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Bohr effect of human hemoglobin A: Magnitude of negative contributions determined by the equilibrium between two tertiary structures

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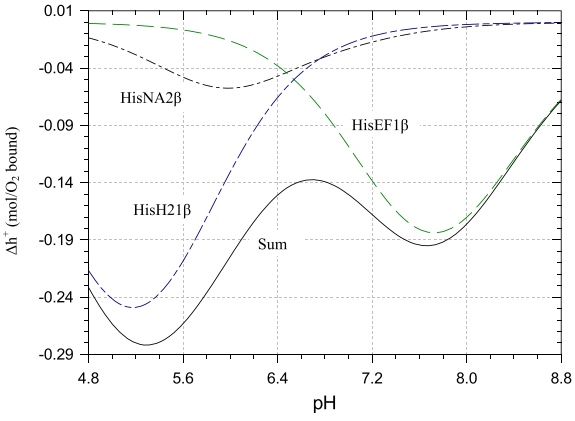
HIGHLIGHTS

- pK_as of groups linked to reaction of DTNB with Hb are lower in deoxyHb than in oxyHb.
- This suggests that these groups make negative contributions to the Bohr effect.
- Reduced Bohr effect of Cys93β modified hemoglobins is explained quantitatively.
- Increased oxygen affinity of Cys93β modified hemoglobins is explained quantitatively.
- Quantitative explanations based on equilibrium between two Hb tertiary conformations

GRAPHICAL ABSTRACT



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ABSTRACT

We have measured the affinity of the CysF9[93]β sulfhydryl group of human deoxyhemoglobin and oxyhemoglobin for 5,5′-dithiobis(2-nitrobenzoate), DTNB, between pH ≈5.6 and 9 in order to understand the basis of the reported reduction of the Bohr effect induced by chemical modification of the sulfhydryl. We analyzed the results quantitatively on the basis of published data indicating that the sulfhydryl exists in two conformations that are coupled to the transition between two tertiary structures of hemoglobin in dynamic equilibrium. Our analyses show that the ionizable groups linked to the DTNB reaction have *lower* pK_as of ionization in deoxyhemoglobin compared to oxyhemoglobin. So these ionizable groups should make negative contributions to the Bohr effect. We identify these groups as HisNA2[2]β, HisEF1[77]β and HisH21[143]β. We provide explanations for the finding that hemoglobin, chemically modified at CysF9[93]β, has a lower Bohr effect and a higher oxygen affinity than unmodified hemoglobin.

1. Introduction

The binding of oxygen to deoxyhemoglobin is accompanied by structural changes at the quaternary and tertiary levels. These structural alterations result in changes in the ionization states of some amino acid residues that are linked to the oxygen binding reaction, giving rise to the release or absorption of protons from the solution. These are referred to as the Bohr effect. Two kinds of Bohr effect arise from this: (i) In the acid Bohr effect, which normally occurs below pH 6, protons are absorbed from the solution as oxygen binds to deoxyhemoglobin. (ii) In the physiologically more important alkaline Bohr effect, which occurs above pH 6, protons are released into the solution on oxygen binding.

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To the best of our knowledge, the contributions of the quaternary and tertiary structure changes to the alkaline Bohr effect have not been delineated.

In efforts to gauge the possible contribution of the CysF9[93]β sulfhydryl group to the Bohr effect [1–3], human hemoglobin A was treated with various sulfhydryl reagents. The Bohr effect of each resulting compound was measured by differential titration as a function of pH as the difference, Δh^+, between the amount of protons bound by deoxyhemoglobin and by oxyhemoglobin upon binding of one O_2 molecule [1–3]. In each compound the acid Bohr effect was enhanced but the alkaline Bohr effect was diminished relative to untreated hemoglobin [1–3]. Although it was concluded that CysF9[93]β does not directly contribute to the Bohr effect, no plausible explanation was given for these observations.

It is now accepted that the tertiary structure transition plays a role in the oxygen binding process [4–10]. Consequently, the Bohr effect results [1–3] raise an interesting question: Do the pK_a decreases leading to positive contributions to the alkaline Bohr effect and the pK_a increases leading to negative contributions arise as a consequence of one structural transition (that is, quaternary only) or do they arise from different structural transitions, that is, quaternary and tertiary, respectively? In order to answer this question it is necessary to employ a reaction system in which the quaternary and tertiary structure transitions are isolated from each other [11,12].

We have demonstrated in kinetic and equilibrium studies of hemoglobins in the R quaternary state that the reaction of CysF9[93]β with 5,5′-dithiobis(2-nitrobenzoate), DTNB, is a reversible process that is coupled to the transition between two tertiary conformations **r** and **t** [13–18]. The DTNB reaction is also coupled to the ionizations of certain amino acid residues whose number and nature can be gauged by determining the equilibrium constant of the DTNB reaction as a function of pH, followed by curve fitting to the resulting pH dependence data [15–17]. To the best of our knowledge, a similar study has not been reported for hemoglobin in the T quaternary state.

The hemoglobin/DTNB system would be ideal for comparing the tertiary structure transition within T-state and R-state hemoglobin and for identifying the nature of the ionizable groups linked to the tertiary structure transition. We report here on the reaction of DTNB with human deoxyhemoglobin and oxyhemoglobin. We find that the groups whose ionizations are linked to the DTNB reaction have *lower* pK_as in deoxyhemoglobin compared to oxyhemoglobin. We identify these groups as HisNA2[2]β, HisEF1[77]β and HisH21[143]β.

2. Experimental

2.1. Determination of K_{equ} for the reaction of human oxyhemoglobin with DTNB

The equilibrium constant for the reaction of oxyhemoglobin with DTNB was determined with methods and under conditions that are identical with those previously described [14–17].

2.2. Preparation of human deoxyhemoglobin

The human hemoglobin A used in the deoxy experiments was first prepared as oxyhemoglobin from blood obtained from non-smoking, adult donors. After passing it through a carboxymethylcellulose (CM-52) column, the solution of oxyhemoglobin obtained was flash-frozen in liquid nitrogen. The flash-frozen oxyhemoglobin pellets were kept in a number of plastic Wheaton bottles and stored in a freezer at −80 °C. Prior to deoxygenation, a few pellets were allowed to thaw slowly on ice. Deoxygenation was carried out as described below.

Humidified nitrogen gas (that is, nitrogen gas that had been passed through deionized water) was used to deoxygenate the thawed oxyhemoglobin. Anhydrous sodium dithionite, $Na_2S_2O_{4(s)}$, was added to the humidifier to completely eliminate all traces of oxygen.

2.3. Determination of K_{equ} for the reaction of human deoxyhemoglobin with DTNB

A 50 μmol (heme) dm^{-3} solution of human oxyhemoglobin (25 μmol dm^{-3} in reactive sulfhydryl groups) was prepared in an appropriate buffer of known pH. A 1 cm^3 portion of this solution was transferred into each of three specially fabricated 0.2 cm × 1 cm cuvettes. Fitted into the mouth of each cuvette was a reservoir compartment, which can hold solutions when tilted and in which mixing of reactants can be carried out. The cuvette was covered with a resealable septum seal and Teflon tapes, which were held tightly in place with an open-top plastic screw cap. Humidified nitrogen gas was introduced into the solution inside the cuvette from a flow meter which has a long narrow pipe fitted with a long thin needle. The needle was inserted into the cuvette through the septum seal. A second, shorter, needle was also inserted to prevent the build-up of pressure inside the cuvette. The flow meter was adjusted to allow N_2 gas to flow through the oxyhemoglobin solution at a rate of about 0.1 dm^3 min-1 until it was completely deoxygenated. This usually lasted between 2 and 3 h. Complete deoxygenation was confirmed by comparing the spectrum of the solution (taken between 300 and 700 nm on a Varian Cary 400 Scan UV/Visible spectrophotometer) with that of a standard spectrum of human deoxyhemoglobin.

After complete deoxygenation was confirmed, a known volume of a deoxygenated 29 mmol dm^{-3} DTNB solution was added into the deoxyhemoglobin solution in each cuvette with a gastight micro-syringe. The mixtures were shaken gently and allowed to equilibrate for between 6 and 8 h under a continuous flow of humidified nitrogen. All the deoxygenation and equilibration processes were carried out at 25 °C in a room thermostated with a CARRÉ refrigerant plant. After equilibrating deoxyhemoglobin with DTNB, the spectrum of each solution was taken between 300 and 700 nm to ensure that no trace of oxyhemoglobin was present. All data were recorded on an external computer recording unit equipped with a Cary® WinUV software.

2.4. Calculation of K_{equ}

Owing to the limited number of the specially fabricated 0.2 × 1 cm cuvettes available, it was difficult to obtain sufficient data points at a single wavelength to determine the equilibrium constant (K_{equ}) for the reaction of DTNB with deoxyhemoglobin. To overcome this limitation we took the absorbance of 5-thio-2-nitrobenzoate (TNB), the chromophoric product of the DTNB reaction, at five wavelengths instead of one: 405, 412, 436, 450 and 470 nm. Eyer et al. [19] have determined the molar absorption coefficients of TNB at these wavelengths to be 13,800, 14,000, 11,000, 8000 and 4000 mol^{-1} dm^3 cm^{-1}, respectively, at 25 °C. The equilibrium constant was calculated for the content of each cuvette from the change in absorbance at each of these wavelengths, using Eq. (2) of the text. A computer program was written on a MicroMath Scientist software (Salt Lake City, Utah) to aid these calculations.

3. Results and discussion

3.1. Purity of deoxyhemoglobin samples

Considering the long equilibration times required for the reaction of DTNB with deoxyhemoglobin, we took considerable care to ensure that our deoxyhemoglobin samples were completely free of O_2. As a check for this we took the spectra of the deoxy samples prior to mixing with DTNB and also after the reaction with DTNB. Both spectra turned out to be the normal spectrum of deoxyhemoglobin, except that the spectrum taken after the DTNB reaction had a peak at 330 nm. The same peak was observed after the reaction of DTNB with oxyhemoglobin. Suspecting that this peak may have arisen

from DTNB, we determined the spectrum of DTNB alone from 200 to 700 nm with a Shimadzu UV 1800 double beam spectrophotometer (Obafemi Awolowo University, Ile-Ife). There was a peak at 330 nm whose intensity was reduced drastically in the presence of an excess of reduced glutathione, with the development of another peak around 412 nm, the absorption maximum of TNB^-, the product of the reduction of DTNB [20–22]. This proved that the 330 nm peak observed after the reaction of DTNB with either deoxyhemoglobin or oxyhemoglobin was due to DTNB. To the best of our knowledge, this is the first time such a peak has been reported for DTNB (cf Eyer et al. [19]; Ellman [20,21]).

3.2. Reversibility of the reaction of deoxyhemoglobin with DTNB

We have already established [13–18] that the reaction of DTNB with various hemoglobins in the R quaternary state is a reversible process. Before proceeding to determine the equilibrium constant of the reaction of DTNB with deoxyhemoglobin, which has the T quaternary structure, it was necessary to satisfy ourselves that this reaction is a reversible process. For this we studied the kinetics of the reaction of DTNB with deoxyhemoglobin at pH 8.0 under pseudo-first order conditions, with DTNB in excess. Fig. 1a reports a pseudo-first order rate plot of the data. The plot is linear for up to 1.5 half-lives. This indicates that the reaction is truly pseudo-first order. Similar plots were obtained at other concentrations of DTNB. In Fig. 1b we report a plot of k_{obs}, the pseudo-first order rate constant, against the DTNB concentration. It is seen that the plot is linear and has a positive intercept on the ordinate axis. This proves that the reaction of DTNB with deoxyhemoglobin is indeed a reversible process. Having demonstrated that the reaction is an equilibrium process, we proceeded to determine the equilibrium constant, K_{equ}, as a function of pH.

3.3. Equilibrium constant for the DTNB reaction

The complete reaction of CysF9[93]β with DTNB may be depicted as [10–15]:

$$\begin{aligned} PSH + DTNB &\overset{Q_{SH}}{\rightleftharpoons} H^+ + PS^- + DTNB \overset{K_{equ}}{\rightleftharpoons} H^+ + PS.ST \\ &+ TNB^- \overset{Q_{TNB}}{\rightleftharpoons} PS.ST + TNBH. \end{aligned} \quad (1)$$

The various species and parameters appearing in Eq. (1) are defined in the Appendix A at the end of this paper. Eq. (2), which relates the equilibrium constant, K_{equ}, with the parameters and species that appear in Eq. (1), was derived previously [14]. In Eq. (2) $[P]_{total}$ is the total concentration of hemoglobin species in terms of reacting sulfhydryl groups, and the values of pQ_{SH} and pQ_{TNB}, the pK_as of ionization of PSH and TNBH, are 8.30 and 5.27, respectively [14].

$$K_{equ} = \frac{[TNB^-]^2\left\{1+\frac{[H^+]}{Q_{TNB}}\right\}\left\{1+\frac{[H^+]}{Q_{SH}}\right\}}{\left\{[P]_{total}-[TNB^-]\left\{1+\frac{[H^+]}{Q_{TNB}}\right\}\right\}\left\{[DTNB]_{total}-[TNB^-]\left\{1+\frac{[H^+]}{Q_{TNB}}\right\}\right\}}. \quad (2)$$

In Fig. 2 we report the pH dependence profiles of $-\log_{10}K_{equ}$ for the reaction of DTNB with deoxyhemoglobin and with oxyhemoglobin. Between pHs 5.7 and 9 the affinity of deoxyhemoglobin for DTNB decreases by 1.6 orders of magnitude; that of oxyhemoglobin decreases by 2 orders of magnitude. These strong pH dependences indicate that the DTNB reaction is linked to the ionization of certain groups on the protein.

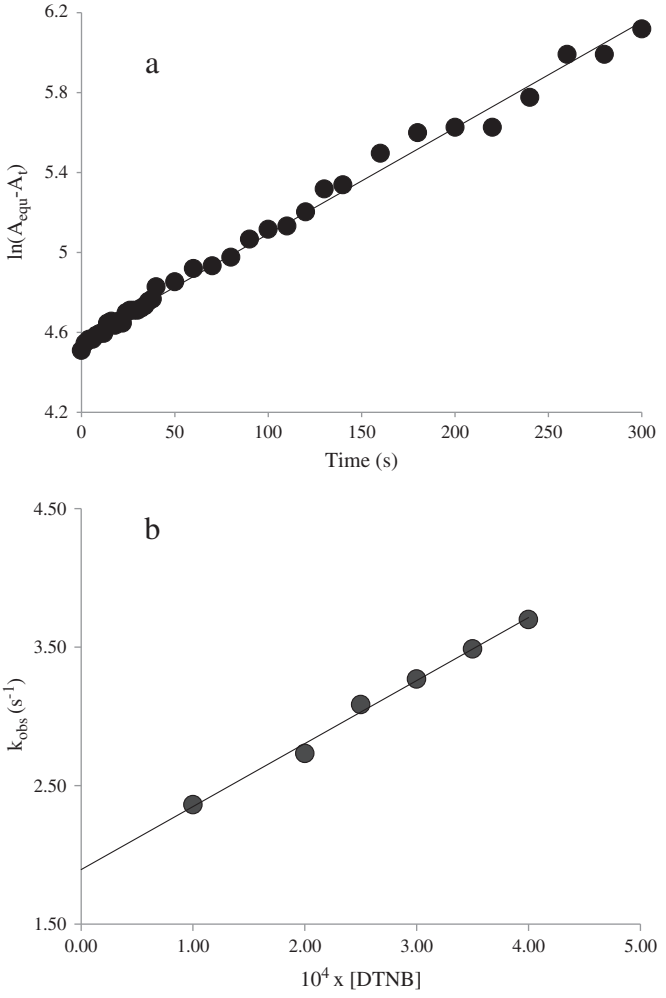


Fig. 1. Reaction of 5,5′-dithiobis(2-nitrobenzoate), DTNB, with the CysF9[93]β sulfhydryl group of stripped human deoxyhemoglobin A. (a) Semi-logarithmic plot of the time course of the reaction. The pseudo-first order rate constant, k_{obs}, calculated from the slope is $(3.4 \pm 0.07) \times 10^{-3}\ s^{-1}$. The plot is linear for 1.5 half-lives. R^2, the square of the correlation coefficient, is 0.993. Conditions: 25 °C; borate buffer pH 8.00; ionic strength 50 mmol dm^{-3}; [deoxyhemoglobin] = 5 μmol dm^{-3} in reacting sulfhydryl groups, that is, 10 μmol (heme) dm^{-3}; [DTNB] = 250 μmol dm^{-3}. (b) Variation of k_{obs}, the pseudo-first order rate constant, with the DTNB concentration. Each experimental point is the mean of three determinations and is subject to a standard error of 10–15% of the mean. Apart from the concentration of DTNB, the conditions are the same as in panel label (a).

3.4. Analyses of pH dependence profiles of $-log_{10}K_{equ}$

To determine the number and the nature of the linked ionizing groups we carried out quantitative analyses on the data in Fig. 2. The analyses were based on the strong pH dependences seen in Fig. 2 and on two previous experimental findings: (i) the CysF9[93]β sulfhydryl exists in two conformations [12]; and (ii) in deoxyhemoglobin, and also in carbonmonoxyhemoglobin, the two sulfhydryl conformations are coupled to the transition between two tertiary structures in dynamic equilibrium [11]. In the experiments reported here DTNB reacted with hemoglobin in each of two fixed quaternary states: deoxyhemoglobin (T-state) and oxyhemoglobin (R-state).

For the determination of the number and the nature of the DTNB-linked ionizing groups we employed Scheme 1 [15–20]. Scheme 1 is an extended form of Eq. (1) in which the ionizations of groups on the various hemoglobin species are taken into consideration. In Scheme 1, the protons arising from the various ionization steps have been omitted for the sake of clarity. The species and parameters that appear in

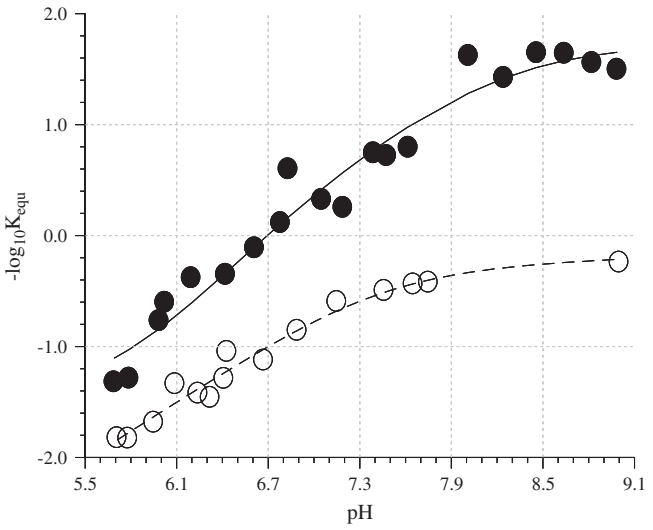


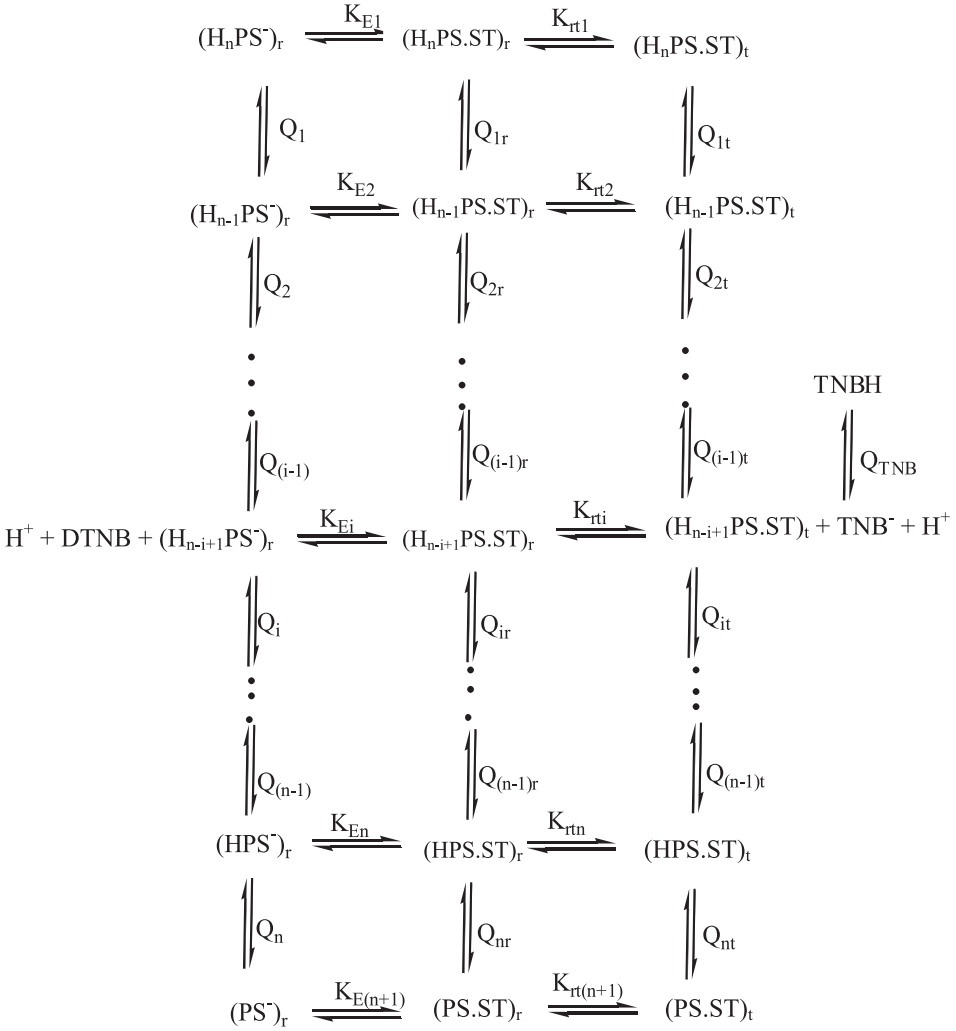
Fig. 2. Reaction of 5,5′-dithiobis(2-nitrobenzoate), DTNB, with the CysF9[93]β sulfhydryl group of human hemoglobin A: Variation of the equilibrium constant with pH. Filled symbols, oxyhemoglobin; open symbols, deoxyhemoglobin. Conditions: 25 °C; phosphate buffers, pH 5.6–7.8; borate buffers, pH 8.0–9.0; ionic strength, 50 mmol dm^{-3}; and hemoglobin concentration, 25 μmol dm^{-3} in reacting sulfhydryl groups, that is, 50 μmol (heme) dm^{-3}. Each data point is subject to a standard error of about ±0.1 in the log. The lines through the data points are the best-fit theoretical lines calculated with Eq. (3) of the text for n = 2 (cf. Scheme 1).

Scheme 1 are defined in the Appendix A at the end of this paper. The relationship between K_{equ} and these parameters is given [15] by Eq. (3).

$$K_{equ} = \frac{K_{E(n+1)}\left\{1+\sum_{i=1}^{n}\left(H^+\right)^{n-i+1}\left(\prod_{j=i}^{n}Q_{jr}\right)^{-1}\right\} + K_{rt(n+1)}\left\{1+\sum_{i=1}^{n}\left(H^+\right)^{n-i+1}\left(\prod_{j=i}^{n}Q_{jt}\right)^{-1}\right\}}{\left\{1+K_{E(n+1)}\left\{\sum_{i=1}^{n}\left(H^+\right)^{n-i+1}\left(\prod_{j=i}^{n}Q_{jr}\right)^{-1}K_{Ei}^{-1}\right\}\right\}} \quad (3)$$

We employed Eq. (3) to fit the data reported in Fig. 2, using different values of n, the number of DTNB-linked ionizable groups. The fits were performed with a computer program written on a MicroMath Scientist software (Salt Lake City, Utah), as previously reported [15]. Our initial fits with an n value of 2 were very good (see the lines through the experimental points in Fig. 2). The best-fit parameters are reported in Table 1. As can be clearly seen in Table 1 the pQs (that is, pK_as) of the ionizable groups are *lower* in deoxyhemoglobin compared to oxyhemoglobin.

The finding that the pK_as of the DTNB-linked ionizable groups are *lower* in deoxyhemoglobin compared to oxyhemoglobin suggested to us that these groups might be identical with the groups that make negative contributions to the Bohr effect. Three such groups were identified by Fang et al. [22] in their ^{1}H NMR studies of deoxyhemoglobin and carbonmonoxyhemoglobin. They are HisH21[143]β, HisNA2[2]β and HisEF1[77]β [22]. On the basis of this finding we re-analyzed the equilibrium data in Fig. 2 with Eq. (3), using an n value of 3. Although we still obtained very good fits to the data, the values of K_{rt} (the equilibrium



Scheme 1. Negative Bohr contributions of histidine residues.

Table 1
Reaction of 5,5′-dithiobis(2-nitrobenzoate), DTNB, with the CysF9[93]β sulfhydryl group of human deoxyhemoglobin A and oxyhemoglobin A: Best-fit parameters employed to fit the data points in Fig. 2 for n = 2 (compare with Scheme 1 and Eq. (3) of the text.).

	pQ_{1r}	pQ_{1t}	pQ_{2r}	pQ_{2t}	$-\log_{10}K_{E3}$	$\frac{K_{E3}}{K_{E2}}$	$\frac{K_{E3}}{K_{E1}}$	K_{rt3}
Deoxy	5.85	6.62	7.30	7.63	−0.136	0.147	2.6×10^{-2}	0.336
Oxy	6.16	7.02	8.00	8.73	1.73	1.86×10^{-2}	1.20×10^{-3}	0.126

constant for the tertiary structure transition) obtained from the n = 3 fits were very low compared to previously reported values [11]: about 400-fold lower for deoxyhemoglobin and 4-fold lower for oxyhemoglobin. By contrast, the values of K_{rt} for the n = 2 fits were in agreement with previously reported values [11]. For this reason we decided to reject the n = 3 fits. Another reason for rejecting the n = 3 fits was that the pQs of the ionizable groups obtained from the fits could not account for the reduction of the Bohr effect of hemoglobin modified at CysF9[93]β (see Section 3.5 below).

3.5. Bohr effect and ionization constants of groups in deoxyhemoglobin and oxyhemoglobin linked to the DTNB reaction

One experimental result that has defied explanation for almost five decades is the finding that the Bohr effect of human hemoglobin A, chemically modified at CysF9[93]β, is diminished compared to the native protein. The results presented in Table 1 are interesting because the values of pQ_{ir}/pQ_{it} (i = 1, 2) are *lower* in deoxyhemoglobin compared to oxyhemoglobin by between 0.3 and 1 pK_a unit. This suggests that the linked groups should make negative contributions to the Bohr effect when deoxyhemoglobin reacts with O_2. In their ^{1}H NMR study of the histidine groups contributing to the alkaline Bohr effect, Fang et al. [22] found that three histidines make negative contributions: HisH21[143]β has a mean pK_a of 4.70 in deoxyhemoglobin and 5.65 in carbonmonoxyhemoglobin; HisNA2[2]β has a mean pK_a of 6.19 in deoxyhemoglobin and 6.40 in carbonmonoxyhemoglobin; and HisEF1[77]β has a mean pK_a of 7.45 in deoxyhemoglobin and 7.76 in carbonmonoxyhemoglobin (see Tables 1 and 2 of Fang et al. [22]).

Our pQ values for the n = 3 fits were all higher than those of Fang et al. [22]. We attempted, without success, to use the pQ_{ir} (and also the pQ_{it} (i = 1–3)) values obtained from these fits to account *quantitatively* for the reduction of the Bohr effect of human hemoglobin A modified at CysF9[93]β. This persuaded us that the pK_as of the negatively contributing groups determined directly by the ^{1}H NMR technique [22] must be more accurate than those (not shown) obtained from our n = 3 fits, which were determined indirectly by curve fitting to DTNB equilibrium data. Consequently, in the following discussion we shall employ the pK_a data of Fang et al. [22]. Although our pQ values for the n = 2 (Table 1) or n = 3 fits could not successfully be employed to give a satisfactory *quantitative* explanation of the reduction of the Bohr effect upon chemical modification, the finding that the pQ_i values for deoxyhemoglobin are *lower* than those of oxyhemoglobin (Table 1) was what pointed the way to a solution of the problem.

3.6. Basis of changes in the Bohr effect arising from chemical modification

In Fig. 3a we compare the Bohr effect, Δh^+, of native human hemoglobin A with that of hemoglobin A modified with cystine. It is seen, in a mathematical sense, that the acid Bohr effect and the physiologically more important alkaline Bohr effect of the modified protein are decreased compared to the native protein. A similar result is seen in Fig. 3b for hemoglobin modified with iodoacetamide. Although these results are similar to those obtained by Taylor et al. [1], the magnitudes of the decreases in the Bohr effect seen in Fig. 3 are lower than those reported in Fig. 8 of Taylor et al. [1] As seen in the top panel of the latter figure, the decrease in the Bohr effect of the cystine-treated hemoglobin is greater at higher ionic strength. While we worked at an ionic strength

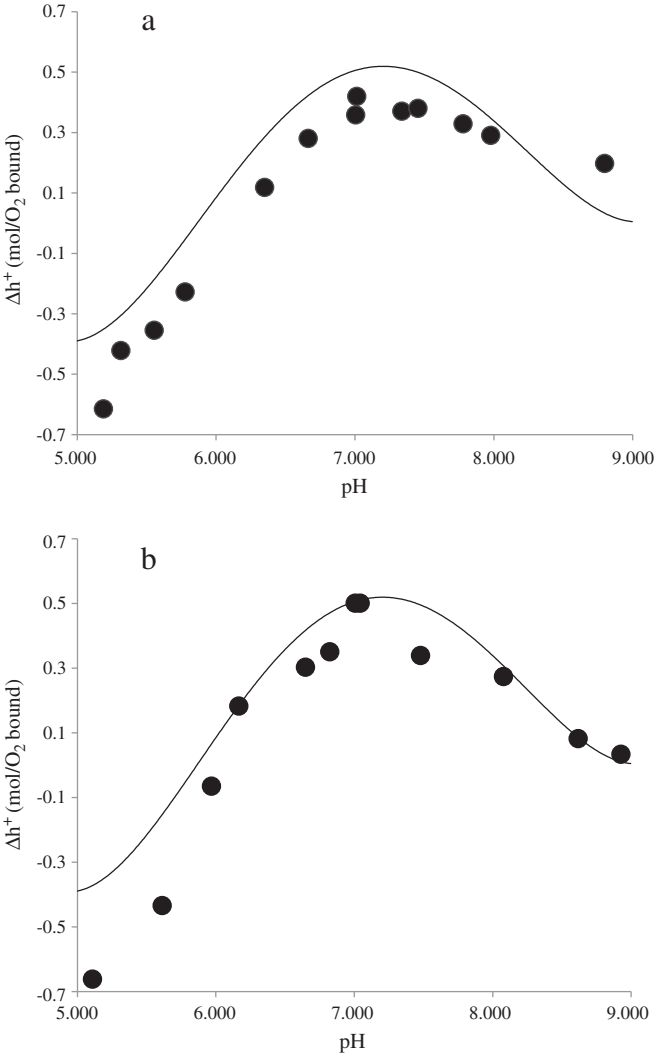


Fig. 3. Bohr effect of human hemoglobin A modified with (a) cystine (b) iodoacetamide. Conditions: 25 °C; ionic strength, 50 mmol dm^{-3} (NaCl). The data points are for the modified hemoglobins; the full line in each case is from the data for *unmodified* hemoglobin A [26].

of 50 mmol dm^{-3}, Taylor et al. [1] carried out their work at an ionic strength of 300 mmol dm^{-3} or higher. This explains why the magnitudes of the changes seen in Fig. 3 are lower than those seen in Fig. 8 of Taylor et al. [1]. (Our data for the cystamine-modified hemoglobin were not consistent with the data of Taylor et al. [1]. For this reason we will confine our discussion to the data for the cystine-modified and iodoacetamide-modified hemoglobins.)

The reduction of the Bohr effect as a consequence of chemical modification [1–3] has remained unexplained for almost five decades. We now attempt to account for this reduction *quantitatively*. As a first step we wished to determine whether the reductions in the Bohr effect upon chemical modification could be associated with the groups that contribute negatively to the Bohr effect. The equation employed by Sun et al. [23] for calculating the Bohr effect contribution of individual histidine residues comes from the work of Wyman [24,25]. We slightly modified this equation to enable the use of pK_a instead of K_a values. The modified equation reads:

$$\Delta h_j^+ = \frac{1}{2}\left\{\frac{10^{-pK_j^R}}{10^{-pH}+10^{-pK_j^R}} - \frac{10^{-pK_j^T}}{10^{-pH}+10^{-pK_j^T}}\right\}. \quad (4)$$

In Eq. (4) Δh_j^+ is the Bohr effect contribution of residue j (per O_2 molecule bound) arising from a change in quaternary structure from the T to the R quaternary state; pK_j^R and pK_j^T are the pK_as of ionization of residue j in the R and T quaternary structures, respectively. Using Eq. (4) we calculated, as a function of pH, the individual contributions of HisH21[143]β, HisNA2[2]β and HisEF1[77]β, and the sum of these contributions, to the Bohr effect. These calculations were aided by a program written on a MicroMath Scientist software (Salt Lake City, Utah, USA). As already stated above, we first attempted to account for the reduction in the Bohr effect (Fig. 3) by employing the pQ_{ir} and pQ_{it} (i = 1, 2) values reported in Table 1. We also attempted to account for the Bohr effect reduction with the pQ_i (i = 1–3) values obtained from the n = 3 fits. Our attempts were qualitatively satisfactory but quantitatively unsatisfactory. For this reason we employed the pK_a values determined by Fang et al. [22]. In Fig. 4 we present the calculated individual contributions of HisH21[143]β, HisNA2[2]β and HisEF1[77]β, and the sum of these contributions, as a function of pH. It is seen that the sum of these contributions is negative throughout the pH range 5.0 to 9.0. The summed contribution (Fig. 4) at the relevant pH of the determination of the Bohr effect of each modified hemoglobin (experimental points in Fig. 3) was read off and subtracted from the experimental Δh^+ for the modified hemoglobin. The results arising from these subtractions are compared with Δh^+ for the native hemoglobin in Fig. 5. For each modified hemoglobin it is seen that the calculated Bohr effect closely matches that of the native protein. Therefore the decrease in the Bohr effect arising from chemical modification has been fully accounted for *quantitatively* by the sum of the negative contributions arising from the ionizations of HisH21[143]β, HisNA2[2]β and HisEF1[77]β.

3.7. Structural basis of negative contributions to the Bohr effect

In their oxygen binding studies on the chemically modified hemoglobins, Taylor et al. [1] found that the Hill coefficient, *n*, was the same as for native hemoglobin A. So the quaternary structure transition and the cooperativity of oxygen binding remained unaffected by treatment with the sulfhydryl reagents. If the Bohr effect were solely determined by the quaternary structure transition it would be logical, from the O_2 binding results, to expect that the Bohr effect would be the same in the native and modified hemoglobins. As seen in Fig. 3, and in Fig. 8 of Taylor et al. [1], it is not. It seems clear, therefore, that chemical modification has given rise to a *non-quaternary* (that is, tertiary) structure change that is linked to HisH21[143]β, HisNA2[2]β and HisEF1[77]β, leading to the reduction in the Bohr effect seen in Fig. 3 and in Fig. 8 of Taylor et al. [1]. We previously associated such a tertiary structure change with the negatively contributing Bohr group HisH21[143]β [11].

In an X-ray crystallographic study of human oxyhemoglobin at 2.1 Å resolution, Shaanan [12] observed that the CysF9[93]β sulfhydryl group exists as a mixture of two conformations: *cis* to the main chain amino group and *cis* to the main chain carbonyl group. He suggested that in solution these two conformations might be in dynamic equilibrium. We subsequently carried out temperature-jump kinetic studies (between pH 5.4 and 7.0) on human deoxyhemoglobin A and on carbonmonoxyhemoglobin A, each mixed with pH indicators in buffer-free solution [11]. We observed a single relaxation process and found that τ^{-1}, the reciprocal relaxation time, varied with pH. Analyses of the pH-dependence of τ^{-1} showed that the relaxation process involved a non-quaternary isomerization linked to an ionizable group with a pK_a of 5.3 in deoxyhemoglobin and 6.0 in carbonmonoxyhemoglobin, that is, an acid Bohr group. We assigned this ionization [11] to HisH21[143]β. The pH dependence of τ^{-1} was abolished in *both* deoxyhemoglobin and carbonmonoxyhemoglobin

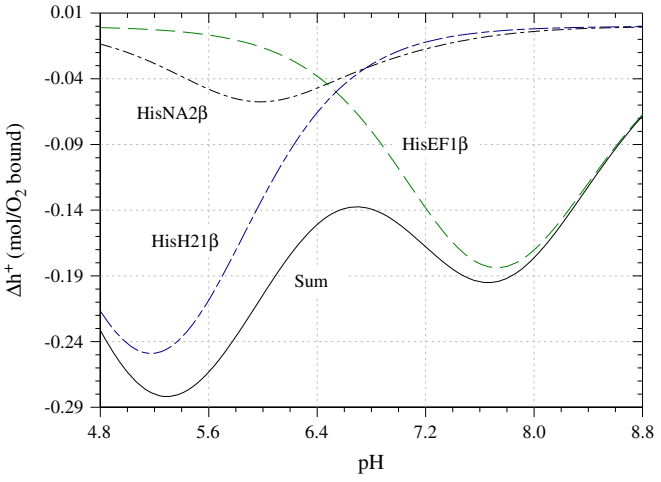


Fig. 4. Individual negative contributions to the Bohr effect by HisH21[143]β, HisNA2[2]β and HisEF1[77]β as a function of pH. "Sum" represents the sum of all contributions. Each contribution was calculated with the appropriate pK_a obtained from the ^1H NMR data of Fang et al. [22].

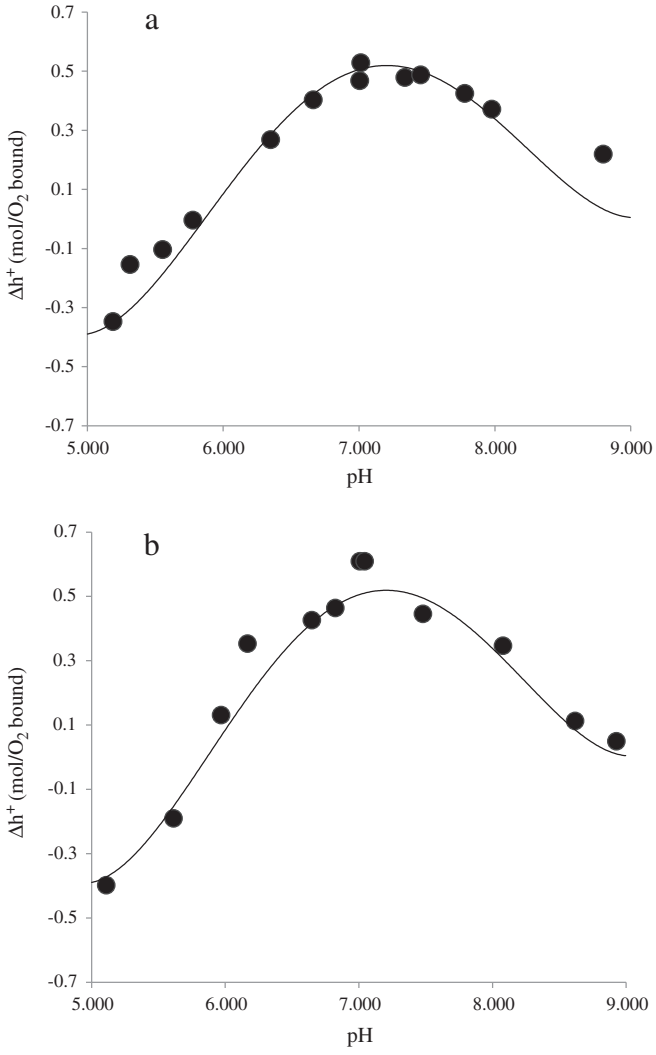


Fig. 5. Bohr effect of human hemoglobin A modified with (a) cystine, and (b) iodoacetamide. The data points are the results obtained after subtracting the sum of the negative contributions of HisH21[143]β, HisNA2[2]β and HisEF1[77]β (see Fig. 4) from the experimental points in Fig. 3. Full lines are from data for native hemoglobin A [26]. Conditions as in Fig. 3.

when the CysF9[93]β sulfhydryl group was modified with iodoacetamide, suggesting that the two sulfhydryl conformations [12] are coupled to the transition, $\mathbf{r} \overset{K_{rt}}{\rightleftharpoons} \mathbf{t}$, between two hemoglobin tertiary conformations, **r** and **t**, in dynamic equilibrium [11]. The abolition of the pH dependence of τ^{-1} in deoxyhemoglobin, and also in carbonmonoxyhemoglobin, arose because the equilibrium between the two tertiary conformations had been shifted fully to the right, in favor of the **t** conformation, by the treatment with iodoacetamide [11].

Both the T-jump [11] and Bohr effect experiments (Fig. 3) were carried out on the same *chemically modified* hemoglobin system, in the complete absence of buffer ions. It is therefore reasonable to extend the conclusions drawn from the T-jump experiments to the Bohr effect data in Fig. 3: that is, chemical modification fixes the CysF9[93]β sulfhydryl in the *cis*-to-carbonyl, that is **t** tertiary, conformation. This fixing in the **t** tertiary conformation is what gave rise to the reduction in the Bohr effect of the modified hemoglobins seen in Fig. 3 and in Fig. 8 of Taylor et al. [1].

We suggest that the negatively contributing Bohr groups (HisH21[143]β, HisNA2[2]β and HisEF1[77]β) make their maximum (negative) contribution to the Bohr effect when hemoglobin is fixed in the **t** tertiary conformation, in both deoxyhemoglobin and oxyhemoglobin, as a result of chemical modification. The Bohr effect of *unmodified* hemoglobin is then the net result of positive Bohr contributions arising from the R⇌T quaternary structure change and (modulated) negative contributions whose magnitude will be determined by the position of the $\mathbf{r} \overset{K_{rt}}{\rightleftharpoons} \mathbf{t}$ tertiary equilibrium: the lower the population of the **t** tertiary conformation, the lower the sum of the negative contributions of HisH21[143]β, HisNA2[2]β and HisEF1[77]β becomes.

The Tertiary-Two-State (TTS) model of Henry et al. [6] predicts that, in the R quaternary structure at equilibrium, the liganded subunits are all in the **r** tertiary conformation, and in the T quaternary structure all unliganded subunits are in the **t** tertiary conformation. However, owing to incomplete coupling between tertiary and quaternary structures, the fractional population of the **t** tertiary conformation is 40% in the *unliganded* R quaternary state and that of the **r** tertiary conformation is about 90% in liganded T quaternary subunits. In a previous report [11] we found that K_{rt}, the equilibrium constant for the $\mathbf{r} \overset{K_{rt}}{\rightleftharpoons} \mathbf{t}$ tertiary transition, has a value of 0.131 in carbonmonoxyhemoglobin and 0.31 in deoxyhemoglobin at 11 °C. The corresponding values at 25 °C are reported in Table 1: 0.126 for oxyhemoglobin and 0.336 for deoxyhemoglobin. These values translate to a fractional **t** population of 11% in the *liganded* R quaternary structure and 75% **r** population (that is, 25% **t**) in the *unliganded* T quaternary structure. It is seen that the results of these calculations are in line with the predictions of Henry et al. [6]. If, for simplicity, we assume a mean **t** population of 18% for R and T state hemoglobin, the negative contributions of HisH21[143]β, HisNA2[2]β and HisEF1[77]β to the Bohr effect would be a mere 18% of what is seen in Fig. 3. For animal species that have these three histidines in their hemoglobin structure, negative Bohr contributions by the three histidines, modulated by the $\mathbf{r} \overset{K_{rt}}{\rightleftharpoons} \mathbf{t}$ tertiary equilibrium, might account, at least in part, for the variability in the Bohr effect of various animal hemoglobins observed by Bailey et al. [26].

3.8. Does chemical modification of CysF9[93]β affect the quaternary structure?

Apart from the effect of chemical modification of CysF9[93]β in reducing the Bohr effect of human hemoglobin A, Taylor et al. [1] also found that it increases the affinity of hemoglobin for O_2. These results are fully supported by the more recent data of Manjula et al. [27] and Cheng et al. [28]. In the classical Monod–Wyman–Changeux model the increase in O_2 affinity would be interpreted to mean that chemical modification has favored the R quaternary structure. To check if this is so, we consider the ^{1}H NMR data of Lukin and Ho [29]. These authors report that HisCD3[45]α of human hemoglobin A, like HisNA2[2]β, HisEF1[77]β and HisH21[143]β, has a lower pK_a in deoxyhemoglobin compared to carbonmonoxyhemoglobin: 5.25 compared to 6.12. This result is very interesting because it provides an opportunity to test the extent to which the chemical modification of CysF9[93]β affects the α chains of hemoglobin, that is, whether it affects the quaternary structure of hemoglobin. In Fig. 5 we demonstrated that if account is taken of the negative Bohr contributions of HisNA2[2]β, HisEF1[77]β and HisH21[143]β, the decrease in the Bohr effect of hemoglobins modified at CysF9[93]β can be fully accounted for.

In Fig. 6 we show, for iodoacetamide-modified hemoglobin, the effect of including the contribution of the α chain negative Bohr contributor, HisCD3[45]α, in calculating the sum of the negative Bohr contributions (cf. Fig. 4). It is clear that upon including HisCD3[45]α the calculated Bohr effect is no longer in agreement with the Bohr effect of unmodified hemoglobin, especially in the low pH range. Therefore while Fig. 5 demonstrates that the chemical modification of CysF9[93]β is connected with the beta chain negative Bohr contributors (HisNA2[2]β, HisEF1[77]β and HisH21[143]β), Fig. 6 shows that it is *not* connected with the event occurring in the alpha chains, namely, the negative Bohr contribution of HisCD3[45]α. This being so, the increase in the oxygen affinity of hemoglobin arising from chemical modification cannot be because the quaternary equilibrium has been shifted in favor of the R quaternary structure. This leaves the increase in oxygen affinity arising from chemical modification still unaccounted for.

3.9. Increase in oxygen affinity and fixing of tertiary conformation in the t state

We demonstrated [11] that in iodoacetamide-modified hemoglobin the $\mathbf{r} \overset{K_{rt}}{\rightleftharpoons} \mathbf{t}$ tertiary equilibrium is shifted entirely to the right in favor of the **t** state. Using the K_{rt} data presented in Table 1 we calculated the values of ΔG_{rt}, the free energy change for the $\mathbf{r} \rightleftharpoons^{K_{rt}} \mathbf{t}$ tertiary conformational transition in deoxyhemoglobin and in oxyhemoglobin. The values so calculated are $+2702$ J mol^{-1} and $+5132$ J mol^{-1}, respectively. So on going from deoxyhemoglobin to oxyhemoglobin we expect a $\Delta(\Delta G_{rt})$ of $+2430$ J mol^{-1}. From the oxygen binding data of Taylor et al. at pH 7.0 (Figs. 2 and 4 of [1]) we make the following (admittedly rough) estimates of the free energies for oxygen binding to unmodified and modified

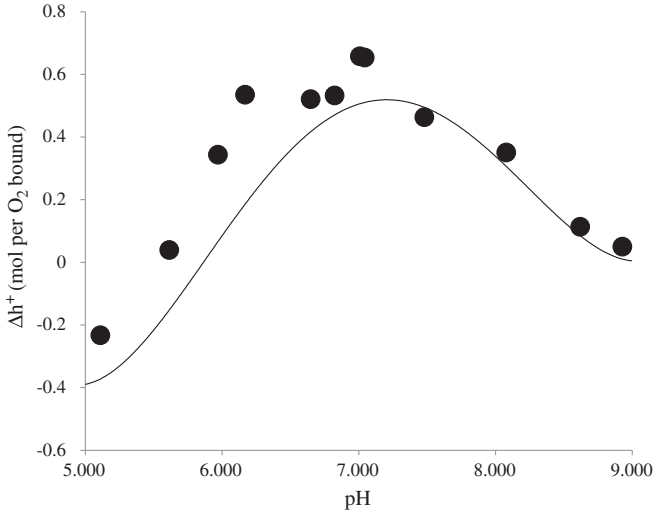


Fig. 6. Bohr effect of human hemoglobin A modified at CysF9[93]β with iodoacetamide. The data points are the results obtained after subtracting the sum of the negative contributions of HisH21[143]β, HisNA2[2]β and HisEF1[77]β, as well as HisCD3[45]α, from the experimental points in Fig. 3. Full lines are from data for native haemoglobin A [26]. Conditions as in Fig. 3. Compare with Fig. 5b.

hemoglobin. Assuming $\log P_{50} = 1$ for unmodified hemoglobin and 0.65 for (iodoacetamide and cystine) modified hemoglobin, we have the following ΔG values:

$$\Delta G(\text{unmodified}) = -2.303RT\log_{10}P_{50} = -2.303RT \times 1 = -5610\ \text{J mol}^{-1};$$

$$\Delta G(\text{modified}) = -2.303RT\log_{10}P_{50} = -2.303RT \times 0.65 = -3647\ \text{J mol}^{-1};\ \text{and}$$

$$\Delta\left(\Delta G_{\text{oxygen binding}}\right) = \Delta G(\text{modified}) - \Delta G(\text{unmodified}) = +1960\ \text{J mol}^{-1}.$$

So the free energy difference for O_2 binding to modified and unmodified hemoglobin A is 1960 J mol^{-1}. This roughly marches the $\Delta(\Delta G_{rt})$ of +2430 J mol^{-1} required for fixing the **r** $\overset{K_{rt}}{\rightleftharpoons}$ **t** equilibrium in the **t** conformation. Considering the different experimental conditions employed for determining $\Delta(\Delta G_{rt})$ and $\Delta(\Delta G_{\text{oxygen binding}})$ [25 °C, ionic strength 50 mmol dm^{-3}; and 20 °C, 200 mmol dm^{-3} phosphate, respectively], the agreement between the two parameters is very good.

We suggest that, on chemical modification, the energy conserved by fixing the **r** $\overset{K_{rt}}{\rightleftharpoons}$ **t** transition in the **t** state (2430 J mol^{-1}) is expended to increase the oxygen affinity of modified compared to unmodified hemoglobin, which requires 1960 J mol^{-1}. In other words, the free energy that (in unmodified hemoglobin) has been expended to transform oxyhemoglobin from the **t** to the **r** tertiary conformation has been conserved in modified hemoglobin and switched to free energy for increasing the oxygen affinity. So the **r** $\overset{K_{rt}}{\rightleftharpoons}$ **t** equilibrium has accounted not only for the decrease in the Bohr effect of hemoglobin modified at CysF9[93]β (compare Figs. 3 and 5) but also for the increase in its O_2 affinity compared to unmodified hemoglobin (Figs. 2 and 4 of Taylor et al. [1]). It is very likely that the increase in oxygen affinity is confined to the beta chains of the modified hemoglobin, just as the decrease in the Bohr effect can only be attributed to the negative Bohr contributors on the beta chains (compare Figs. 5 and 6).

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Appendix A

A.1. Definitions of species and parameters appearing in Eq. (1) of the text

In Eq. (1) PSH is hemoglobin with CysF9[93]β in its protonated form, which does not react with non-mercurial sulfhydryl reagents [30–32]; PS^- is the corresponding anion form, which reacts with DTNB; PS.ST is the mixed disulfide formed after reaction with DTNB; TNB^- is 5-thio-2-nitrobenzoate, the chromophoric product of the DTNB reaction, which is used to monitor the reaction spectrophotometrically; TNBH is the protonated form of TNB^-; Q_{SH} and Q_{TNB} are the ionization constants of CysF9[93]β and TNBH, respectively; K_{equ} is the equilibrium constant for the formation of the mixed disulfide (PS.ST), that is, the DTNB reaction step.

A.2. Definitions of species/parameters appearing in Scheme 1 and Eq. (3) of the text

$H_{n-i+1}PSH$ (i = 1, 2, …, n + 1) are hemoglobin species in which the sulfhydryl group is in its protonated form, which does not react with DTNB. These species are therefore omitted from Scheme 1. $H_{n-i+1}PS^-$ (i = 1, 2, …., n + 1) are species in which the sulfhydryl is in its thiolate anion form, the form that reacts with DTNB; $H_{n-i+1}PS.ST$ (i = 1, 2, …., n + 1) are the mixed disulfide species formed after the reaction of the sulfhydryl with DTNB. Species marked with subscripts r or t are those in which the sulfhydryl is in the **r** or **t** tertiary isomeric form of hemoglobin. The various proton ionization constants are represented as Q_i, Q_{ir} and Q_{it} (i = 1, 2, …, n) to differentiate them from K_{Ei} (i = 1, 2, …., n + 1), the equilibrium constants for the reaction of the various $H_{n-i+1}PS^-$ species with DTNB; and $K_{rt(n+1)}$ is the equilibrium constant at high pH for the **r** ⇌ **t** tertiary conformational transition.

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