The Oxygen-Linked Zinc-Binding Site of Human Haemoglobin

By JOHN G. GILMAN* and GEORGE J. BREWER†

Cattedra di Enzimologia, Università di Milano, Via G. Celoria 2, 20133 Milano, Italy

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Zn^{2+} is known to increase the O_2 affinity of human haemoglobin. Previous data suggested that Zn^{2+} exerts its effect by directly binding to haemoglobin, rather than by competing with or binding to 2,3-bisphosphoglycerate. It was also shown that there are two O_2 -linked zinc-binding sites in haemoglobin, and that Zn^{2+} does not significantly alter haemoglobin co-operativity or the alkaline Bohr effect. The effect of Zn^{2+} on O_2 affinity of haemoglobin can also be observed for other haemoglobins as diverse as those of cow and chicken. This paper presents new data on the haemoglobin–zinc interaction for normal haemoglobin, des-His^{146β}-haemoglobin and *N*-ethylsuccinimide–haemoglobin of humans. For normal haemoglobin (0.05 mM in tetramers), at 20°C in buffer containing 0.1 M-Cl[−], O_2 -dissociation-curve experiments showed that the addition of 0.4–0.5 mM- ZnSO_4 did not change the Bohr effect between pH 6.71 and 7.29. Similar experiments, with ‘zinc-ion buffers’, showed that the value of the Hill coefficient, *h*, decreased only slightly if the concentration of free Zn^{2+} was held constant. For *N*-ethylsuccinimide–haemoglobin, Zn^{2+} caused less increase in O_2 affinity than for normal haemoglobin. These studies, together with data on the equilibrium binding of Zn^{2+} to oxy-, deoxy- and des-His^{146β}-haemoglobins, suggest that zinc is chelated in oxyhaemoglobin by at least three amino acids, two of which are histidine-146β and cysteine-93β.

While studying zinc deficiency in sickle-cell anaemia, Oelschlegel *et al.* (1973) found that Zn^{2+} increases the O_2 affinity of human haemoglobin. In the presence of 2,3-bisphosphoglycerate at 37°C, this ‘left-shifting’ of the O_2 -dissociation curve by Zn^{2+} did not alter the alkaline Bohr effect. Zn^{2+} appeared to bind strongly to the haemoglobin and not to exert its effect by competing with 2,3-bisphosphoglycerate binding (Oelschlegel *et al.*, 1973, 1974).

Gilman *et al.* (1975) demonstrated the presence of two strong zinc-binding sites in both human and cow haemoglobin. They showed that Zn^{2+} increases the O_2 affinity of cow haemoglobin at 37°C (in the absence of 2,3-bisphosphoglycerate) to about the same extent as for human haemoglobin (in the presence of 2,3-bisphosphoglycerate). Zn^{2+} did not appear to cause any significant decrease in haemoglobin co-operativity. Their data also showed that Zn^{2+} increases the O_2 affinity of chicken haemoglobin, which suggested the relative lack of evolutionary variability at the O_2 -linked zinc-binding site.

Abbreviations used: Bistris, 2-[bis-(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol; zincon, *o*-(2-[α-(2-hydroxy-5-sulphophenylazo)benzylidene]hydrazino)benzoic acid, sodium salt.

* To whom reprint requests should be sent, at the Department of Chemistry, University of Indiana, Bloomington, IN 47401, U.S.A.

† Permanent address: Department of Human Genetics, University of Michigan, Ann Arbor, MI 48104, U.S.A.

We here provide data concerning the effect of Zn^{2+} on the O_2 -binding behaviour of human normal and *N*-ethylsuccinimide–haemoglobin, in the absence of 2,3-bisphosphoglycerate at 20°C. A study of the equilibrium binding of Zn^{2+} to normal human oxy- and deoxy-haemoglobins, and to human carbon-monoxide-des-His^{146β}-haemoglobin, is also presented. Our results are consistent with the hypothesis that Zn^{2+} is chelated in normal human oxyhaemoglobin by at least three amino acids, two of which are histidine-146β and cysteine-93β.

Experimental

Haemoglobin

Haemoglobin was prepared as described by Perrella *et al.* (1972), with the addition of an equilibration step in 1 mM-KOH followed by deionization on a mixed-bed ion-exchange resin column. The haemoglobin (1.5–2 mM-tetramers, in 0.1 M-KCl) was stored under N_2 in Pyrex bottles in the cold (under these conditions, the methaemoglobin content remained virtually zero for more than 2 months).

The des-His^{146β}-haemoglobin (Kilmartin *et al.*, 1975) was generously provided by Dr. J. V. Kilmartin, Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, U.K.

N-Ethylsuccinimide–haemoglobin was prepared by reaction of *N*-ethylmaleimide with cysteine-93β,

by a method similar to that of Riggs (1961): *N*-ethylmaleimide was added to the haemoglobin (in Bistris buffer, pH 7.02) to a concentration of 1.0 mM, and the reaction was allowed to proceed for 1 h at room temperature (23°C).

Throughout this paper, haemoglobin concentrations are given for tetramers (and generally are either 0.05 mM or 0.1 mM).

Buffers

The pH 7.02 Bistris buffer (0.05 M-Bistris/0.1 M-Cl⁻) for measuring O₂-dissociation curves was made with a 1:5 dilution of stock concentrated buffer (0.25 M-Bistris/0.081 M-HCl containing 0.019 M-NaCl); after addition of concentrated haemoglobin (in 0.1 M-KCl), final volume was reached by adding 0.1 M-NaCl solution. For the pH 7.29 buffer (0.05 M-Bistris/0.1 M-Cl⁻), dilutions were made as for the pH 7.02 buffer, but with the addition of 60 µl of 1 M-NaOH per 10 ml of buffer. The pH 6.71 buffer (0.05 M-Bistris/0.1 M-Cl⁻) was made by mixing equal quantities of the pH 7.02 buffer with a pH 6.38 buffer (0.05 M-Bistris/0.034 M-HCl/0.068 M-NaCl). All pH values were checked at 20°C, and were always within 0.015 pH unit of the expected value.

All solutions containing Zn²⁺ were made from stock 0.1 M-ZnSO₄ (Merck Titrisol).

For O₂-dissociation curves made with buffers containing 5 mM-*NN*-bis(carboxymethyl)glycine, the buffers were made as for pH 7.02 above, but with the addition of a 1:10 dilution of *NN*-bis(carboxymethyl)glycine stock solution. Two *NN*-bis(carboxymethyl)glycine stock solutions at pH 7 were used, one with zinc (0.05 M-*NN*-bis(carboxymethyl)glycine/0.045 M-ZnSO₄/0.1 M-NaCl/0.15 M-NaOH) and the other without zinc (0.05 M-*NN*-bis(carboxymethyl)glycine/0.1 M-NaCl/0.1 M-NaOH). The final pH of the *NN*-bis(carboxymethyl)glycine-containing solutions was 7.06.

O₂-dissociation curves

The method of Riggs (1951) was used on 0.05 mM-haemoglobin, in buffers containing 0.1 M-Cl⁻. Details are as described by Kilmartin & Rossi-Bernardi (1971). Equilibration was for 20 min at 20°C, and the *A*₅₅₈ was read with a Cary 118 spectrophotometer.

For experiments carried out in the presence of Zn²⁺, small quantities (up to 100 µl) of 20 mM-ZnSO₄ solution (containing 0.1 M-NaCl) were injected into the 4 ml of deoxyhaemoglobin in the glass tonometer.

For experiments in *NN*-bis(carboxymethyl)glycine-containing buffers, small quantities of 20 mM-ZnSO₄ were also injected. Since *NN*-bis(carboxymethyl)glycine is an effective zinc chelator, the free concentration, *A*, of Zn²⁺ is given approximately by $A = [Zn_T]K_x / ([NN\text{-bis(carboxymethyl)glycine}] -$

$[Zn_T]$], where $[Zn_T]$ is the total concentration of Zn²⁺ present, and K_x is the apparent dissociation constant for the binding of Zn²⁺ to *NN*-bis(carboxymethyl)glycine, as discussed by Cohen & Wilson (1966). These authors quote the p*K* for the dissociation of the third hydrogen of *NN*-bis(carboxymethyl)glycine as 9.73, and the p*K* for the dissociation of the chelate of Zn²⁺ with unprotonated *NN*-bis(carboxymethyl)glycine as 10.67 (both values measured at 20°C in 0.1 M-KNO₃); they also present a formula that allows calculation of K_x as $10^{-pH} \cdot (10^{-10.67} / 10^{-9.73})$. Then, K_x may be calculated at 10⁻⁸ M at the pH of our experiments (pH 7.06). However, this value may not be precise for the conditions of our experiments, because they were conducted in the presence of 0.095 M-Na⁺ and 0.003 M-K⁺ [for buffers made with the zinc-containing *NN*-bis(carboxymethyl)glycine stock solutions], rather than in 0.1 M-K⁺.

Zinc-binding studies

Two different methods were used for measuring the equilibrium binding of Zn²⁺ to haemoglobin, namely equilibrium dialysis and a ΔpH method that is not in principle very different from the ΔH⁺ method of Benesch *et al.* (1976).

For equilibrium dialysis experiments, ZnSO₄ was added to the external buffer, in which was immersed a dialysis sac containing 4 ml of haemoglobin (in the same buffer); equilibration proceeded for 48 h at 20°C. For 0.05 mM-carbonmonoxy normal human haemoglobin, 30 ml of CO-saturated external buffer (0.01 M-Bistris/0.1 M-NaCl) was either at pH 6.6 or pH 7.0 (pH adjusted with HCl). For 0.05 mM-carbonmonoxy-des-His^{146β}-haemoglobin, 30 ml of CO-saturated external buffer (0.01 M-Bistris/0.25 M-NaCl) was at pH 7.0. For experiments on 0.1 mM-deoxy normal haemoglobin, the haemoglobin was first reduced under N₂ in an IL237 tonometer (Instrumentation Laboratory, Lexington, MA, U.S.A.). It was quickly placed in a dialysis sac, which was then immersed in 250 ml of N₂-bubbled external buffer (0.01 M-Bistris/0.25 M-NaCl, pH 7.3). After waiting several hours to ensure thorough deoxygenation, if the haemoglobin was judged deoxy (on the basis of its colour), ZnSO₄ was then added to the external buffer.

After dialysis, zinc was analysed by the zincon method (McCall *et al.*, 1958), with absorption measured at 620 nm (haemoglobin was precipitated with trichloroacetic acid). Haemoglobin concentrations were determined spectrophotometrically, at 540 nm, on cyanmethaemoglobin, by the method of Drabkin (1950), by using the absorption coefficient reported by van Kampen & Zijlstra (1965). Methaemoglobin was measured by the method of Kilmartin & Rossi-Bernardi (1971), and was always less than 10% of total haemoglobin, at the end of the experiment.

For the ΔpH method of measuring Zn^{2+} binding to haemoglobin, small amounts of ZnSO_4 were added to unbuffered haemoglobin solutions (containing 0.25M-NaCl). The binding of Zn^{2+} to haemoglobin displaces protons, and also causes a charge change for the haemoglobin molecule, so that the pH of the solution changes. If the pH change associated with stoichiometric binding is known, the concentration of zinc that has bound can be calculated. This is simply the ratio of the pH change observed to that expected for stoichiometric binding, multiplied by the concentration of Zn^{2+} added. The Zn^{2+} that is not bound is free, so that, by making successive additions of ZnSO_4 and recording the pH change, one can construct binding curves.

To determine the change in pH with Zn^{2+} stoichiometrically bound ($\Delta\text{pH}/\Delta\nu$), it is necessary to add small amounts of ZnSO_4 to haemoglobin solutions that are as concentrated as possible. The reason is seen by considering the equation $\nu = 2KA/(1+KA)$, for the binding of zinc to two identical non-interacting sites on the haemoglobin tetramer (see Edsall & Wyman, 1958); ν is the ratio of mol of zinc bound per mol of haemoglobin, A is the concentration of unbound zinc, and K is the association constant for the binding. For oxy and deoxy normal haemoglobins at a concentration of 0.75mM, 0.5 zinc atom per haemoglobin tetramer was added, and the pH change was recorded. Under these conditions, if one assumes that $K = 1.5 \times 10^5 \text{M}^{-1}$ (the lowest value of K determined in this paper), then more than 99% of the added zinc would have been bound. Thus the ΔpH observed should have been that for stoichiometric binding. For des-His^{146 β} -haemoglobin, however, 0.1mM-haemoglobin was used, and 0.35 zinc atom per haemoglobin tetramer was added; in this case, one expects 4% of the added zinc to be free, so that the determination of $\Delta\text{pH}/\Delta\nu$ was not as precise, and K may have been somewhat overestimated.

For the experiment on 0.75mM-deoxy and oxy normal haemoglobins, they were first equilibrated with N_2 or O_2 respectively in the IL237 tonometer. 0.1mM-Carbonmonoxy-des-His^{146 β} -haemoglobin was equilibrated with O_2 . Then 2.2ml of this haemoglobin was placed in a glass cell, maintained at 20°C, into which the appropriate gas (N_2 or O_2) was flowing. A pH electrode (GK2321C; Radiometer, Copenhagen, Denmark) was immersed in the gently stirred solution, and the pH was monitored with a Vibron model 33B-2 electrometer. Initially, the pH slowly increased, but it stabilized after about 30min.

Determinations of $\Delta\text{pH}/\Delta\nu$ were as described above for 0.75mM-oxy and deoxy normal haemoglobin and 0.1mM-carbonmonoxy-des-His^{146 β} -haemoglobin. For oxyhaemoglobin, $\Delta\text{pH}/\Delta\nu$ was approximately constant between pH 7.5 and pH 7.25,

at -0.125 , and decreased as pH decreased, to -0.133 at pH 6.96 and -0.157 at pH 6.33. For deoxyhaemoglobin, $\Delta\text{pH}/\Delta\nu$ was practically constant at -0.152 between pH 7.42 and pH 7.07, and fell to -0.162 at pH 6.82. For carbonmonoxy-des-His^{146 β} -haemoglobin, $\Delta\text{pH}/\Delta\nu$ was -0.114 at pH 7.40, -0.116 at pH 7.17 and -0.124 at pH 6.99.

After values of $\Delta\text{pH}/\Delta\nu$ had been determined, the association constant K could be measured by using relatively unconcentrated haemoglobin (0.1mM or less). For this, 2.2ml of 0.05mM-oxyhaemoglobin or 0.1mM-deoxy normal or carbonmonoxy-des-His^{146 β} -haemoglobin was handled as for the determination of $\Delta\text{pH}/\Delta\nu$. Haemoglobins were then titrated with 1 or 2 μl increments of 20mM- ZnSO_4 , up to a ratio of zinc atoms added per haemoglobin tetramer of about 2.5. By comparing the pH change observed for each increment with that expected from the values of $\Delta\text{pH}/\Delta\nu$, the Scatchard (1949) plot could be constructed.

Low values of A are impossible to measure accurately with the ΔpH method. This is because A is proportional to the difference between two ΔpH values, and the relative error is largest when the two values differ by very little. This limits the values of ν for which data can be obtained. Thus, for the deoxyhaemoglobin data plotted in Fig. 3(b) below, haemoglobin was initially at pH 7.42, and was titrated to pH 7.10 with ZnSO_4 . However, the eight points shown in Fig. 3(b) are only for the pH range 7.25 to 7.12, for values of ν from 1.10 to 1.96.

Results and Discussion

Effects of zinc on the O_2 -dissociation curve of haemoglobin

Fig. 1 shows the effect of zinc on the O_2 affinity ($\log P_{50}$) and co-operativity (h) of normal human haemoglobin. Data were obtained for 0.05mM-haemoglobin in the presence of various concentrations of ZnSO_4 at three pH values, 7.29, 7.02 and 6.71 (buffers were 0.05M-Bistris/0.1M-Cl⁻).

The data confirm the claim of Oelshlegel *et al.* (1974) that zinc does not alter the alkaline Bohr effect of haemoglobin. The difference between $\log P_{50}$ for pH 7.29 and 6.71 is 0.324 in the absence of Zn^{2+} , and 0.316 for the maximum concentrations of Zn^{2+} used.

The data of Fig. 1 may be used to estimate the ratio of the association constants of Zn^{2+} for oxy- and deoxy-haemoglobin (K_o and K_d respectively). For a given free- Zn^{2+} concentration A , the decrease in $\log P_{50}$ (relative to zero zinc) is given by $\Delta\log P_{50} = -0.5\log(1+K_oA)/(1+K_dA)$ (see Baldwin, 1975). If A is very large, $\Delta\log P_{50} = -0.5\log(K_o/K_d)$. For the maximum concentrations of Zn^{2+} that were used, $\Delta\log P_{50}$ (averaged for the three pH values tested) was -0.678 , giving the estimate of K_o/K_d as 22.7.

Fig. 1. O_2 -dissociation-curve data for haemoglobin in the presence of Zn^{2+}

Values of the Hill coefficient h (a) and $\log P_{50}$ (b) are plotted against the total zinc concentration, which is given as the molar ratio of total Zn^{2+} (represented by $[Zn_T]$) to haemoglobin tetramers (represented by $[haemoglobin]$); P_{50} is the partial pressure of oxygen (in torr) at which the haemoglobin is 50% saturated with O_2 . Human haemoglobin concentration was 0.05 mM in 0.05 M-Bistris buffers containing 0.1 M- Cl^- . Δ , pH 6.71; $\blacksquare$, pH 7.02; $\bullet$, pH 7.29. For the inset, data on more concentrated haemoglobin solutions are taken from Gilman *et al.* (1975). Those experiments were performed at 37°C and pH 7.4 in bicarbonate buffer in the presence of 5.5% CO_2 : ∇ , cow haemoglobin (0.76 mM, no 2,3-bisphosphoglycerate); $\blacklozenge$, human haemoglobin (0.87 mM, approximately equimolar amount of 2,3-bisphosphoglycerate present). Means $\pm$ S.D. are given when more than two experiments were performed for any experimental condition, and solid lines connect the means of the data for the various zinc concentrations at a given pH. For zero zinc concentration, the means are for six, six and five experiments for pH 6.71, 7.02 and 7.29 respectively; at maximum zinc concentrations used, the means are for five, six and four experiments respectively. For clarity, standard deviations are omitted for values of h , but are less than 0.14 for zero and maximum zinc concentrations. The dotted line is a theoretical curve, computed assuming two identical zinc-binding sites, with the association constant K_o for oxyhaemoglobin of $1.5 \times 10^6 M^{-1}$, and the ratio of K_o to K_d (for deoxyhaemoglobin) of 22.7 (see the text). Then, for a given free Zn^{2+} concentration A , $\Delta \log P_{50} = -0.5 \log(1 + K_o A)/(1 + K_d A)$ (see the text). It was necessary to estimate A from the known values of total added Zn^{2+} ($[Zn_T]$). This was done by assuming that the haemoglobin was always in the oxy state, so that the equation $v = 2K_o A/(1 + K_o A)$ could be used. This procedure gave a minimum estimate for A , since oxyhaemoglobin binds Zn^{2+} better than does deoxyhaemoglobin. The theoretical curve thus derived corresponds fairly well to the experimental data, except for low values of $[Zn_T]$, where the error, owing to estimating A for oxyhaemoglobin only, would be the greatest.

The inset to Fig. 1 presents data on more-concentrated solutions of cow (0.76 mM) and human (0.87 mM) haemoglobin, the latter in the presence of an approximately equimolar amount of 2,3-bisphosphoglycerate (these data are taken from Gilman *et al.*, 1975). Zinc decreases $\log P_{50}$ in a similar manner for human haemoglobin (with 2,3-bisphosphoglycerate) and cow haemoglobin (without 2,3-bisphosphoglycerate) at 37°C and pH 7.4 in the presence of CO₂. The values of $\log P_{50}$ show an approximately linear decline as Zn²⁺ concentration increases from zero to $[Zn_T]/[haemoglobin] = 2$, followed by a levelling-off; this suggests the presence of two O₂-linked zinc-binding sites per haemoglobin tetramer. (The data on 0.05 mM-haemoglobin show a less-steep decline in $\log P_{50}$ as ZnSO₄ is added, because $[Zn_T]/[haemoglobin]$ represents a much lower Zn²⁺ concentration in those experiments than in the experiments with more concentrated haemoglobins.)

The upper part of Fig. 1 shows that the Hill coefficient, h , the measure of co-operativity, initially declines as ZnSO₄ is added to the haemoglobin, but then recovers to close to the original value as ZnSO₄ concentration increases. A similar effect was noted by Tomita & Riggs (1971) for the interaction of haemoglobin with 2,3-bisphosphoglycerate. They suggested that the low value of h observed could be due to the fact that the concentration of allosteric effector varied during the course of an experiment. For example, oxyhaemoglobin binds zinc more than 20 times as effectively as does deoxyhaemoglobin, as shown above; therefore the free Zn²⁺ concentration is highest for deoxyhaemoglobin, and gradually decreases as the haemoglobin becomes oxygenated.

To test this explanation, O₂-dissociation curves were obtained for haemoglobin in 'zinc ion buffers'. Fig. 2 shows a plot of $\log P_{50}$ and h against A/K_x . A (the free Zn²⁺ concentration) was held constant as the result of the interaction between Zn²⁺ and the chelating agent *NN*-bis(carboxymethyl)glycine (see the legend to Fig. 2 and the Experimental section for the definition of K_x and for a description of the principles involved). The upper part of Fig. 2, which gives the plot of h against A/K_x , shows that h was almost constant as zinc concentration increased. This demonstrates that most of the decline in apparent h , for the experiments in Fig. 1, was due to the variation in free Zn²⁺ concentration that occurred during the course of an experiment.

Zinc-binding studies

Equilibrium binding of Zn²⁺ to haemoglobin was investigated for normal and des-His^{146β}-haemoglobin after the effect of Zn²⁺ on O₂ affinity was known, but before we studied the interaction of Zn²⁺ with *N*-ethylsuccinimide-haemoglobin (see the next sec-

Fig. 2. O₂-dissociation-curve data for haemoglobin in zinc-ion buffers at pH 7.06

Values of h (a) and $\log P_{50}$ (b) are plotted against A/K_x , where A is the free Zn²⁺ concentration. In these experiments, A was kept constant by the presence of 5 mM-*NN*-bis(carboxymethyl)glycine, a chelating agent, and K_x represents the apparent dissociation constant of Zn²⁺ for *NN*-bis(carboxymethyl)glycine. K_x should be of the order of 10⁻⁸ M at pH 7.06, and values of A/K_x were computed by using the approximation $A/K_x = [Zn_T]/[NN\text{-bis(carboxymethyl)glycine}] - [Zn_T]$, as described in the Experimental section. The averages $\pm$ s.d. are given for $A/K_x = 0$ and $A/K_x = 9$, for five and four experiments respectively (other points represent individual experiments). The dotted line is a theoretical curve, constructed in a manner similar to that for the theoretical curve of Fig. 1 (see the legend to Fig. 1), except that A/K_x is given as described above, and the assumption is made that $K_o K_x = 6.74 \times 10^{-2}$.

tion). The data in the present paper are thus being given in roughly chronological order, to illustrate the development of the hypothesis concerning the location of the O₂-linked zinc-binding site.

Fig. 3 shows Scatchard plots for Zn²⁺ binding to oxy- and carbonmonoxy-haemoglobin, deoxyhaemoglobin and carboxy-des-His^{146β}-haemoglobin. Filled symbols represent data obtained by equilibrium dialysis, and open symbols are for data obtained by the Δ pH procedure (described in the Experimental section). The data were analysed as described in the legend to Fig. 3. Equilibrium-dialysis data for carbonmonoxy-haemoglobin gave values for the association constant of $1.1 \times 10^6 \text{ M}^{-1}$ at pH 7.0 and $5 \times 10^5 \text{ M}^{-1}$ at pH 6.6, whereas Δ pH data for oxyhaemoglobin gave the value of $1.9 \times 10^6 \text{ M}^{-1}$ for the pH range

Fig. 3. Scatchard plots of equilibrium zinc binding to haemoglobin

Zinc-binding data were obtained by both equilibrium dialysis (filled symbols) and a Δ pH method (open symbols) (see the Experimental section for a detailed description of the Δ pH method). v is the ratio of mol of zinc bound to mol of haemoglobin, and A is the free Zn^{2+} concentration. (a) Oxy and carbonmonoxy normal haemoglobins; $\blacksquare$, pH 7.0, 0.1 M-KCl, carbonmonoxyhaemoglobin (equilibrium dialysis); $\blacktriangledown$, pH 6.6, 0.1 M-KCl, carbonmonoxyhaemoglobin (equilibrium dialysis); $\square$, pH 7.11–6.82, 0.25 M-NaCl, oxyhaemoglobin (Δ pH). (b) Deoxy normal, and oxy- and carbonmonoxy-des-His^{146β}-haemoglobins. $\bullet$, pH 7.0, 0.25 M-NaCl, carbonmonoxy-des-His^{146β}-haemoglobin (equilibrium dialysis); $\circ$, pH 7.17–6.97, 0.25 M-NaCl, oxy-des-His^{146β}-haemoglobin (Δ pH); $\blacktriangle$, pH 7.3, 0.25 M-NaCl, deoxy-haemoglobin (equilibrium dialysis); $\triangle$, pH 7.42–7.10, 0.25 M-NaCl, deoxyhaemoglobin (Δ pH). The straight lines in these Figures have been drawn through equilibrium-dialysis data points, estimating by eye (for Fig. 3b, the line was drawn for des-His^{146β}-haemoglobin only, because the data for deoxyhaemoglobin were too scattered to allow the drawing of any meaningful line). Values of the association constant K were then computed for the strongest binding sites, by using those straight lines, according to the method of Scatchard *et al.* (1957) for estimating K values for multiple classes of binding sites; the assumption

covered, 7.11–6.83 (Fig. 3a). For deoxyhaemoglobin, the Δ pH data gave a value for the association constant of $1.8 \times 10^5 \text{ M}^{-1}$ for the pH range 7.42–7.10 (Fig. 3b). For carbonmonoxy-des-His^{146β}-haemoglobin, equilibrium dialysis gave an association constant of $1.5 \times 10^5 \text{ M}^{-1}$ (pH 7.0), whereas the Δ pH data gave a value of $2 \times 10^5 \text{ M}^{-1}$ for the pH range 7.17–6.97 (Fig. 3b). For all haemoglobins, the data indicate two identical principal binding sites for zinc per tetramer.

These binding data thus demonstrate that deoxyhaemoglobin has a considerably smaller association constant for Zn^{2+} than does oxyhaemoglobin, in qualitative agreement with deductions from O_2 -dissociation-curve data (see the legend to Fig. 1). Carbonmonoxy-des-His^{146β}-haemoglobin binds Zn^{2+} approximately as weakly as does deoxyhaemoglobin; this suggests that histidine-146 β is crucial to the strong binding of Zn^{2+} observed for oxyhaemoglobin.

In deoxyhaemoglobin, histidine-146 β , an alkaline Bohr group, is involved in a salt bridge to aspartic acid-94 β of the same chain (Perutz, 1970). This fact could account for the weaker binding of Zn^{2+} to deoxy- than to oxy-haemoglobin: histidine-146 β would be less free to participate in the chelation of Zn^{2+} in deoxy- than in oxy-haemoglobin, since in deoxyhaemoglobin that residue is constrained, whereas in oxyhaemoglobin it is relatively free to move.

One may ask, at this point, why Zn^{2+} does not alter the alkaline Bohr effect of haemoglobin, if zinc is binding to an alkaline Bohr group. The hypothesis just presented suggests the reason, namely that Zn^{2+} binding to deoxyhaemoglobin may interfere only minimally with the salt bridge between histidine-146 β and aspartic acid-94 β . In deoxyhaemoglobin, Zn^{2+} binding may occur as chelation to two other amino acids, but not significantly to histidine-146 β . [Two other amino acids are suggested by the order of magnitude of the zinc-binding constant for deoxyhaemoglobin (10^5 M^{-1}); a single histidine side chain would be expected to bind Zn^{2+} with an association constant of only $10^{2.5} \text{ M}^{-1}$ at pH 7, from data of Gurd & Goodman (1952).]

Thus Zn^{2+} may be reasonably strongly chelated to deoxyhaemoglobin despite the minimal participation of histidine-146 β in the chelate. The very switch to oxyhaemoglobin that liberates the alkaline

was made that there are two identical strong binding sites, which appears reasonable, judged from the data. For the Δ pH data, a computer program was devised to fit the data points for each experiment to a straight line, by the method of least squares; the program then calculated values of K by the above procedure. Values of K thus calculated, for the data of this Figure, are given in the text.

Bohr protons would permit histidine-146 β to swing into position and provide the third side chain of the chelate, thereby raising the association constant for Zn²⁺ by some 20-fold.

Effects of zinc on the O₂-dissociation curve of N-ethylsuccinimide-haemoglobin

If Zn²⁺ does bind to histidine-146 β in oxyhaemoglobin, examination of the three-dimensional oxyhaemoglobin model (Perutz *et al.*, 1968) suggests three possible locations for the additional amino acids of the chelate: in the vicinity of histidine-143 β or cysteine-93 β of the same β -chain, or histidine-2 β of the other β -chain.

The last possibility is effectively ruled out by the data of the insert to Fig. 1. Zinc increases the O₂ affinity of cow haemoglobin, in the absence of 2,3-bisphosphoglycerate, in a manner similar to that for human haemoglobin in the presence of 2,3-bisphosphoglycerate, even though cow haemoglobin lacks histidine-2 β . These same data also suggest that it is unlikely that histidine-143 β is involved in the binding of Zn²⁺, since that residue is at the 2,3-bisphosphoglycerate-binding site (see Perutz, 1970). If histidine-143 β were involved in the binding of both 2,3-diphosphoglycerate and Zn²⁺, then the presence of 2,3-bisphosphoglycerate might be expected to enhance the degree to which Zn²⁺ causes a left-shift of the O₂-dissociation curve. One concludes that the O₂-linked zinc-binding site may be in the vicinity of cysteine-93 β . The experiments on N-ethylsuccinimide-haemoglobin test this possibility.

Blockage of cysteine-93 β , by reaction with N-ethylmaleimide, is known to prevent the formation of the salt bridge between histidine-146 β and aspartic acid-94 β . For N-ethylsuccinimide-haemoglobin, therefore, the alkaline Bohr effect is decreased by 50%, O₂ affinity increases, and co-operativity diminishes, though not drastically (see Baldwin, 1975). If one accepts the premise that Zn²⁺ binding involves histidine-146 β in oxy- but not in deoxy-haemoglobin, one might expect N-ethylsuccinimide-deoxyhaemoglobin to bind Zn²⁺ about as well as does oxy normal haemoglobin. This is because much of the constraint on histidine-146 β in deoxyhaemoglobin would be removed in N-ethylsuccinimide-haemoglobin, and histidine-146 β should therefore be freer to swing into position and participate in Zn²⁺ chelation. So, for N-ethylsuccinimide-haemoglobin, Zn²⁺ would be expected to bind very strongly ($K > 10^6 \text{ M}^{-1}$) to both the deoxy and oxy states, unless cysteine-93 β (or groups close by) was directly involved in the chelation of the Zn²⁺, in which case both the oxy and deoxy forms of N-ethylsuccinimide-haemoglobin would bind Zn²⁺ rather weakly.

Fig. 4. O₂-dissociation-curve data for N-ethylsuccinimide-haemoglobin at pH 7.02 in the presence of Zn²⁺

Values of h (a) and $\log P_{50}$ (b) are plotted against the total Zn²⁺ concentration, which is given as the molar ratio of total Zn²⁺ ([Zn_T]) to haemoglobin tetramers ([haemoglobin]). Means $\pm$ s.d. are given when more than two experiments were performed for any experimental condition, and otherwise the actual points for the data are given. For zero Zn²⁺, the mean is for eight experiments; for [Zn_T]/[haemoglobin] equal to 2.0, 4.0 and 6.0, the means are for three experiments each. The broken and dotted lines are theoretical curves constructed by the procedure used to compute the dotted line of Fig. 1 (see the legend to Fig. 1). For the broken line, the association constant for oxyhaemoglobin is assumed to be 10^6 M^{-1} and the ratio of association constants for oxy- to deoxy-haemoglobin is 2.71, whereas for the dotted line the association constant for the oxy state is assumed to be $3 \times 10^4 \text{ M}^{-1}$ and the ratio of the constant for the oxy state to that of the deoxy state is 3.0.

Fig. 4 shows the effect of Zn²⁺ on the O₂ affinity (given as $\log P_{50}$) and co-operativity (represented by h) of human N-ethylsuccinimide-haemoglobin at pH 7.02. Comparison with Fig. 1 shows that, in the absence of Zn²⁺, the N-ethylmaleimide treatment decreased $\log P_{50}$ from 0.70 to 0.22, and h from 2.86 to 2.33. Zinc affected the O₂ affinity of N-ethylsuccinimide-haemoglobin much less than that of normal haemoglobin. The addition of 0.5 mM-ZnSO₄ to the N-ethylsuccinimide-haemoglobin decreased $\log P_{50}$ by 0.217, whereas the addition of 0.4–0.5 mM-ZnSO₄ to normal haemoglobin decreased $\log P_{50}$

by 0.678 (the average for the three pH values of Fig. 1). This decrease in $\log P_{50}$ for normal haemoglobin was interpreted as showing that the ratio of the Zn^{2+} association constants for oxy- and deoxy-haemoglobin was 22.7. The same analysis applied to the *N*-ethylsuccinimide-haemoglobin data of Fig. 4 gave a value for this ratio of only 2.71.

The dotted and broken lines in Fig. 4 are theoretical curves that were computed by the procedure used for the dotted curve of Fig. 1 (see the legend to Fig. 1). For Fig. 1, the theoretical curve was computed by assuming that the association constant of Zn^{2+} for oxyhaemoglobin was $1.5 \times 10^6 \text{ M}^{-1}$ and reasonably good agreement with the data was obtained. For Fig. 4, however, the assumption of a high value of the association constant for *N*-ethylsuccinimide-oxyhaemoglobin (10^6 M^{-1}), was in poor agreement with the data (broken line). Only by assuming a low value for the association constant of *N*-ethylsuccinimide-oxyhaemoglobin was it possible to obtain reasonably good agreement with the experimental data, as shown by the dotted line (for which the value was taken as $3 \times 10^4 \text{ M}^{-1}$).

It is concluded that the data of Fig. 4 imply that groups in the vicinity of cysteine-93 β are involved at the O_2 -linked zinc-binding site. The fact that cysteine is one of the strongest zinc-binding amino acids (Albert, 1961) suggests that cysteine-93 β may itself be involved in the chelation of Zn^{2+} , along with histidine-146 β , and at least one other (unknown) residue located nearby.

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