8.2). After the addition of 2mol of $Na_2S_2O_4$/mol of haem to protect the haemoglobin from autoxidation and bacterial infection, 100μl of ^3H_2O (20mCi) was injected into the incubation tube with an Agla Micrometer Syringe. The final specific radioactivity of the water monitored on part of each sample, was 270±20c.p.m./nmol. A Beckman model LS-250 scintillation counter was used for all ^3H counting. The final haemoglobin concentration determined spectrophotometrically was 1.2mM-haem.

A 1ml sample of carbonmonoxy- or deoxyhaemoglobin solution was incubated at 37°C for 38h at various pH values (4.9–8.2). After incubation, the pH of each sample was recorded at 37°C with a Radiometer pH-meter 20. To terminate the reaction, 50μl of acetic acid and 100μl of 0.25M-HCl were added to the reaction mixture.

Isolation of the peptide. Protein was separated from the incubation buffer and the ^3H_2O by passage through a Sephadex G-25 (medium grade) column (2.5cm×100cm) equilibrated with 2mM-HCl in a cold-room at 5°C. The haemoglobin solution was freeze-dried twice from 2mM-HCl, redissolved in 0.5ml of the same buffer, and denatured in a boiling-water bath for 5min. About 20mg of haemoglobin was digested successively for 3h with 0.2mg of trypsin and 0.2mg of chymotrypsin in 0.5ml of 0.1M-ammonium carbonate buffer, pH7.98 at 37°C. One drop of acetic acid was added to end the digestion after 3h incubation. The digest was freeze-dried and subjected to electrophoresis and paper chromatography. The whole sample was mounted on chromatographic paper (Whatman 3MM) as a 12cm strip, and the peptides were resolved first by electrophoresis (acetic acid/pyridine/water, 1:33:300, by vol.; pH6.5; 45V/cm; 100min). The locations of the peptides containing histidine were found by fluorescamine staining and by the Pauli reaction. Each band was cut out and sewn on another sheet of chromatographic paper, and two-phase descending chromatography (butan-1-ol/acetic acid/water/pyridine, 15:3:12:10, by vol.; pH3.5) was carried out. Before the tritium-exchange experiment, the amino acid composition of each peptide containing histidine was first determined by amino acid analysis of the peptide separated from the control peptide 'map'. All analyses were run on a Durrum D-500 amino acid analyser. The *N*-terminal amino acid of the peptide was confirmed by the dansyl method. The isolated and identified peptides containing histidine-122α and histidine-2β were cut out and eluted with 10% (v/v) formic acid. Histidine-2β was chosen so as to compare its exchange rates and p*K* values with those of histidine-122α.

Determination of the tritium exchange rate constant. The radioactivity of 75% of the sample was counted in 10ml of scintillation solution mixture [6.25g of 2,5-diphenyloxazole and 375mg of 1,4-bis(5-phenyloxazol-2-yl)benzene in 1 litre of toluene+250ml of Beckman Bio-Solv]. The remaining 25% of the sample together with 50nmol of isoleucine was freeze-dried, hydrolysed with 6M-HCl at 110°C for 20h, and analysed for amino acids to determine the amount of histidine present. Several peptides that did not contain histidine were extracted in the same way and used to measure the background radioactivity. A first-order rate constant *k* for tritium exchange was calculated from the following equation (Krieger *et al.*, 1976):

$$k = -\frac{1}{t}\ln\left(\frac{e-sa}{e}\right) \quad (1)$$

where *sa* is the observed specific radioactivity after an incubation time *t* (38h) and *e* is the specific radioactivity of the ^3H_2O used in each incubation (270±20c.p.m./nmol).

Results and Discussion

Identification of peptide

After incubation for 38h in ^3H_2O at various pH values, each sample was enzymically digested, and the peptides were separated by electrophoresis and chromatography. Two kinds of peptides containing histidine-122α (peptide A and peptide B) and one peptide containing histidine-2β were identified by amino acid analysis and by the dansyl method. The

Table 1. *Amino acid compositions in chymotryptic–tryptic peptides of haemoglobin*
The relative molar ratios of amino acids in the peptides are listed. The numbers expected (Braunitzer *et al.*, 1961) are given in parentheses.

	His-122α in peptide A	His-122α in peptide B	His-2β peptide	Control peptide
Asx	0.07	0.57 (1)	0.22	0.09
Thr	1.02 (1)		1.00 (1)	1.00 (1)
Ser	0.11	0.97 (1)	0.22	
Glx	0.14	0.06	2.12 (2)	1.06 (1)
Pro	0.96 (1)		1.07 (1)	0.07
Gly	0.20	0.07	0.19	0.11
Ala	1.10 (1)	1.14 (1)	0.38	0.07
Val	1.00 (1)		0.78 (1)	0.05
Leu	0.06	1.17 (1)	1.00 (1)	
His	0.81 (1)	1.00 (1)	0.74 (1)	0.03
Lys	0.08	0.61 (1)	1.11 (1)	0.05
Arg			0.11	0.89 (1)
N-Terminal amino acid	Thr	His	Val	Thr
Sequence assignment	Thr118–His122α	His122–Lys127α	Val1–Lys8β	Thr38–Arg40β

Table 2. *Pseudo-first-order rate constants of the tritium exchange with histidine of haemoglobin*
Pseudo-first-order rate constants for the exchange, k, were calculated from eqn. (1) by using the observed specific radioactivity of each histidine and the specific radioactivity of the ^3H_2O used for each incubation (270±20c.p.m./nmol). The values in parentheses correspond to $k_{max.}$ for histidine-122α and histidine-2β, which were obtained from the pH-independent maximum part of the curves of Figs. 1 and 2.

		$10^4 \times k$ (h^{-1})			
	pH	His-122α in peptide A	His-122α in peptide B	His-2β	Control* (Thr-38–Arg-40β)
Carbonmonoxy-haemoglobin	5.06	0.39	2.27	3.92	0.13
	5.63	0.65	3.79	6.89	0.10
	6.26	0.83	10.42	22.61	0.29
	6.83	1.01	13.65	31.16	0.31
	7.46	0.80	14.39	38.22	0.30
	7.86	0.69	14.30	41.71	0.25
			(14.5)	(40.1)	
Deoxyhaemoglobin	5.12	0.26	0.79	4.61	0.16
	5.59	0.17	1.25	8.48	0.07
	6.26	0.32	4.59	18.97	0.20
	6.71	0.58	8.70	34.70	0.13
	7.48	0.39	13.90	46.45	0.11
	7.89	0.61	14.42	45.88	0.22
			(14.6)	(46.0)	

* The values for the control peptide were calculated on the assumption that there was one position in it able to exchange with ^3H.

amino acid compositions of these peptides and the results of the *N*-terminal analyses are shown in Table 1. The results for the β-chain peptide Thr-38–Arg-40β are also listed as an example of a control peptide that contains no histidine.

Measurement of first-order rate constant

Pseudo-first-order rate constants for the exchange, k, were calculated from eqn. (1). Some of the results are shown in Table 2. The rate constants of histidine-2β and histidine-122α in peptide B are high enough to permit determination of accurate pK values, but the rate constant of histidine-122α in peptide A is only slightly above that of the control and too low to assign a pK value. The back-exchange rate of tritium from the C-2 position of histidine-122α during the preparation of the peptide must have been faster in peptide A than in peptide B, but the cause of that difference is unclear. Only the results of peptide B were used for the measurement of the pK values of histidine-122α.

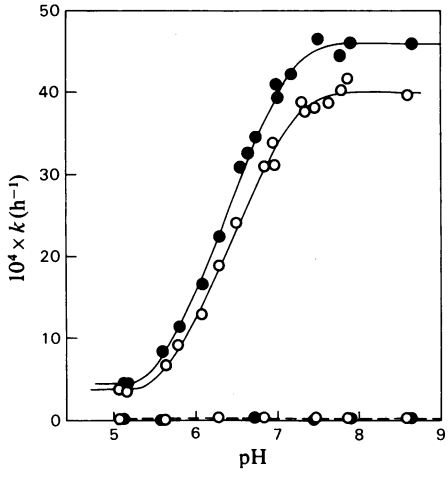

Fig. 1. *pH-dependence of the pseudo-first-order rate constants for tritium exchange with the C-2 proton in the imidazole ring of histidine-2β at 37°C*

The hydrogen–tritium exchange was measured in 0.1 M-sodium cacodylate/HCl in 0.1 M-NaCl (pH 4.9–5.5), 0.05 M-Bistris/HCl in 0.1 M-NaCl (pH 5.5–7.5) and 0.1 M-Tris/HCl in 0.1 M-NaCl (pH 7.5–8.2). The concentration of haemoglobin was 1.2 mM in haem. The solid curves are standard curves for histidine-2β chosen to give the best fit to the data. The dashed curve represents those of the control peptide, Thr-38–Arg-40β. ○, Carbonmonoxyhaemoglobin; ●, deoxyhaemoglobin. The p*K* values (6.4) of histidine-2β in carbonmonoxy- and deoxy-haemoglobin are obtained from the pH at the midpoint of each sigmoidal curve.

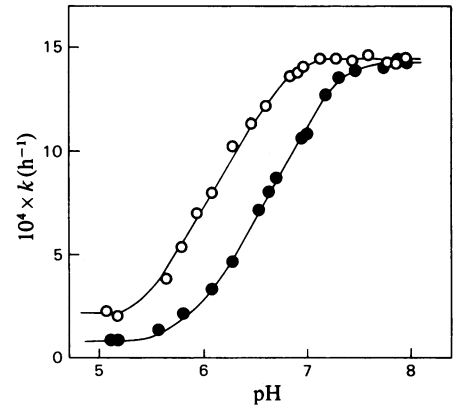

Fig. 2. *pH-dependence of pseudo-first-order rate constants for tritium exchange with the C-2 proton in the imidazole ring of histidine-122α at 37°C*

The conditions used were the same as those in Fig. 1. ○, Carbonmonoxyhaemoglobin; ●, deoxyhaemoglobin. The curves are standard titration curves chosen to give the best fit to the data. The p*K* values of histidine-122α in carbonmonoxyhaemoglobin (6.1) and deoxyhaemoglobin (6.6) are obtained from the pH at the midpoint of each sigmoidal curve.

Measurement of the pK values of histidine-2β and histidine-122α

The experimental data for the pH-dependence of the exchange rates of histidine-2β in carbonmonoxy- and deoxy-haemoglobin, together with the curves chosen to give the best fit to the data, are illustrated in Fig. 1. In Fig. 1 results for the peptide Thr-38–Arg-40β, which contains no histidine, are also shown. As the rate of tritium exchange of the control peptide was negligible, the pH-dependence curves of the histidine residues were not corrected for background. The results for histidine-122α of peptide B are shown in Fig. 2. The $k_{max.}$ (pH-independent maximum of k) values for histidine-122α and histidine-2β were obtained as the k value corresponding to the flattened part of the pH-dependence curve in the alkaline region (Table 2). $k_{max.}$ of histidine-122α is only just one-third of that of histidine-2β in both carbonmonoxy- and deoxy-haemoglobin, suggesting that histidine-2β is much more accessible to water than histidine-122α.

The p*K* values were taken as the pH at the midpoint of each sigmoidal curve. The p*K* of histidine-2β is 6.4 at 37°C in both carbonmonoxy- and deoxy-haemoglobins (Fig. 1). Ohe & Kajita (1977) measured the p*K* value of histidine 2β by the mass-spectrophotometric method by using the hydrogen–deuterium exchange reaction and reported a p*K* of 6.9 at 36.5°C for both oxy- and deoxy-haemoglobins, which is a little higher than the value I obtained. The difference might be due to the different conditions used for removing the organic phosphate from the haemoglobin solution, because histidine-2β is a part of the binding site for organic phosphate (Arnone, 1972) and its p*K* value is sensitive to the presence of organic phosphate (Ohe & Kajita, 1977). The p*K* value of histidine-122α in carbonmonoxyhaemoglobin is 6.1, and that in deoxyhaemoglobin is 6.6, at 37°C (Fig. 2). The decrease in the p*K* value from the deoxy to the carbonmonoxy form suggests that histidine-122α contributes to the alkaline Bohr effect, whereas the identity of the two p*K* values of histidine-2β indicates that this residue is not involved in the Bohr effect.

Measurement of the oxygen-equilibrium curve

To confirm that the haemoglobin was not denatured during the long incubation, samples in non-radioactive media at pH 5.0 and 8.0 were treated in the same way as in the tritiation experiment. After the incubation, carbonmonoxy- and deoxy-haemoglobin were isolated from the incubation buffer by passage through a Sephadex G-25 column (2.5 cm × 50 cm)

equilibrated with 1mM-Tris/HCl, pH8.0. Carbon monoxide was removed from carbonmonoxyhaemoglobin (Kilmartin & Rossi-Bernardi, 1971). The oxygen equilibrium curves of these samples in 0.1M-sodium phosphate buffer, pH6.5, pH7.0, and 0.1M-Tris/HCl buffer, pH8.0, were measured by using an automatic recording apparatus (Imai *et al.*, 1970), and the functional properties were found to be the same as those of native human haemoglobin.

Stereochemical mechanism of the pK change of histidine-122α on oxygenation

Perutz (1970) proposed first that histidine-122α might be involved in the alkaline Bohr effect, because the electron-density maps then available suggested that it lay nearer the guanidinium group of arginine-30β in the oxy form and nearer to the carboxy group of aspartate-126α in the deoxy form. Recent high-resolution maps have shown that histidine-122α is one link in a long chain of hydrogen bonds, which extends through much of the tetramer in both the quaternary oxy (R) and deoxy (T) structures; glutamate-26β_1-arginine-30β_1-histidine-122α_1-H_2O-tyrosine-35β_1-aspartate-126α_1 (Ladner *et al.*, 1977; Fermi, 1975). [The subscripted numbers 1 and 2 are used to distinguish between two like subunits in the haemoglobin tetramer (Perutz, 1965).] In the T structure this chain is further extended to arginine-141α_2-anion-valine-1α_1 (Arnone *et al.*, 1976), but it is not clear why that extension of the hydrogen-bonding chain should influence the p*K* of histidine-122α. If there are some movements that change the environment and therefore the p*K* of histidine-122α on oxygenation, such movements are too small to be detectable at the present resolution of the electron-density maps.

Quantitative analysis of the alkaline Bohr effect

I next tried to estimate the contribution of histidine-122α to the alkaline Bohr effect. The measured Bohr effect at 25°C (Kilmartin, 1973) can be accounted for theoretically by contributions of two alkaline Bohr groups per αβ dimer with p*K* values of 6.7 in oxy- and 7.8 in deoxy-haemoglobin, and two acid Bohr groups with p*K* value of 5.5 in oxy- and 4.9 in deoxy-haemoglobin (Kilmartin *et al.*, 1973*a*). In Fig. 3 the broken line represents the change in the charge on oxygenation of the theoretical alkaline Bohr groups. The p*K* values of histidine-146β were measured directly by using proton magnetic resonance of the proton at the C-2 position in the imidazole ring and found to be 7.2 in carbonmonoxy- and 8.1 in deoxy-haemoglobin at 25°C (Greenfield & Williams, 1972; Kilmartin *et al.*, 1973*a*). The curve representing the changes in charge due to the p*K* changes of histidine-146β is shown in Fig. 3. Several authors have reported the p*K* values for

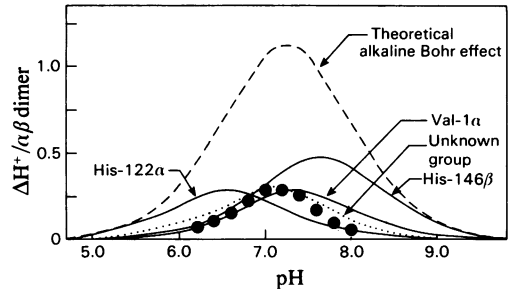

Fig. 3. *Relative contributions to the alkaline Bohr effect by the alkaline Bohr groups*

The dashed curve represents the change on oxygenation due to two alkaline Bohr groups per αβ dimer with p*K* values 6.7 and 7.8 at 25°C in oxy- and deoxy-haemoglobin (Kilmartin *et al.*, 1973*a*). The curves marked Val-1α, His-146β and His-122α show the change in charge of these groups on oxygenation, with the p*K* values given in the text. Values represented by solid filled circles were obtained by the subtraction of these three curves from the total alkaline Bohr effect (broken line). The dotted curve was fitted to the circles and represents the change in charge of the last unknown alkaline Bohr group, with p*K* values of 6.9 and 7.4 in oxy- and deoxy-haemoglobin at 25°C.

Table 3. *Balance sheet of the alkaline Bohr effect*

The total contribution of each group to the alkaline Bohr effect was estimated as the ratio of the area under the curve of each alkaline Bohr group to the area under the curve of the total alkaline Bohr effect in Fig. 3. All values were rounded off to the nearest 5%.

Residue	Total contribution to the alkaline Bohr effect (%)	Contribution at pH7.4 (%)
His-146β	40	40
Val-1α	20	25
His-122α	20	10
Unknown (possibly Lys-82β)	20	25

valine-1α. Kilmartin *et al.* (1973*a*) estimated the p*K* values as 7.1 and 7.6 at 25°C in oxy- and deoxy-haemoglobin respectively by subtraction of the Bohr curves for normal human haemoglobin from those of human haemoglobin treated with cyanate (Kilmartin *et al.*, 1973*b*). Garner *et al.* (1975) measured the pH-dependence of the reaction between valine-1α and cyanate and reported the p*K* values of 7.0 for carbonmonoxy- and 7.8 for deoxy-haemoglobin, at 26.6°C and 25.6°C respectively. Matthew *et al.* (1977) obtained the p*K* values of 7.2 for carbonmonoxy- and 7.8 for deoxy-haemoglobin at 30°C by the analysis of the nuclear-magnetic-resonance

intensity of valine-1α carbamoylated with $^{13}CO_2$ at various pH values. In Fig. 3, the contribution of valine-1α to the alkaline Bohr effect is illustrated by using the pK values reported by Kilmartin *et al.* (1973*a*). The values reported by the other authors give larger contributions, especially at the high-pH region. Histidine-146β and valine-1α contribute about 40 and 20% of the total alkaline Bohr effect and about 40 and 25% at pH 7.4 respectively (Table 3).

To compare my results for histidine-122α measured at 37°C with those for histidine-146β and valine-1α at 25°C, the pK values of histidine-122α were converted into those at 25°C by van't Hoff's formula. There is no justification in this instance to use different heats of ionization for the oxy and deoxy forms (Rossi-Bernardi & Roughton, 1967), since the environment of histidine-122α is closely similar in the two forms. A value of $\Delta H^0 = 28900$ J/mol characteristic for the imidazole moiety in histidine (Edsall & Wyman, 1958) was therefore assumed. The correction gives pK 6.3 and 6.8 for histidine-122α at 25°C in carbonmonoxy- and deoxy-haemoglobin. The change in charge on oxygenation due to this pK change is shown in Fig. 3. Histidine-122α thus contributes to the alkaline Bohr effect in a more acid pH region than histidine-146β or valine-1α. Its contribution to the alkaline Bohr effect is about 20% of the total and 10% at pH 7.4 (Table 3). Since this accounts for only part of the alkaline Bohr effect which is still unknown (40%), the combined changes in charge on oxygenation due to histidine-146β, valine-1α and histidine-122α are subtracted from the broken line representing the total alkaline Bohr effect to estimate the protons released from another unknown alkaline Bohr group at different pH values. In Fig. 3 the result is illustrated by the solid circles together with the dotted line which fits to the plots and represents the theoretical curve of the change in charge of an unknown alkaline Bohr group per $\alpha\beta$ dimer with pK 6.9 in oxyhaemoglobin and 7.4 in deoxyhaemoglobin. This group would contribute 20% of the total alkaline Bohr effect and 25% at pH 7.4 (Table 3). The Bohr effects of several abnormal haemoglobins in which basic groups near the 2,3-diphosphoglycerate-binding site in the internal cavity have been replaced by neutral residues were measured and their alkaline Bohr effects near pH 7.4 found to be diminished by up to 30% (M. F. Perutz, J. V. Kilmartin, K. Nishikura, J. H. Fogg & P. J. G. Butler, unpublished work). This depended on the concentration of Cl^-. They have proposed that lysine-82β, together with other basic groups near the 2,3-diphosphoglycerate-binding site, contributes to the unknown part of the alkaline Bohr effect by binding more diffusible anions in deoxy- than in oxy-haemoglobin. The last contribution to the Bohr effect shown as the dotted curve in Fig. 3 must therefore come from that source.

Alkaline Bohr effects of the abnormal haemoglobins substituted at position 122α

In embryonic human Hb Portland ($\zeta_2\gamma_2$) the alkaline Bohr effect is nearly halved (Tuchinda *et al.*, 1975). In the ζ-chain histidine-122α is replaced by aspartate and valine-1α may be acetylated (H, Lehmann, personal communication). Since the acetylation of valine-1α must decrease the alkaline Bohr effect by about 20%, the remaining about 30% may be due to the loss of histidine-122α. A decrease by 13% in the alkaline Bohr effect at pH 7.4 compared with those of human Hb A has been also found for Hb Llama (His-122α → Asp) (M. F. Perutz, J. V. Kilmartin, K. Nishikura, J. H. F. Fogg & P. J. G. Butler, unpublished work). However, there is one observation which conflicts with my findings. Histidine-122α is present in trout haemoglobin component I, which has been shown to exhibit no Bohr effect (Brunori, 1975). I can offer no explanation for this anomaly.

In summary, all of the alkaline Bohr groups have now been identified, and their relative contributions to the alkaline Bohr effect determined.

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References

Arnone, A. (1972) *Nature (London)* **237**, 146–149
Arnone, A., O'Donnell, S. & Schuster, T. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 1604
Bohr, C., Hasselbalch, K. A. & Krogh, A. (1904) *Skand. Arch. Physiol.* **16**, 402–412
Braunitzer, G., Gehring-Muller, R., Hilschmann, N., Hilse, K., Hobom, G., Rudloff, V. & Wittmann-Liebold, B. (1961) *Hoppe-Seyler's Z. Physiol. Chem.* **325**, 283–286
Brunori, M. (1975) *Top. Cell Regul.* **9**, 1–39
Edsall, J. T. & Wyman, J. (1958) *Biophysical Chemistry*, vol. 1, pp. 464, Academic Press, New York
Fermi, G. (1975) *J. Mol. Biol.* **97**, 237–256
Garner, M. H., Bogardt, R. A., II & Gurd, F. R. N. (1975) *J. Biol. Chem.* **250**, 4398–4404
Greenfield, N. J. & Williams, M. N. (1972) *Biochim. Biophys. Acta* **257**, 187–197
Imai, K., Morimoto, H., Kotani, M., Watari, H., Hirata, W. & Kuroda, M. (1970) *Biochim. Biophys. Acta* **200**, 189–196
Kilmartin, J. V. (1973) *Biochem. J.* **133**, 725–733
Kilmartin, J. V. & Rossi-Bernardi, L. (1971) *Biochem. J.* **124**, 31–45
Kilmartin, J. V. & Rossi-Bernardi, L. (1973) *Physiol. Rev.* **53**, 836–890
Kilmartin, J. V., Breen, J. J., Roberts, G. C. K. & Ho, C. (1973*a*) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1246–1249
Kilmartin, J. V., Fogg, J., Luzzana, M. & Rossi-Bernardi, L. (1973*b*) *J. Biol. Chem.* **248**, 7039–7043

Krieger, M., Koeppe, R. E., II & Stroud, R. M. (1976) *Biochemistry* **15**, 3458–3464
Ladner, R. C., Heidner, E. J. & Perutz, M. F. (1977) *J. Mol. Biol.* **114**, 385–414
Matthew, J. B., Morrow, J. S., Wittebort, R. J. & Gurd, F. R. N. (1977) *J. Biol. Chem.* **252**, 2234–2244
Nozaki, Y. & Tanford, C. (1967) *Methods Enzymol.* **11**, 715–734
Ohe, M. & Kajita, A. (1977) *J. Biochem.* (*Tokyo*) **82**, 839–845
Ohe, M., Matsuo, H., Sakiyama, F. & Narita, K. (1974) *J. Biochem.* (*Tokyo*) **75**, 1197–1200
Perutz, M. F. (1965) *J. Mol. Biol.* **13**, 646–668
Perutz, M. F. (1970) *Nature* (*London*) **228**, 726–739
Perutz, M. F., Muirhead, H., Cox, J. M. & Goaman, L. C. G. (1968) *Nature* (*London*) **219**, 131–139
Perutz, M. F., Muirhead, H., Mazzarella, L., Crowther, R. A., Greer, J. & Kilmartin, J. V. (1969) *Nature* (*London*) **222**, 1240–1243
Rossi-Bernardi, L. & Roughton, F. J. W. (1967) *J. Biol. Chem.* **242**, 784–792
Tuchinda, S., Nagai, K. & Lehmann, H. (1975) *FEBS Lett.* **49**, 390–391
Vaughan, J. D., Mughrabi, Z. & Chung Wu, E. (1970) *J. Org. Chem.* **35**, 1141–1145
Wyman, J. (1939) *J. Biol. Chem.* **127**, 581–599