The interaction of inositol hexakisphosphate (IHP) with oxygenated human adult hemoglobin (Hb) was investigated at 25 °C. The affinity of IHP for oxygenated Hb is strongly pH-dependent, and potentiometric measurements of proton uptake and release upon IHP addition have shown that over the range between pH 8.0 and pH 6.0 in oxygenated Hb there are three groups of residues that change their pKa values after IHP addition, likely because of their interaction with negative charges of the hetrotropic effector. On the basis of previous calculations on the electrostatic properties of human Hb (Matthew, J. B., Hanania, G. I. H., and Gurd, F. R. N. (1979) Biochemistry 18, 1919–1928; Lee, A. W.-m., Karpplus, M., Poyart, C., and Bursaux, E. (1988) Biochemistry 27, 1285–1301), two of these groups might be Val1β and His143β, which are located in the ββ dyad axis, where they have been also proposed to interact with 2,3-diphosphoglycerate, whereas the third group does not appear easily identifiable. Calorimetric measurements of the heat associated with IHP binding at different pH values over the same range indicate that IHP binding is mostly enthalpy-driven at pH < 7 and mostly entropy-driven at pH > 7.

Human hemoglobin (Hb) is functionally modulated by several non-heme ligands, such as organic phosphates (i.e., 2,3-diphosphoglycerate (2,3-DPG) and myo-inositol hexakisphosphate (IHP)), protons, and chloride ions (1–5), which bind at hetrotropic interaction sites, topologically distinct from the heme at which hetrotropic ligands bind.

The structure of this binding pocket has been determined for the interaction of 2,3-DPG (6), which has been shown to bind at the interface between the two β-chains, mainly involving three residues from either one of β-subunits (i.e. HisNA2(β2), Lys-EF6(β2), and HisH21(β143)), see Ref. 6).

In more recent years, another organic phosphate, namely IHP (closely related to the inositol pentaphosphate, which is the physiological effector in avian erythrocytes; see Ref. 7), has often been employed to study the modulation of functional properties of human Hb (8, 9). It possesses additional negative charges with respect to 2,3-DPG, and it displays a much larger effect, which suggests the occurrence of additional electrostatic interactions with respect to 2,3-DPG, as from early model building studies on deoxy Hb (10). Therefore, the enhanced functional effect of IHP on the O2 binding properties of human Hb with respect to 2,3-DPG could be related to a more widespread interaction surface, with the possibility of modulating ligand-linked conformational changes taking place over a larger portion of the whole tetramer.

However, a comprehension of the origin for this enhanced effect starts from the characterization of the IHP interaction energy with deoxyHb and with oxyHb. Previous studies have shown that IHP binds HbO2, and its binding properties are pH-dependent (11, 12). In this study, we have carried out a detailed analysis of the interaction of IHP with human HbO2, measuring the effect on (a) proton titration, (b) O2 dissociation kinetics from fully liganded tetramer, and (c) heat associated to the reaction in order to give a quantitative description of the system and of the interplay between IHP and proton interaction with human HbO2.

EXPERIMENTAL PROCEDURES

Human HbO2 was obtained from the blood of healthy volunteers and stripped of anions according to the procedure reported by Rigg (13). Cells were washed three times with iso-osmotic NaCl solutions by centrifugation at 1000 × g, and packed cells were lysed by adding 2 volumes of cold bidistilled water. Stroma were removed by centrifugation at 12,000 × g for 30 min. Hemolysate was first filtered through a Sephadex G-25 column, equilibrated with 0.01 M Tris/HCl buffer, pH 8.0, and EDTA 10−5 M, and afterward it was passed through a column of mixed bed ion-exchange resin (Bio-Rad AG501-X8). For proton titration experiments, Hb solution was concentrated on Amicon YM10 (Bio-Rad) membranes. For all other experiments, the sample was then extensively dialyzed versus the desired buffer. All experiments were performed at 25 °C in the presence of 0.1 M NaCl.

Titrations were performed at 25 °C using a thermostatted autotitration (Radiometer, Copenhagen, Denmark) equipped with a SAM90 sample station, ABUS triburette unity, and VIT90 titration controller, adding automated 100-μl aliquots of 2 mM NaOH (prepared from 0.1 M Normex and checked by acid titration). For each experiment, three solutions (between 0.75 and 2.00 ml) were titrated, namely (a) HbO2 ranging between 1.0 and 1.5 mM tetramer, (b) IHP ranging between 20 and 25 mM, and (c) IHP plus HbO2 at the same concentrations employed in a and b. We also carried out experiments at 0.2 mM heme concentration (i.e. the concentration at which kinetic experiments were performed; see below), but no appreciable difference was noticed, indicating that the dimer-tetramer equilibrium does not affect these results to a detectable extent. From titration curves, composed of more than 150 experimental points and elaborated by our own programs in order to express constant pH increments, the proton buffering capacities (Δ

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The abbreviations used are: Hb, hemoglobin; IHP, inositol hexakisphosphate; 2,3-DPG, 2,3-diphosphoglycerate; MES, 4-morpholineethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; Bis-Tris, 2-[bis(2-hydroxyethyl)amin]-2-hydroxyethyl-propane-1,3-diol; MOPS, 4-morpholinepropanesulfonic acid.
Interaction of IHP with HbO₂

μmol/8 pH) were obtained. Buffering capacities of IHP-bound HbO₂ were computed by subtracting the contribution of HbO₂ from the overall buffering capacities measured on IHP plus HbO₂ solution. The integration of this differential buffering capacity gave the corrected titration curve of IHP-bound HbO₂, the position of which relative to IHP-free HbO₂ should be independently determined. Therefore, proton uptake for the formation of the IHP-HbO₂ (i.e. ΔZ) was obtained at several fixed pH values by measuring the moles of HCl per mole of HbO₂ needed to recover the starting pH value after the addition to oxyHb of a saturating amount of concentrated IHP (IHP/HbO₂ molar ratio 20:1 with [HbO₂] = 1.5 mM tetramer) and the correction for IHP dilution effects (obtained by IHP blank titration). These values allowed us to establish the relative position of the titration curves and thus to obtain experimental ΔZ values over the whole pH range investigated (i.e. between 6.0 and 8.0). Outside this pH range, the reproducibility of data decreased dramatically, and thus the errors were too large to allow any meaningful analysis of experimental curves. The value of ΔZ as a function of pH (see Fig. 1A) is related to the pH dependence of the IHP binding equilibrium constant K according to the following equation (14).

\[ \text{dlogK/dpH} = -\Delta Z \] (Eq. 1)

Upon integration, this relation becomes the following equation.

\[ \log K(pH) - \log K(pH_0) = \int_{pH_0}^{pH} \Delta Z \text{d}pH \] (Eq. 2)

The value of K at a given pH value (in our case, pH = 7.1) was determined by subsequent additions of subsaturating amounts of IHP to HbO₂ and measuring after each addition the moles of HCl needed to maintain a constant pH value. The knowledge of the moles of HCl, of ΔZ at that pH, of the moles of HbO₂, and of the moles of IHP added allows one to determine the moles of free IHP after each addition and the saturation degree of the IHP-HbO₂ complex (Y). If one assumes a single binding site for IHP to the tetrameric HbO₂ (under these experimental conditions; see Ref. 12), it is then possible to fit values of Y as a function of x moles of free IHP (see Fig. 1B), according to the following equation.

\[ \hat{Y} = K_x / (1 + K_x) \] (Eq. 3)

where x is given by the equation,

\[ x = \left( \frac{C_{\text{HIPC}} \cdot V_{\text{HIPC}} - (\mu \text{mol of HCl/ΔZ})/V_{\text{tot}}}{C_{\text{HIPC}}} \right) \] (Eq. 4)

where C_{HIPC} is the IHP concentration of the stock solution employed and V_{HIPC} and V_{tot} are the volume added of IHP stock solution and the total volume of the sample solution, respectively. The extent of IHP binding \( \hat{Y} \) is given by the following equation.

\[ \hat{Y} = \mu \text{mol of HCl/ΔZ}/\mu \text{mol of HbO}_2 \] (Eq. 5)

Thus, using Equation 2, the value of K at pH 7.1 (by Equation 3), and ΔZ dependence on pH, we were able to calculate K over the pH range between 6.0 and 8.0 (see Fig. 2).

Kinetically, O₂ dissociation in fully liganded Hb was undertaken employing a Hi-Tech SF-51 stopped-flow apparatus with a 2-cm path length observation cell that was interfaced with a desktop computer for fast data acquisition. Oxygen dissociation was followed by mixing HbO₂ (0.2 mM heme after mixing) with a CO-saturated buffer containing sodium dithionite and following the conversion of HbO₂ to HbCO at λ = 563 nm (15). No CO concentration dependence was observed for these kinetics, down to a concentration of 50 μM, a value 10 times lower than that employed for all observations reported in this study (i.e. 0.5 mM after mixing). The amount of free IHP was calculated, implying that the IHP-dependent effect on the O₂ dissociation rate constant is linearly dependent on the percentage of IHP-HbO₂ complex with respect to the total concentration of tetrameric HbO₂.

Calorimetric measurements were performed using a high-precision twin titration isothermal microcalorimeter (16).

The Hb solution was kept inside the sample cell (total cell volume, 184 μl), and the injection syringe was filled with the concentrated IHP solution. In order to reduce the heat of dilution, small volumes of IHP solution (i.e. 2 μl) were added each time, and corrections were made for the heat effects due to stirring and dilution (16). Calibration experiments were carried out, employing HCl/NaOH titrations and electrical calibrations (16).

Calorimetric IHP titration experiments of human HbO₂ were carried over the pH 6.0–8.0 range, employing an IHP concentration range that was enough to fully saturate the HbO₂, and this occurrence was determined when no heat was produced upon further addition of IHP. The data analysis is based on a titration in which IHP concentration is increased at each step from \( x_{i-1} \) to \( x_i \), and the quantity of heat \( q_{i-1} \) is associated with the binding of IHP to HbO₂ in this step. The value of \( q_{i-1} \) is then given by the equation,

\[ q_{i-1} = m_b ( \hat{H} - \hat{H}_b ) - ( \hat{H} - \hat{H}_b )_{i-1} \] (Eq. 6)

where \( m_b \) is the moles of HbO₂ employed in each calorimetric experiment.

The excess enthalpies (\( \hat{H} - \hat{H}_b \)) depend on the ligand concentration \( x_i \) according to the following equation.

\[ ( \hat{H} - \hat{H}_b ) = -R \ln( P / \beta_1 / T) \] (Eq. 7)

The latter expression is a van’ Hoff formulation in terms of the binding polynomial \( P \) (17).

The heat \( q_{i-1} \) is the experimentally measurable quantity in isothermal titration calorimetry. If one assumes only one site for the

![Fig. 1. A, observed pH dependence at 25 °C of proton uptake or release (ΔZ) upon binding of saturated amounts of IHP to HbO₂. The error bars refer to the distribution of errors based on five different measurements of the same sample. The data presented are limited to the pH range between 6.0 and 8.0 because outside this range, data become very unreliable. The continuous line corresponds to the behavior expected for Equation 10, employing the parameters reported in Table I. Dashed line corresponds to the fit of data employing only two protonating groups. For further details, see text. B, saturation function (T) of HbO₂ as a function of pH 7.1 and 25 °C. The continuous line was obtained by nonlinear least-squares fitting of experimental data according to Equation 3. The fitted limiting values for Y = 1.0 (i.e. under saturating amounts of IHP) have been employed to calculate ΔZ at the given pH. For further details, see text.](http://www.jbc.org/content/15330/1/A1)
interaction of IHP with HbO₂, the van’t Hoff expression reduces to the following equation.

\[
(\Delta H - \Delta H_{pK}) = -R(\ln P/\ln T) = \Delta H \cdot K_b/(1 + K_b)
\]  

(Eq. 8)

The value of the observed enthalpy change \(\Delta H_{pK}\) as calculated from the van’t Hoff expression, can be dissected into two main contributions, one related to the IHP binding phenomenon itself and the other one ascribable to the ligand-linked proton equilbria in the buffer. Therefore, because there is a linkage between IHP binding to HbO₂ and proton release or uptake, the observed \(\Delta H_{pK}\) is represented by the equation,

\[
\Delta H_{pK} = \Delta H_{1} + \Delta H_{2} + \Delta H_{3} = \Delta H_{1} + \Delta H_{2} + \Delta H_{3}
\]  

(Eq. 9)

where \(\Delta H_{1}\) is the buffer-corrected enthalpy change for IHP interaction with HbO₂, which still contains the contribution arising from the ionization enthalpy of HbO₂ (18). The second term in Equation 9 refers to the apparent enthalpy change obtained when 3 or 4 moles of protons are released or taken up to a buffer with a \(\Delta H_{pK}\) ionization enthalpy change. The value of \(\Delta H_{pK}\) was determined at every pH investigated, carrying out the same calorimetric experiment in buffers with different ionization enthalpy, such as MES, HEPES, PIPES, Bis-Tris, and MOPS, and extrapolating to \(\Delta H_{pK} = 0\) (see Equation 9 and Ref. 19).

All experiments were performed either in distilled H₂O in the presence of 0.1 M NaCl (potentiometric experiments) or in 0.1 M MES (between pH 5.5 and 7.0) or HEPES (between pH 6.5 and 8.0) in the presence of 0.1 M NaCl (calorimetric and kinetic experiments).

RESULTS AND DISCUSSION

Fig. 1A shows that the total proton uptake of human HbO₂ at 25 °C in the presence of 30 mM IHP, a concentration sufficiently high to guarantee the full saturation of the higher affinity site for IHP in the liganded hemoglobin (11, 12), is pH-dependent, approaching 0 at pH \(\approx 8.0\), attaining a maximum value of \(\Delta Z \approx 2.8\) at pH \(\approx 7\), and then decreasing upon pH lowering. It is important to note that over the same pH range, the buffering capacity (and thus the amount of protons exchanged with bulk solvent) of a 30 mM solution of IHP alone was much less than that observed for a solution of 1.5 mM tetrameric HbO₂, clearly indicating that the phenomenon reported in Fig. 1A is mostly related to the proton exchange involving the Hb molecule and not the IHP molecule. Because \(\Delta Z\) is the derivative of the proton-linked effect on the IHP binding constant to HbO₂ (see Equation 1), a quantitative analysis of \(\Delta Z\) as a function of pH (Fig. 1A) allows the determination of the linkage between IHP binding and shifts of \(pK_a\) values for groups affected by IHP interaction with HbO₂. The analysis of these data requires the involvement of (at least) three classes of residues, according to the following relationship.

\[
\Delta Z = ((K_{eq} + 2K_{eq}^3 + 3K_{eq}^5)/P_b) - ((K_{eq} + 2K_{eq}^3)/P_b) + 3K_{eq}^5/P_b
\]  

(Eq. 10)

where \(x = 10^{-pH}\) and \(K_{eq} = 10^{-pK_i}\) (i = 1–3) are the proton binding association constants of the three groups, and the superscript b and f refer to IHP-bound and IHP-free HbO₂, respectively. \(P_b\) and \(P_f\) are the proton binding polynomials for proton binding to IHP-bound and IHP-free HbO₂, respectively.

\[
P_b = (1 + K_{eq}[H^+] + K_{eq}^2[H^+] + K_{eq}^3[H^+])
\]  

(Eq. 11)

and

\[
P_f = (1 + K_{eq}[H^+] + K_{eq}^2[H^+] + K_{eq}^3[H^+])
\]  

(Eq. 12)

It is important to note that Equations 10–12 imply that the three groups are protonating in a concerted way; that is, the protonation of the first group alters the protonation of the second group, and the protonation of both the first and the second group affects the protonation of the third group. In other words, groups 2 and 3, which would not protonate in the range investigated, change their proton affinity upon protonation of group 1. Therefore, by virtue of the cooperative behavior, the values of \(K_a\) may be indeed treated as intrinsic binding constants, and they can be immediately referred to the \(pK_a\) values of the various residues involved. The \(pK_a\) values of groups involved in the IHP binding to HbO₂ resulting from the fit of data in Fig. 1A according to Equation 10 are reported in Table I, and they correspond to the continuous line in Fig. 1A. It is important to note that in Table I the \(pK_a\) for IHP-free HbO₂ is reported only as being <4.5, because any value below 4.5 gives an equally good fit of data, and we can consider its value as partially undetermined.

Fig. 1B displays the fitting of pH-stat data on the equilibrium titration of human HbO₂ with IHP at pH 7.1 according to Equation 3, which allows one to calculate the affinity of IHP for HbO₂ at this pH. Combination of the information obtained from the experiments reported in the two panels of Fig. 1, namely (a) \(\Delta Z\) as a function of pH (Fig. 1A), and (b) the equilibrium IHP binding constant at a given pH value (Fig. 1B), allows one to calculate, according to Equation 2 (14), the \(logK\) for IHP binding to human HbO₂ over the pH range covered by the proton titration reported in Fig. 1A. In Fig. 2, the pH dependence of the equilibrium IHP binding constant to human HbO₂ is reported at 25 °C. The continuous line reported in Fig. 2 was obtained employing the following equation.

\[
K_{obs} = K_0 \cdot P_b/P_f
\]  

(Eq. 13)

where \(K_{obs}\) is the observed IHP equilibrium binding constant, \(K_0\) is the IHP equilibrium binding constant to unprotonated HbO₂, and \(P_b\) and \(P_f\) are the proton binding polynomials to IHP-bound and IHP-free human HbO₂, respectively, (see Equations 11 and 12), employing the values of \(K_a\) reported in Table I. Therefore, the interrelationship between IHP and proton linkage can be represented by the following Scheme.

\[
\begin{align*}
\text{PH} + \text{H}^+ &\rightleftharpoons \text{PH}^+ \\
P &\rightleftharpoons \text{P}^+ \\
\text{IHP}^+ &\rightleftharpoons \text{IHP}^++ \text{H}^+ \\
\text{P}_{1}^+ &\rightleftharpoons \text{P}_{1}^++ \text{H}^+ \\
\text{P}_{2}^+ &\rightleftharpoons \text{P}_{2}^++ \text{H}^+ \\
\text{P}_{3}^+ &\rightleftharpoons \text{P}_{3}^++ \text{H}^+
\end{align*}
\]

Scheme I

\[
\begin{align*}
\text{IHP} &\rightleftharpoons \text{IHP}^+ + \text{H}^+ \\
\text{P}_{1}^+ &\rightleftharpoons \text{P}_{1}^+ + \text{H}^+ \\
\text{P}_{2}^+ &\rightleftharpoons \text{P}_{2}^+ + \text{H}^+ \\
\text{P}_{3}^+ &\rightleftharpoons \text{P}_{3}^+ + \text{H}^+
\end{align*}
\]  

Scheme I and the \(pK_a\) values reported in Table I deserve some further comment. As a matter of fact, the behavior observed in Table I underlies a cooperative proton-linked process, such that protonation of one residue facilitates the protonation of another residue. This concerted process may envisage the occurrence of a pH-dependent conformational transition in liganded human Hb, as also suggested by previous observations (15). Furthermore, Scheme I indicates that IHP and protons act.
Fig. 2. pH dependence of IHP binding constant to HbO₂ in 0.1 M NaCl, at 25 °C, according to Equation 2 and employing data reported in Fig. 1. The line represents the least-squares fitting of data, employing Equation 13, with \( K_Q \) values reported in Table I and \( K_0 = 5.13 \times 10^5 \) M \(^{-1}\). For further details, see text.

synergistically to facilitate the conformational transition, raising the \( pK_a \) of interacting groups upon binding of the negatively charged IHP. Obviously, with our experimental approach, we cannot absolutely rule out a contribution arising, in addition, from a change in the protonation state between Hb-free and Hb-bound IHP, even though the small amount of proton exchanged by IHP alone (see above) indicates that this contribution is not relevant. This conclusion is further supported by a previous observation on deoxy Hb and on HbCO by \(^{31}\)P NMR, where a change in the protonation state of IHP upon binding Hb indeed was detected, but it turned out to be pH-independent between pH 5.2 and 8.5 (20). Therefore, the observed pH dependence for IHP binding to HbO₂ (see Fig. 2) can be almost completely attributed to a pH-dependent difference in protons bound by IHP-free and IHP-bound oxyHb.

This proton-linked behavior is calculated on the basis of the proton titration carried out on IHP-free and IHP-bound human HbO₂, but a confirmation of its validity may come from an independent measurement of IHP binding to fully liganded HbO₂. This can be carried out by investigating the effect of IHP on the displacement kinetics of oxygen by CO. Thus, in this experimental approach, the rate of CO binding is rendered much faster than the \( O_2 \) dissociation process, and the observation concerns a fully liganded protein, allowing a direct determination of IHP binding to HbO₂. In Fig. 3, the values of rate constants for \( O_2 \) dissociation from fully liganded Hb are reported as a function of free IHP concentration at different pH values. It is important to note that the continuous lines in Fig. 3 are not fit to experimental points; instead, they simply show the correlation between the predicted pH dependence of \( K \) (see Fig. 2) and the observed pH dependence of the \( O_2 \) dissociation rate constant from fully liganded Hb. Therefore, they are constrained to the expected IHP dependence on the basis of the IHP binding equilibrium constant at the same pH according to the parameters reported in Table I, employed to fit the data reported in Fig. 1A and used to obtain the continuous line in Fig. 2. The agreement is quite impressive and allows a very strong degree of confidence in the correctness of the prediction based on data in Fig. 1A and on Equation 2, and thus in the accuracy of parameters in Table I, as well as in the pH dependence described in Fig. 2, to quantitatively describe the linkage between proton and IHP binding to human HbO₂.

Parameters in Table I clearly indicate that the pH-dependence of IHP binding constant depends on the \( pK_a \) shift of three classes of residues that increase their \( pK_a \) values by \( \approx 0.96, 0.92, \) and \( >3.7 \), respectively, upon interaction with negative charges of IHP. It is important to note that in free HbO₂ the \( pK_a \) values of at least two of these residues turn out to be low enough to rule out the relevant role in the “alkaline” Bohr effect, whereas a third residue displays a \( pK_a \) of 6.72 (see Table I) in the IHP-free HbO₂, which makes it a good candidate for a contribution to the alkaline Bohr effect (21–23). On the other hand, such \( pK_a \) values for IHP-free HbO₂ are within the pH range of a conformational transition, which has been detected in human HbO₂ in the absence of anions (15) and which is characterized by an enhancement of the \( O_2 \) dissociation rate constant in the fully liganded form. A similar behavior was observed in the presence of 0.1 M Cl\(^-\) (Fig. 4), and it can be accounted for by employing the three \( pK_a \) values reported in Table I for IHP-free HbO₂, as from the continuous line reported in Fig. 4. The same consideration can be applied to the pH dependence of the \( O_2 \) dissociation rate constant for fully liganded Hb in the presence of saturating amounts of IHP (i.e. 30 mM), which also is fully described employing the \( pK_a \) values reported in Table I for IHP-bound human HbO₂ (see Fig. 4).

Altogether, these data strengthen our confidence in the possibility of giving a quantitative description of the thermodynamics of IHP interaction with human HbO₂. Therefore, we can claim that (a) the protonation of three residues, the \( pK_a \) of which values range in IHP-free HbO₂ between \( \approx 4.0 \) and 6.7 (see Table I), brings about a conformational transition of a fully liganded human Hb, (b) this event is closely related to the pH-dependent enhancement of the IHP equilibrium binding constant to HbO₂ (Fig. 2), and (c) IHP binding is accompanied by a more or less marked raising of \( pK_a \) values of these three classes of residues.

The identification of the three residues involved in the proton-linked IHP binding to HbO₂ is not easy, but previous observations indicated that some potentially important residues...
However, it must be noticed that in the case of IHP-bound HbO$_2$, the pKa to be not true for His$_2$ substituted by Arg; see Ref. 26), in which the IHP effect on oxygen-residue in the $b$ $k$ values of for the IHP-free and IHP-bound HbO$_2$. For IHP-free HbO$_2$, the continuous curve was obtained using $K_{O2}=K_{O2,obs}$ from HbO$_2$, in 0.1 M NaCl, at 25 °C, from IHP-free (25) and which might be tentatively recognized in the residue characterized by a $pK_a$ below 6.5 (25). However, it must be pointed out that a fairly low $pK_a$ because its taken into account in our analysis of the pH dependence simply displayed low $pK_a$ values in oxyHb in the absence of organic phosphates (24). In particular, a fairly low $pK_a$ value (i.e. $pK_a < 4.5$) has been reported by several authors for His$^{439}$ in HbO$_2$ (25), a residue that has been already proposed to be involved in the binding of organic phosphate (6, 10). A second residue might be Val$^{16}$, which has been proposed to display a $pK_a$ value $7.8$ in IHP-free HbO$_2$ (25) and which might be tentatively recognized in the residue characterized by a $pK_a$ of 6.72 (see Table 1). The residue characterized by a $pK_a = 5.96$ in IHP-free HbO$_2$, see Table 1) is very difficult to identify, even with some uncertainty, and we cannot rule out at this stage that the effect attributed to this IHP-linked group is instead attributable to a widespread small effect on several residues, such as His$^{720}$ and His$^{770}$, which have been reported to have in HbO$_2$ $pK_a$ values below 6.5 (25). However, it must be pointed out that a fairly low $pK_a < 6.5$ has also been proposed for His$^{240}$ (25, 24), another residue in the $\beta$-dyad axis cavity where organic phosphates bind (6, 10). The possibility of a role by His$^{240}$ in the interaction of IHP with HbO$_2$ is not in contradiction with the observation on a mutant, namely Hb Deer Lodge (where His$^{240}$ is substituted by Arg; see Ref. 26), in which the IHP effect on oxygenation appears unmodified. Thus, (a) Arg may substitute reasonably well for His in the interaction, and (b) the effect of the substitution is substantially reduced, and (b) in the oxygenation, an effect is observed only if there is a difference in the IHP binding mode between deoxy- and oxyHb, and this seems to be not true for His$^{240}$ (25, 24). The role of Lys$^{321}$ has not been taken into account in our analysis of the pH dependence simply because its $pK_a$ is much too high to come into play over the pH range investigated (25, 27), but its contribution to the free energy of IHP binding is probably a major one in determining the affinity for pH > 8.0.

The linkage relationship between proton and IHP interaction with human HbO$_2$ can be described in quantitative energetic terms by calorimetric measurements of the heat that accompanies binding of IHP at different pH values. In this way, information concerning $\Delta H$ of the interaction allowed us to attempt a correlation between (a) protonation of residues in IHP-free and IHP-bound HbO$_2$, (b) the free energy involved in the interaction, and (c) the entropic contribution to the binding process. Fig. 5 shows such relationships in the pH range between 6 and 8, from which it was concluded that (a) at pH < 7.0, the IHP binding is essentially enthalpy-driven ($\Delta H$ being strongly exothermic and pH-independent, with a value of $\approx -59$ kJ/mol), whereas the pH dependence of $\Delta G$ is completely attributable to the pH dependence of $\Delta S$, which is always positive for values of pH $\leq 7.0$; (b) at pH > 7.0 there is a progressive decrease of $\Delta S$, which becomes negative at pH > 7.4, accompanied by a decrease of the exothermicity of the process, which becomes endothermic at pH > 7.5. Therefore, at pH > 7.0, the entropy role in determining the affinity of IHP for HbO$_2$ becomes progressively predominant as pH increases, and a proton-linked enthalpy-entropy compensation comes into play in regulating the pH dependence of the free energy for IHP binding. Therefore, it appears as if two different interaction modes are operative in modulating the IHP binding, one predominating at pH < 7.0 and the other predominating at pH > 7.0. We must stress at this point that a previous calorimetric investigation of the interaction of IHP with HbCO at few pH values gave results fully compatible with ours, at least at the corresponding pH values (28).

Altogether, this behavior may be tentatively correlated with the $pK_a$ shifts reported above (see Table 1). Thus, at very alkaline pH (i.e. $\geq 8.0$), IHP interaction is not accompanied by any proton release or uptake, and it appears to be an endothermic process, displaying a negative $\Delta S$. As the pH is decreased toward 7.0, the three IHP-linked protonating groups take up protons when IHP interacts with HbO$_2$, increasing the $\Delta S$ (see Fig. 1B). The proton uptake during IHP binding also brings about a progressive decrease of the endothermicity, with a $\Delta H < 0$ at pH < 7.5, mirrored by a parallel increase of $\Delta S$, which becomes positive at pH < 7.4. (see Fig. 5). As the pH is
lowered below 7.0, the three IHP-linked protonating groups begin to take up protons in IHP-free HbO₂ as well, corresponding to a decrease of ΔZ (Fig. 1B). Such a process seems to affect the ΔS of IHP interaction, decreasing its positive value and thus increasing the ΔG of binding, whereas ΔH appears not to depend on the protonation of these groups in IHP-free HbO₂ (Fig. 5).

Therefore, it seems that the progressively increasing exothermicity of IHP interaction upon pH lowering indeed may be related to the heat released by the groups that take up protons when IHP binds (29). However, the pH-independent value of ΔH at pH < 7.0, over a range in which the groups are already protonated in IHP-free HbO₂ and the extent of IHP-linked proton uptake (i.e. ΔZ) decreases, seems to suggest that additional factors might come into play to determine the observed exothermicity of IHP binding at low pH, such as the electrostatic interaction between the positive charges of HbO₂ and the negative charges of IHP.

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