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Heterotropic effectors control the hemoglobin function by interacting with its T and R states—a new view on the principle of allostery

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Abstract

Careful analyses of precise oxygenation curves of hemoglobin (Hb) clearly indicate that, contrary to the common belief, allosteric effectors exert a dramatic control of the oxygenation characteristics of the protein by binding not only to the T (unligated), but also to the R (ligated) state, in a process that is proton-driven and involves proton uptake. The most striking functional changes were obtained when the allosteric effectors were bound to the fully ligated Hb: the oxygen affinity decreased dramatically, Bohr effect was enhanced, and cooperativity of oxygen ligation was almost absent, emulating a Root effect-like behavior. However, structural analysis, such as Cysβ93 sulfhydryl reactivity and ultraviolet circular dichroism, confirmed that the ligated Hb was in fact in the R state, despite its extremely low affinity state features. These findings provide a new global view for allosteric interactions and invoke for a modern interpretation of the role of allosteric effectors and a reformulation of the Monod–Wyman–Changeaux model for control of allosteric systems, and other complementary models as well.

Keywords: Human adult hemoglobin; Allostery; Allosteric effectors; Oxygen equilibrium curves; Bohr effect; Root effect

1. Introduction

Hemoglobin (Hb) is the paradigm par excellence among allosteric proteins. It is composed by two αβ dimers, each α- or β-subunit carrying a heme group to which a single molecule of oxygen (homotropic effector) binds. A close examination of the oxygen binding characteristics suggests the existence of a positive interaction between the heme sites called cooperativity. This phenomenon

Abbreviations: Hb, human adult hemoglobin i.e. HbA_0; MWC model, the two-state allosteric model proposed by Monod–Wyman–Changeaux; UV-CD, ultraviolet circular dichroism; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; BZF, bezafibrate; IHP, inositol hexaphosphate; 4-PDS, 4,4′-dithiopyridine.

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is manifested in a manner as if these binding sites are somehow 'communicated' to each other, so that when one site becomes ligated, the rest of hemes are more prone to undergo successive ligation. In addition to hemes, Hb has other binding sites to which other ligands (heterotropic effectors) such as protons, chloride, and 2,3-diphosphoglycerate, bind. These effectors operate as inhibitors, since they interact indirectly with the hemes by reducing the affinity for homotropic effectors. Wyman [1] established that the binding function of one component is related to the binding function of any other component by a thermodynamical relationship that he termed 'linked function'.

One of the most remarkable examples involving the combined interaction of homotropic and heterotropic effectors with Hb is the so-called (alkaline or normal) Bohr effect, in which the oxygen binding process is linked to the release of protons from Hb, and vice versa. Conversely, it can be said that an increase in the proton concentration will facilitate the release of oxygen from Hb due to a decrease in its oxygen affinity. This is because protons will bind preferentially to the unligated form of Hb. Binding of heterotropic effectors to Hb is also accompanied by an uptake of protons [2]; the number of protons that are uploaded onto Hb varies and depends on the nature of such an interaction. The effect of the binding of homotropic effectors (for instance, oxygen) to Hb was adequately interpreted according to the two-state model proposed by Monod et al. [3], and complemented by the stereochemical model of Perutz [4,5], and the Szabo–Karplus [6] and Szabo–Karplus–Lee models [7–9] with the inclusion of heterotropic effectors. The proposed rationale was that the stabilization of a constrained or T (tense) state in the unligated Hb was due to the formation of new salt bridges between these heterotropic effectors and the protein moiety, and extra H-bonds born out from the binding of additional protons to specific aminoacid residues (Bohr groups) in the protein. Comparative analysis between Hb in the T (tense) and R (relaxed) states had shown that these salt bridges were in fact broken in the latter state [4]. At this point, the T and R states were equated to the unligated and ligated forms of Hb, respectively. It has been postulated since then that in the presence of strong allosteric effectors and under conditions that favored their interaction, the stabilization of the T (unligated) state could be so great that the allosteric transition towards the R (ligated) state was greatly impaired, which translated in a decreased affinity for homotropic effectors, namely, low oxygen affinity. This T-like oxygen affinity behavior has been considered by many as the criterion for a T state, even in fully ligated species [10], without the corroboration for such a claim from structural data obtained by other techniques, such as NMR spectroscopy. It then followed that the stronger the interaction with the heterotropic effectors, the greater the T character of Hb, and thence the more enhanced the proton upload.

In the present work, we have conducted a thorough and systematic analysis of the oxygenation properties of Hb under a wide range of pH, in the presence of chloride, inositol hexaphosphate (IHP), bezafibrate (BZF), and a combination of IHP and BZF, and contrasted the results against stripped conditions. BZF is a rather new potent synthetic allosteric effector that binds to Hb to a different site from 2,3-diphosphoglycerate or IHP. This compound potentiates the effect of decreasing the oxygen affinity of Hb [11,12]. A careful analysis of the proton release upon stepwise oxygenation for each solution condition indicated that the proton binding capacity depended on the strength of the interaction with the effector and that this process was proton-driven. In addition, binding analysis hinted at the prospect that these allosteric effectors also influenced fully ligated Hb. Studies using structural probes for quaternary conformation, such as sulfhydryl reactivity of Cysβ93 towards 4,4′-dithiopyridine (4-PDS) and ultraviolet circular dichroism (UV-CD), clearly indicated that, contrary to what was previously assumed, allosteric effectors interacted not only with Hb in the T, but also in the R state in a superlative fashion, contradicting the prevailing tenet. These findings call for a new scrutiny of the accepted doctrine of current allosteric models for Hb.

2. Experimental

All reagents and buffers were of analytical grade or the purest degree available. IHP and BZF were from Sigma-Aldrich (St. Louis, MO), and were used without further purification.

2.1. Preparation of hemoglobin samples

Human adult Hb was purified from freshly outdated red cells, as described previously [13], except that the protein in the CO form was further stripped from any salt by dialysis against deionized water, passed through Amberlite MB-1 and then Sephadex G-25 in 10 mM HEPES, pH 7.4. The protein solution was then concentrated by ultrafiltration with an Omega 10 K disc membrane (Pall Filtron Corporation, Northborough, MA), and kept in the cold until use. CO-Hb was converted to its oxy form as described previously under a strong illumination under a stream of pure oxygen placed in a flask immersed in iced water. Complete CO removal was checked by spectrophotometry by the ability to be fully converted to the deoxy form upon oxygen removal. The Hb solution was then centrifuged at $10\,000 \times g$, and filtered with a disposable 0.2 μm HT Tuffryn membrane (Gelman, Pall Filtron Corporation) before use.

2.2. Oxygen binding studies

Oxygenation of Hb under a wide variety of solution conditions and presence of several heterotropic effectors was measured, as previously described [13], using a newly improved version of the Imai's automatic apparatus [14]. Before measurements were started, pure oxygen (grade 4.4) was flushed into the cell compartment containing the Hb solution to increase oxygen saturation. Gradual deoxygenation was then achieved by flushing a low stream of pure nitrogen (grade 5.0) onto the continuously stirred solution, and the changes in optical absorbance were monitored at 560 nm. All measurements were carried out at 15 °C to assure highest oxygen saturation of the samples possible and minimize oxidation of hemes. Sample concentration was 60 μM on heme in 0.1 M HEPES (pH range 6.6–9.0), containing no effectors (stripped condition), chloride (0.1 M), IHP (2 mM), or BZF (10 mM), or a combination of IHP and BZF (2 mM and 10 mM, respectively). Small amounts of catalase and SOD were added to the solutions to minimize metHb formation. Hb concentration and metHb content were determined before and after any oxygenation measurement. MetHb content was always kept to a minimum in the data used for analysis.

2.3. Analysis of oxygen equilibrium curves

Oxygen binding isotherms were analyzed by a least-square curve fitting method according to a 4-binding step Adair scheme, in which the partial saturation with oxygen, Y, is expressed as

$$Y = \frac{K_1 p + 3K_1K_2p^2 + 3K_1K_2K_3p^3 + K_1K_2K_3K_4p^4}{1 + 4K_1p + 6K_1K_2p^2 + 4K_1K_2K_3p^3 + K_1K_2K_3K_4p^4},$$

where p is the partial pressure of oxygen, and $K_1, \ldots, K_4$ are the intrinsic equilibrium association constants at oxygenation steps 1 to 4.

2.4. Reactivity of Cysβ93 sulfhydryl groups towards 4-PDS

A standard solution of 4-PDS was prepared by dissolving 10 mg in 10 ml of deionized water heated at 60 °C. The concentration of 4-PDS was calculated using the extinction coefficient of 16.3 $mM^{-1}\,cm^{-1}$ at 247 nm and pH 7.0. Two milliliters of 40 M on heme of oxyHb or CO-Hb in 0.1 M HEPES buffer of a specific pH value was placed in a quartz cuvette with an attachment for anaerobic conditions, containing a small stirring bar. Once the sample was equilibrated at 15 °C, an amount equivalent to 160 μM final concentration of 4-PDS was added into the cuvette and the reaction was monitored by the increase in absorbance at 324 nm using a Hewlett-Packard 8452A diode array spectrophotometer. For measurements with deoxyHb, pure argon was flushed into the cuvette at 4 °C until complete deoxygenation was achieved. The cell was then transferred to the temperature-controlled holder until it reached 15 °C, at which the reaction was started by adding a deoxygenated solution of 160 μM final concentration of 4-PDS using a gas-tight syringe.

The pH dependence of the reversible conversion of the product, 4-dithiopyridone, to its tautomeric form (absorbance peak at 286 nm increases under alkaline conditions) was also studied to estimate the total absorbance increase upon completion of the reaction over a wide pH range. For this, a solution of known concentration of the product compound (Sigma) was used.

2.5. UV circular dichroism

Circular dichroism was measured with an AVIV 62DS spectrophotometer (AVIV Instruments, Inc., Lakewood, NJ) with a temperature-controlled bath at 15 °C. Sample concentration was 0.5 mM on heme in the same buffer conditions used for oxygenation experiments.

3. Results

To assure the highest possible oxygen saturation of Hb, measurements were carried out at 15 °C in the presence of pure oxygen. Since most of the previous studies found in literature have been performed at 25 °C, and more importantly, were not carried out systematically in the presence of heterotropic effectors different from protons, we completed for this study the measurements thoroughly at this temperature and over a wide range of pH to facilitate comparison. Special attention was given to the study of stripped Hb, that is, Hb in the absence of any allosteric effector.

Oxygen equilibrium curves of Hb in the absence and presence of allosteric effectors are shown in Fig. 1 as Hill plots. The pH dependence of the oxygen affinity of Hb (expressed as $\log P_{50}$), and of its cooperativity (as n_{max}) are shown in Fig. 2a and b, respectively, in the absence and presence of allosteric effectors. As previously reported, the combination of BZF and IHP resulted in a potentiated effect. Under acidic conditions, they produced a stronger effect on the binding properties of Hb, that is, an extreme low affinity for oxygen and almost abolished cooperativity. A more detailed analysis of these measurements is shown in Fig. 2a–e, in terms of intrinsic association equilibrium constants K_i as a function of pH under different solution conditions. The allosteric effectors chosen for this study in ascending order of the magnitude of the effect they exert on the oxygenation properties of Hb were: protons $<$ chloride $<$ BZF $<$ IHP $<$ (BZF + IHP). Fig. 3A shows the intrinsic Bohr effect of Hb. The effect of pH was rather modest and affected primarily both K_1 and K_4 in a comparable magnitude, and seemed associated to an ionization of a group with an apparent pK_a of 7.4. Addition of chloride enhanced the proton effect by reducing values of K_1 to K_3 (Fig. 3B), while K_4 remained practically invariant. The major effect chloride exerted can be seen on K_3 values; they decreased more than one order of magnitude as pH was dropped from 9 to 6.6. Addition of BZF or IHP reduced even more K_1, K_2, and K_3 values; at pH 6.6 their values became almost indistinguishable from each other. K_4 was also affected at low pH. The most striking behavior was observed with the combined effect of BZF and IHP at pH 6.6. Although these two effectors separately showed considerable effects on the oxygenation properties of Hb, the effect when both were present was synergistic. At pH 6.6, the stepwise oxygen binding affinity of Hb reached the lowest affinity state ($P_{50} \sim 116$ torr). This was produced primarily by a strong diminishing effect on the K_4 value at this pH value, since K_1, K_2, and K_3 values reached practically a minimum in the presence of either BZF or IHP.

By inspecting the degree of symmetry (W) of the oxygenation curves obtained (Fig. 3f), it is clear that the strength of the effector(s) correlates well with the magnitude of the shift of the symmetry maxima towards the alkaline side. The stronger the depressing effects of the effector on K_i values, the more to the alkaline side the peak position of symmetry moved. Accordingly, the same increasing order in strength observed from Fig. 3a–e was reflected as a progressive shift to the right of the respective peaks seen in Fig. 3f.

It is also clear that in the absence of any other allosteric effector, protons did not seem to modulate in great extent the oxygenation characteristics of Hb. The P_{50} value only increased 2.7-folds when the pH was decreased from 9 to 6.6. In contrast, for the same pH drop, a substantial enhancement in P_{50} values occurred when allosteric effectors were present. The P_{50} increased 7,

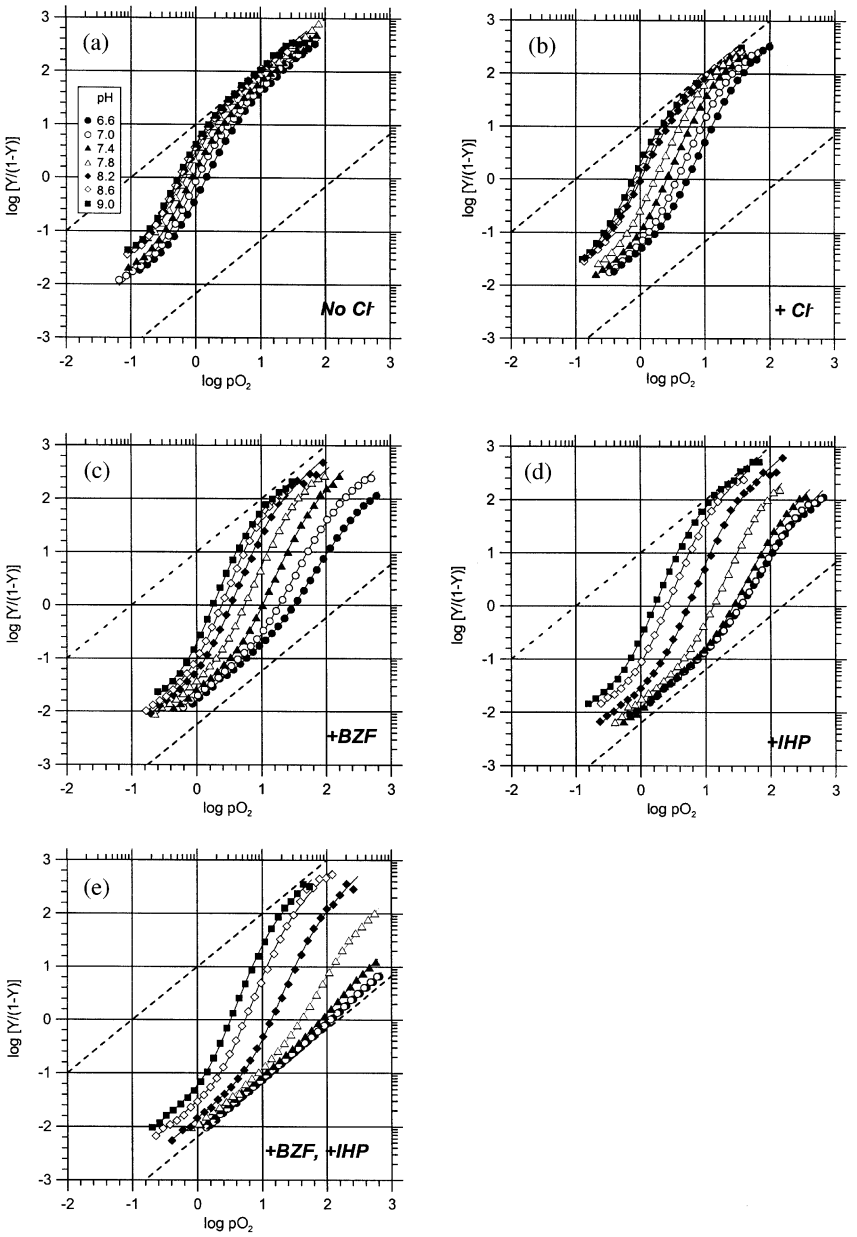

Fig. 1. The effect of pH on the oxygen equilibrium curves, as Hill plots, of Hb in the absence and presence of allosteric effectors. (a) Stripped Hb, no chloride; (b) in the presence of 0.1 M chloride; (c) in the presence of 10 mM BZF; (d) in the presence of 2 mM IHP; (e) in the presence of 2 mM IHP and 10 mM BZF. Conditions were: pH 6.6 (*closed circles*), 7.0 (*open circles*), 7.4 (*closed triangles*), 7.8 (*open triangles*), 8.2 (*closed rotated squares*), 8.6 (*open rotated squares*), and 9.0 (*closed squares*). Hb concentration was 60 μM on heme in 0.1 M HEPES buffer. Measurements were carried out at 15 °C. Plotted data correspond to one every three experimental points. Top and bottom dashed asymptotes correspond, respectively, to the highest (0.00706 $torr^{-1}$) and lowest (11.24 $torr^{-1}$) oxygen affinities experimentally determined.

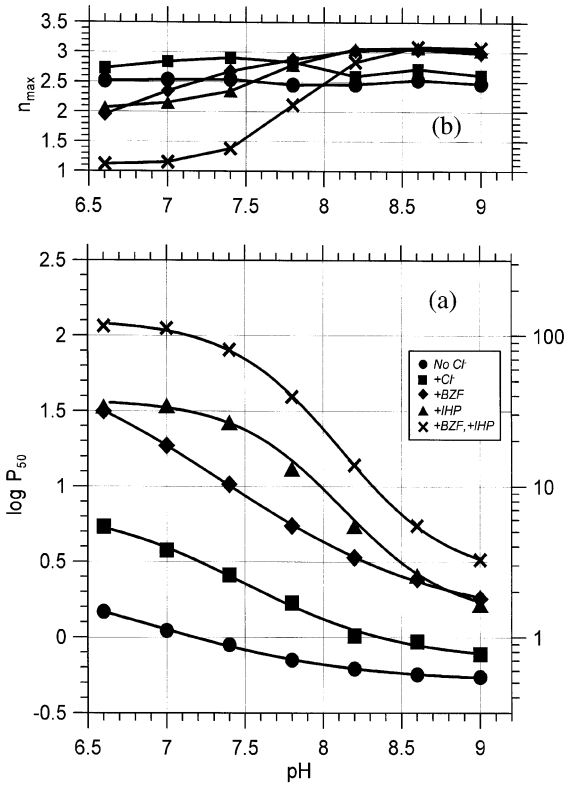

Fig. 2. pH dependence of the oxygen affinity, as log P_{50} (a); and cooperativity, as n_{max} (b); of Hb in the presence of allosteric effectors. Conditions were: no chloride (*circles*); with 0.1 M chloride (*squares*); with 10 mM BZF (*rotated squares*); with 2 mM IHP (*triangles*); and with 2 mM IHP and 10 mM BZF (*rotated crosses*). Hb concentration was 60 μM on heme in 0.1 M HEPES buffer. Measurements were carried out at 15 °C.

18, 20 and 35 times when chloride, BZF, IHP and (BZF + IHP) were added, respectively. From Fig. 3a–e is evident that this is mainly due to a dramatic drop in K_4 values. At pH 6.6, the combined effect of BZF and IHP decreased K_1 approximately 6 times, while K_4 was reduced 406 times.

A stepwise analysis of the Bohr proton release is shown in Fig. 4a–e. The maximum Bohr effect was achieved when IHP and BZF were present, and 4.7 protons were released per tetramer. This is a substantial increase from the maximum of 1.4 protons at pH 7.4 observed for the case of stripped Hb. By comparing Fig. 3 and Fig. 4, a general picture comes to light: the stronger the depressing effect of effectors on the oxygenation properties of Hb upon pH drop, the more enhanced the release of Bohr protons. When IHP was not present, the peak of Bohr protons was centered at physiological pH. However, when IHP was present, the peak position shifted to pH 8.5.

Interestingly, in the case of stripped Hb, the release of protons was practically suppressed at alkaline pH (<0.18 protons per tetramer above pH 8.2). Yet, the molecule of Hb underwent complete allosteric transition upon oxygen ligation ($\Delta G_{41} = 2064 \pm 94$ cal mol^{-1}), while exhibiting relatively high cooperativity at any pH studied ($n_{max} \sim 2.5$).

The exaggerated impairment of the oxygen affinity on Hb at acidic pH in the presence of BZF and IHP certainly reminds the Root effect found in some fish Hbs. This effect has been explained in terms of the Monod–Wyman–Changeaux (MWC) model as the pronounced shifting of the T↔R allosteric equilibrium towards the T (low affinity) state produced by the interaction with allosteric effectors, in this case, BZF and IHP [10]. Therefore, we investigated whether this assumption held water by inspecting the quaternary conformation of Hb, such as the sulfhydryl reactivity of the Cysβ93 towards 4-PDS, as well as the circular dichroism characteristics in the absence and presence of BZF and IHP. Fig. 5 shows the results from the sulfhydryl reactivity for oxyHb (a) and CO-Hb (b) towards 4-PDS. In both cases, the presence of BZF and IHP at acidic pH produced a slight decrease in their reactivities when compared to the case when the effectors were absent, but they were definitively above those typical for deoxyHb. Fig. 6 shows the circular dichroism of Hb in the presence of BZF and IHP at pH 6.6 in the UV region. At this pH value, the effects of BZF and IHP over Hb are strong. The ellipticity values for the oxyHb, CO-Hb, and even air-equilibrated Hb were clearly different from that for deoxyHb (characterized by a large negative trough approx. 284 nm). These results are in total conflict with the commonly accepted notion that fully ligated Hb in the presence of strong effectors is in T state [10], but are fully consistent with our results obtained from sulfhydryl reactivity and indicate that the ligated forms of Hb in the pres-

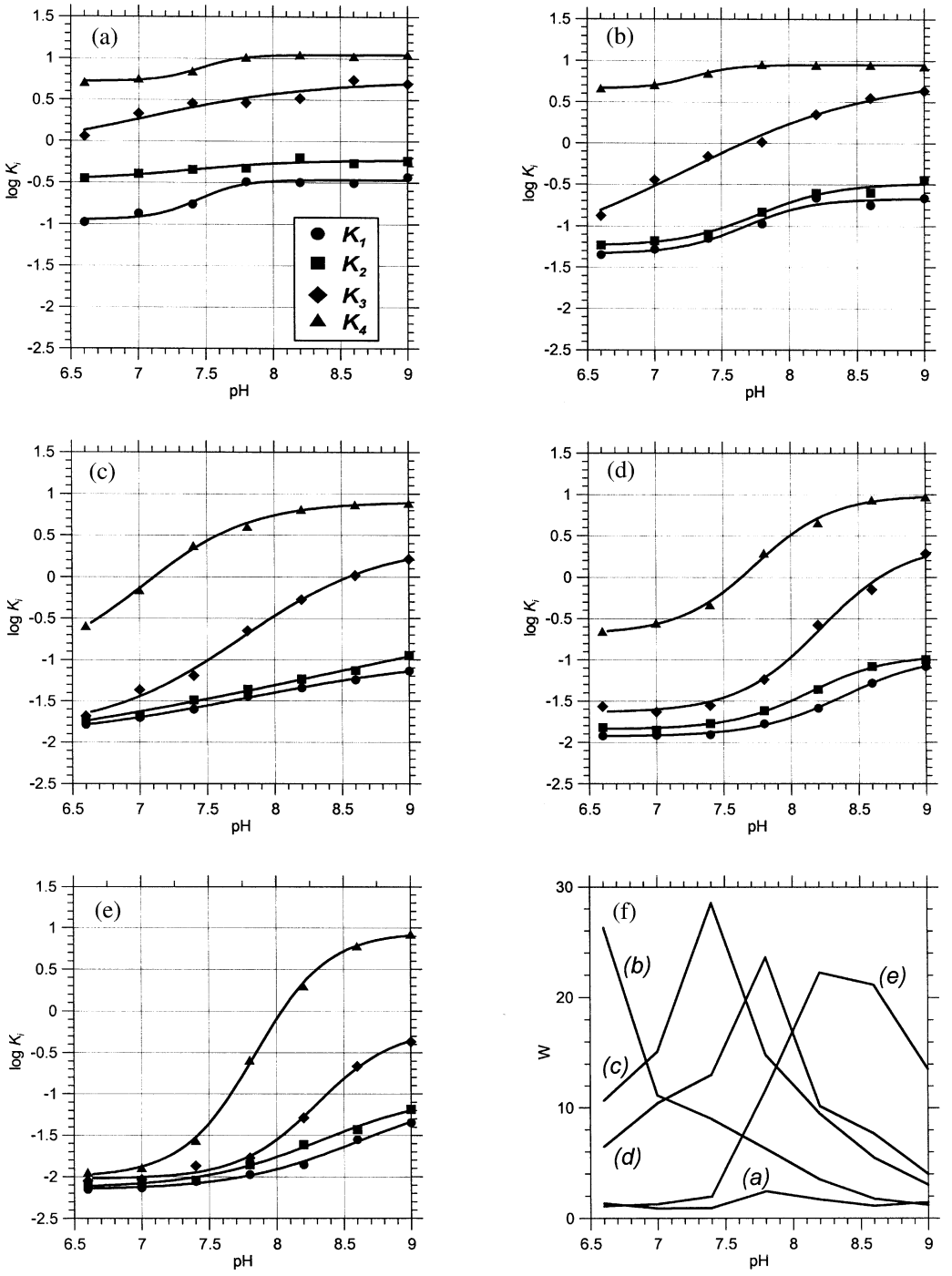

Fig. 3. Effect of pH on the intrinsic oxygen association equilibrium constants, K_1 to K_4, of Hb in the presence of several allosteric effectors. K_1 (*circles*); K_2 (*squares*); K_3 (*rotated squares*); K_4 (*triangles*). (a) Stripped Hb, no chloride; (b) in the presence of 0.1 M chloride; (c) in the presence of 10 mM BZF; (d) in the presence of 2 mM IHP; (e) in the presence of 2 mM IHP and 10 mM BZF. Hb concentration was 60 μM on heme in 0.1 M HEPES buffer. Measurements were carried out at 15 °C. Inset (f) shows the pH dependence of the symmetry index, *W*, expressed as (K_1K_4/K_2K_3) for each set of measurements. Solution conditions were: no chloride (a); with 0.1 M chloride (b); with 10 mM BZF (c); with 2 mM IHP (d); and with 2 mM IHP and 10 mM BZF (e).

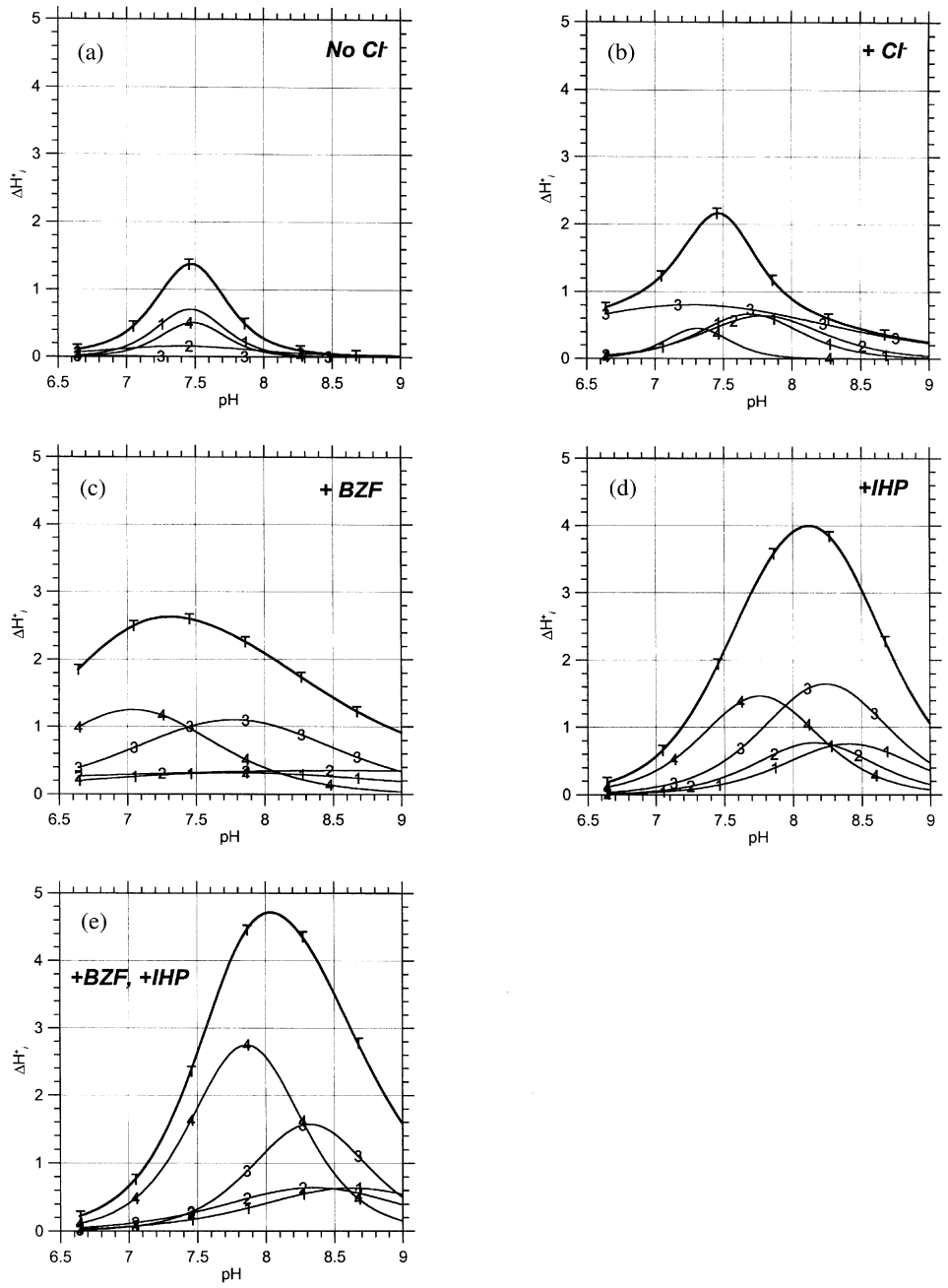

Fig. 4. pH dependence of the release of Bohr protons upon stepwise oxygen binding in the presence of several allosteric effectors. The number on each curve corresponds to the oxygenation step in which the protons are released. The total number of Bohr protons is indicated by the curve with T, and is equivalent to the sum of the all other four curves. (a) Stripped Hb; no chloride; (b) in the presence of 0.1 M chloride; (c) in the presence of 10 mM BZF; (d) in the presence of 2 mM IHP; (e) in the presence of 2 mM IHP and 10 mM BZF. Curves were calculated from the parameters obtained from the oxygenation curves shown in Fig. 1.

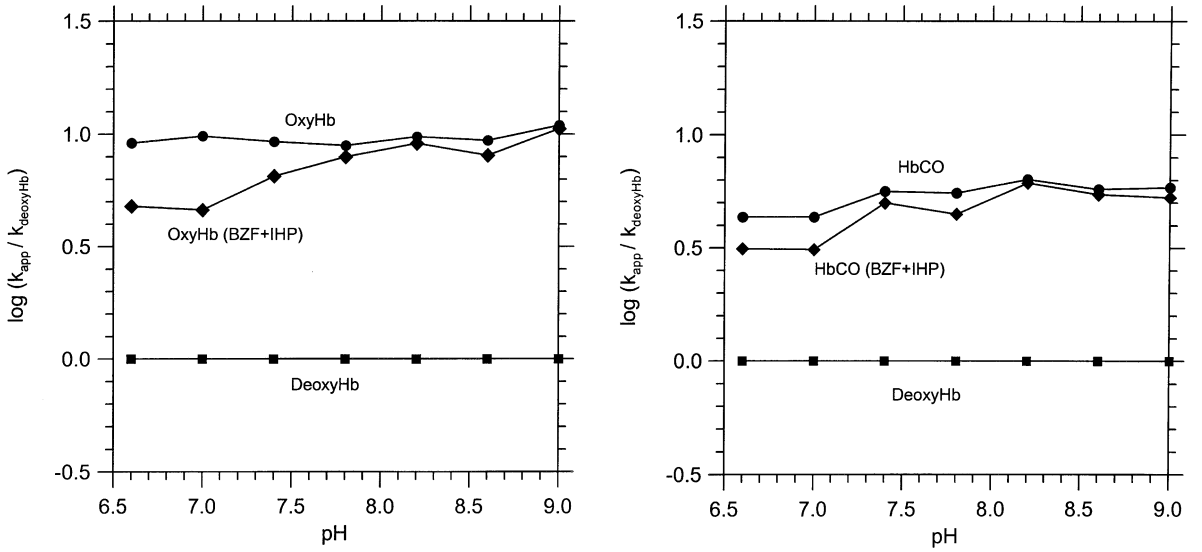

Fig. 5. Effect of the presence of strong allosteric effectors, IHP and BZF, on the pH dependence of the reactivity of the sulfhydryl residue from Cysβ93 towards 4-PDS from some ligation species of Hb. (a) The ligated species is oxyHb; (b) the ligated species is CO-Hb. Ligated Hb species under stripped conditions (*circles*); unligated (deoxy) Hb under stripped conditions (*squares*); ligated Hb species in the presence of IHP and BZF (*rotated squares*). Sample concentration was 40 μM on heme, in 0.1 M HEPES, containing 2 mM IHP and 10 mM BZF, at 15 °C.

ence of BZF and IHP at acidic pH, despite their extremely low oxygen affinity, are unquestionably different from the T state. In sum, the assumption that the quaternary conformation of Hb in the presence of strong allosteric effectors is that of the T structure seems to be inconsistent with our results. Moreover, our conclusions are totally consistent with those obtained from CD measurements at neutral pH previously reported [12], and are valid over a wide pH range, even in the acidic side where the effects of IHP and BZF are stronger.

4. Discussion

Analyses of the oxygen equilibrium curves measured for the present paper were carried out according to the Adair scheme. The purpose was deliberate because this provides a model-independent description of the ligation properties of Hb and therefore no previous assumptions are to be made on how the ligation in Hb proceeds.

Our results for stripped Hb (Figs. 1–3a, and Fig. 4a) clearly show that in the absence of allosteric effectors, the oxygenation process of Hb, or more precisely, the allosteric transition from a low to a high affinity state seems not to be strictly and necessarily linked to the release of protons, in the so-called alkaline Bohr effect. Stripped Hb showed an almost constant cooperativity ($n_{max} \sim 2.5$) over the entire pH range studied. Despite the increase of 250 times in proton concentration (corresponding to a pH change from 9 to 6.6), the oxygen affinity increased only 2.7 times. At pH 9, the total Bohr protons released upon full oxygenation of stripped Hb were merely 0.01 per tetramer (i.e. approx. 0.7% of the maximum number released at pH 7.4). Yet, the molecule was capable of undergoing a normal T→R transition with relatively high cooperativity. The release of Bohr protons approximately pH 7.4 is thought to be due to a difference between release and uptake of protons upon oxygenation. This is consistent with the original treatment of the alkaline Bohr effect on Hb [15] in which two hypothetical ionizable residues could be linked to each ligation state on the alkaline side (pH >6). In essence, all K_i values remained practically constant over the entire pH range studied, and all oxygen-

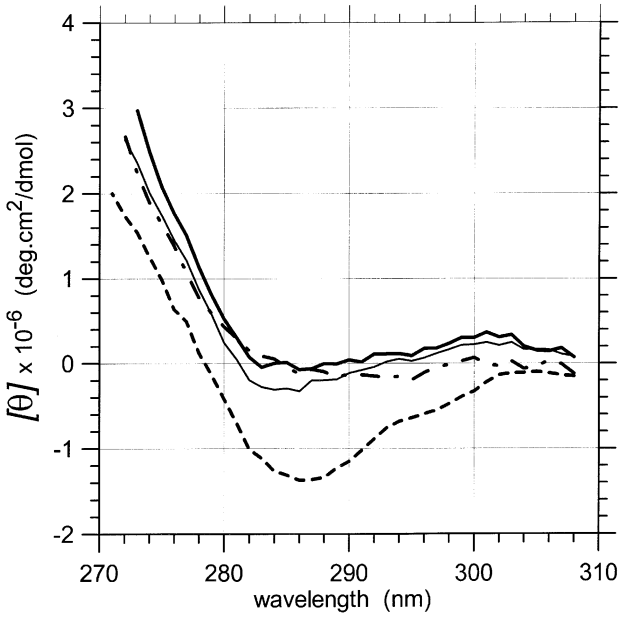

Fig. 6. Effect of the presence of strong allosteric effectors, IHP and BZF, on the circular dichroism characteristics of Hb ligation species in the UV region. OxyHb, (*solid broad line*); CO-Hb, (*solid fine line*); deoxyHb, (*broken line*); Hb equilibrated with air at atmospheric pressure, (*dot-and-dash line*). Sample concentration was 0.5 mM on heme, in 0.1 M HEPES, containing 2 mM IHP and 10 mM BZF, at 15 °C. Measurements were performed with an anaerobic quartz cuvette with 2 mm lightpath length.

ation curves showed considerable symmetry ($W = 1.4 \pm 0.5$). Perhaps most of the inconsistencies found in the study of the Bohr effect in the past can be attributed to the arbitrary use of solutes and buffers. From the very beginning, chloride, with no doubt, has been the most extensively used anion [16–19]. To complicate things even more, HCl was used to adjust the pH of common buffers like bis-Tris or Tris, introducing in this way chloride into the solutions in variable amounts. To standardize results, the reasoning that the presence of chloride (usually 0.1 M) helped to avoid the non-specific binding of other effectors such as 2,3-diphosphoglycerate, was tacitly adopted. The presence of allosteric effectors in the presence of chloride clearly showed the existence of a linkage between oxygenation, proton release, and decrease in the oxygen affinity in Hb, but then the interactions of these effectors were apparent, since the competitive effects of chloride were also included.

As allosteric effectors come to scene, the release of Bohr protons upon ligation is enhanced in a fashion that correlates with the magnitude of their interaction with Hb. Since the effect we observed in the case of stripped Hb was rather modest, the primary role of protons is to facilitate Hb to interact with these effectors, rather than directly with Hb itself. This was previously interpreted in terms of the formation of additional bonds (salt bridges and H-bonds) at molecular level between these molecules and Hb [5]. Therefore, the most plausible explanation for the decrease in oxygen affinity caused by the interaction between effectors and Hb was that these effectors interacted by stabilizing the unligated form, namely, the T state of Hb. In terms of the MWC model this implies that the effectors would shift the allosteric equilibrium, L, to become even larger, since $L = [T]/[R]$, where [T] and [R] are the number of Hb molecules in the T and R state, respectively.

However, as the strength of the effectors increased, K_4 values were unquestionably affected. Now, the contention that the strength of the interaction between the effector and Hb in the T state is such that the transition to R is shunned i.e. the Hb molecule remains in the T state even after full ligation or T_4, is not consistent with the structural data, namely, sulfhydryl reactivity of Cysβ93 and UV-CD studies presented in this paper (Figs. 5 and 6), and refutes the notion that $L \rightarrow \infty$ as [T] progressively increases over a decreasing population of [R]. Additional structural evidences, including NMR data, supporting our contention has been also given by Yonetani et al. [37]. Fully ligated Hb in the presence of the combined effect of IHP and BZF at low pH is unequivocally that of the R state, yet the oxygenation properties are those of a typical low affinity species. This finding confirms previous results obtained at neutral pH [12]. From this finding two important corollaries can be inferred: (1) the Hb functions are primarily modulated by allosteric effectors; this process takes place even after full ligation (R state) and is facilitated by protons; and (2) the interaction with allosteric effectors with Hb, either in the T or R state, is accompanied by proton uptake in a quan-

tity that is proportional to the affinity of the effector for Hb.

If we compare the curves shown in Fig. 4 corresponding to total number of Bohr protons released in the presence of effectors, we notice that the increasing part (acidic side) of the curves going from acidic to alkaline begin at much higher values and adopt a much sharper positive slope as the strength of the effector increases. This implies that as much as the effector uploads protons upon interaction with Hb in the T state (totally unligated state) on grounds of the stereochemical explanation given by Perutz [4,5], it also uploads protons as the transition to the R state is completed. The large release of protons observed in Fig. 4 is due to a net difference between the hypothetical pK_a values of the Bohr groups associated with the T (fully unligated) and R (fully ligated) states. The number of observed released protons is then an indirect evidence of the interaction between effectors and Hb in T and R state, which originate from a mismatch in the rebinding of the released protons upon full ligation in the pH range that corresponds to the peak. If Hb did not bind allosteric effectors in the acidic side of the curve, then the net number of released protons on this side (the difference with respect to the peak) should show a decrease comparable to that observed in the case of stripped Hb. In the case of BZF+IHP, it is noticeable that the interactions with Hb are more pronounced below pH 8.2 than above this value; the number of protons released decreases rapidly as pH is dropped, indicating that protons are taken more actively as pH is decreased and, as a result, an enhanced interaction with effectors is expected in this pH region. Careful inspection of the oxygenation characteristics of Hb with these effectors (Fig. 2) confirms this prediction.

Perhaps the most dramatic finding from the present work is the fact that allosteric effectors do bind to Hb in the R (fully ligated) state and consequently alter its oxygenation functions. To facilitate comparison, oxygenation parameters obtained according to the Adair analysis were translated into terms of the MWC model by means of the appropriate conversion equations. The following approximations were taken: $K_1 \approx K_T$, $K_4 \approx K_R$, $L_0' = (K_4 P_{50})^4 \approx L_0$, and $L_4' = L_0'(K_1/K_4)^4 \approx L_4$. Results from the analysis were plotted as log L_i' vs. log K_i, as shown in Fig. 7. Strikingly, this plot matches the corresponding part of that obtained from analyzing the data according to the MWC equation (see [37], and also Imai et al. in the present issue), except that in our case chloride was present in only one set of measurements ($+Cl^-$). Since the effects of chloride are much reduced than those exerted by BZF or IHP, both plots look almost indistinguishable from each other. In this plot, each oxygenation curve is described by two points, one for the initial oxygenation state (log L_0', log K_1) and the other for the final state (log L_4', log K_4). In the absence of any allosteric effector except protons, data points cluster in two areas (circumscribed to both circles with solid line

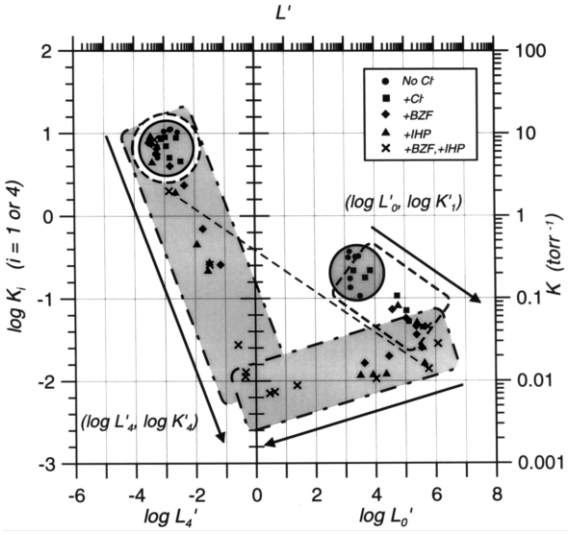

Fig. 7. Global allosteric behavior of Hb, expressed in terms of the log L' vs. log K plot, in the absence and presence of allosteric effectors over a wide pH range. Paired data points (log L_0', log K_1) and (log L_4', log K_4) were calculated for each oxygenation curve obtained under several solution conditions and previously analyzed according to the Adair scheme (see text for details). As an illustration, a dashed line interconnecting two of such points is shown (for pH 7.4, in the presence of 2 mM IHP and 10 mM BZF). Arrows indicate the direction of the shift of paired points as pH decreases. Conditions were: no chloride (*circles*); with 0.1 M chloride (*squares*); with 10 mM BZF (*rotated squares*); with 2 mM IHP (*triangles*); and with 2 mM IHP and 10 mM BZF (*rotated crosses*). Hb concentration was 60 μM on heme in 0.1 M HEPES buffer. Measurements were carried out at 15 °C.

in Fig. 7). The reason is that neither K_1 nor K_4 values varied much with pH change. This condition truly satisfies the original MWC model in which K_T, as well as K_R, are constant and corresponds to the ideal paradigm for the model. As chloride is introduced, a shift on the right extreme of the plot is observed (bottom right end in the rectangle with dashed lines), indicating that now K_1 values are affected and decrease progressively. The arrow indicate the direction of the shift due to increasing acidic conditions. In the presence of BZF, IHP, or a combination of both, L_0' reaches a maximum value of approximately 1.2×10^6, which corresponds roughly to the ratio between T_0/R_0. Interestingly, an additional increase in the acidic condition prevents L_0' from increasing further but instead diminishes progressively as pH drops. At the same time, L_4' values are also affected and simultaneously begin to increase from a minimum value of 3×10^{-3} (top left end on Fig. 7). While K_1 values varied from 0.3689 to 0.00706 $torr^{-1}$ (approx. 50-fold decrease in affinity), those for K_4 changed from 11.24 to 0.01128 $torr^{-1}$ (approx. 1000-fold decrease). Thus it can be said that the oxygen affinity of Hb can be modulated in such a superlative manner because allosteric effectors interact also with Hb in the R state.

As acknowledged by other authors, a common pitfall in the measurement of oxygenation curves has been the unreliable estimation of K_4 due to the decreased affinity of Hb in the presence of strong allosteric effectors [10], as the sample could not get fully saturated even in the presence of pure oxygen. Thus the anticipated contention to our work might be how reliable the estimation of the partial saturation with oxygen of Hb was in the presence of strong effectors, since 100% ligation could not be achieved. Concerns about the reliability of our calculated K_4 values from oxygenation experiments can be ruled out. The total change in optical absorbance due to the transition from fully oxy to fully deoxy forms of Hb can be easily determined in the absence of effectors. The validity of any oxygen binding measurement technique relies on the condition that effectors do not affect directly the optical absorbance of Hb, especially in the visible region where the monitoring takes place, in any other way than by affecting its degree of saturation with oxygen. Favorably, this was the case for BZF + IHP. Therefore, if the Hb concentration in the solution is known, the estimation of oxygen saturation becomes a trivial matter. As an additional precaution, the temperature of the measurements was dropped to 15 °C to increase oxygen saturation, and a strict and systematic measurement per solution condition was carried out, as seen in Fig. 1. The results show that in fact, K_4 values are decreasing not because of an apparent increase in levels of unsaturated Hb. The most challenging condition was when BZF and IHP were present at pH 6.6. The oxygen saturation of Hb in the higher end was approximately 80%. Nevertheless, the obtained oxygenation isotherm described the typical behavior of a low oxygen affinity, non-cooperative system. After analyzing the distribution of ligation species at 80% saturation, we found that 80% of the species present in solution were ligated with three or four oxygen molecules (not shown). This finding suggested that even when full saturation with oxygen was not achieved, a high percentage of species were ligated and the oxygen binding experiments should have reasonably reflected the functional characteristics of the sample, namely, an extremely low affinity for oxygen.

On a more technical aspect, our measurements were conducted with an improved version of the Imai's machine, in which a new low-noise oxygen electrode and custom-made pre and main amplifiers were used. This setting allowed reliable measurements at low, as well as high oxygen tensions, in a continuous manner with ease. This method shows obvious advantages over different measurement techniques employed by others.

In parallel to the Adair analysis, a full MWC analysis, in which K_T, K_R, and L_0 were allowed to float, was conducted on each oxygenation curve we obtained, in a similar manner as indicated by Imai et al. (see article in present issue). For this analysis, we did not input arbitrary or rather convenient values of L_0, as some studies apparently have done in the past. On the contrary, L_0 values were obtained altogether with K_T and K_R from each curve fitting. As a result, a practically identical distribution as those data points shown in Fig. 7 was obtained when data points corresponding to ($\log L_0$, $\log K_T$) and ($\log L_4$, $\log K_4$) were plotted

(not shown). This fact assures the quality of our experimental data, and the validity of the approximations we previously assumed. In sum, variations of K_1 as well as K_4 values (and, as an extension, those of L_0, K_T, L_4, and K_R) due to solution conditions were indeed factual, irrespective of the numerical methods we employed to analyze the oxygenation curves.

In apparent contrast to the oxygenation experiments, sulfhydryl reactivity of Cysβ93 towards 4-PDS and UV-CD experiments (present work), and NMR measurements [37] clearly indicate that ligated Hb adopts the R state even in the presence of strong effectors. Recently, conclusive evidence was obtained from the X-ray crystallographic analysis of the CO-ligated form of Hb in the presence of IHP and BZF, carried out by some of us; the precise location of the binding site for BZF in ligated Hb was elucidated. The molecule of this effector rests on the E-helix of the α-subunit with its chloride group interacting with the edge of the porphyrin ring (unpublished results). This fact demonstrates unequivocally that BZF interacts with totally ligated Hb (R state) by binding to the external surface of the α-subunits, that is, two molecules of BZF are attached to one ligated Hb tetramer.

Our results show that those results that are in accordance with the original MWC model constitute only a fragment of the total view of the Hb physiology. Thus in order to understand the entire set of functional features of Hb under a global view, a serious and thorough reexamination is essential at this point, especially, those aspects involving interactions between allosteric effectors and Hb in the R state. The functional and structural evidences presented in the present work do not invalidate completely previous conclusions. However, they attempt to redefine the notion that strong heterotropic effectors impede Hb from switching to the R state. This challenges the concept universally accepted that in all cases Hb in the R state has high affinity for the ligand (or Hb in the T state has low affinity for the ligand). With no doubt, the contradiction between our functional (oxygen binding) and the structural (sulfhydryl reactivity, UV-CD) studies originate from the above-mentioned equivalence: R state always implies high affinity state. Instead, the synonym that we favor for the R state is 'ligated' form, and for the T state, 'unligated' form.

The present findings should also shed new light on several issues that remained exotic or foreign to the human Hb physiology. The effects found with BZF+IHP on human Hb remind those of fish Hbs featuring the so-called Root effect (refer to [20] for a review), for which several attempts have been made to explain the origin of such an effect at molecular level [21–25]. It is possible that the secret to the origin of the Root effect does not rely completely on the amino acid sequence per se of the Hb, but in the way this Hb interacts with specific effectors in both the T and R states. That is, the key element relies primarily on the effectors and their interaction with both the fully unligated and fully ligated forms of these fish Hbs, so that under acidic conditions these effectors are able to modulate more efficiently the oxygen affinity of the Hb [37]. Among mammalian Hbs, the case of rat Hb merits further analysis. Three of the Hb components react with IHP to show a behavior similar to that of the Root effect [26]. Remarkably, when compared to human Hb, all the aminoacid substitutions found in rat Hb are located on the external surface, thus making inapplicable the stereochemical explanation provided by Perutz [4], as this does not consider the interactions of heterotropic effectors with Hb in the R state, nor involves any type of interaction on the surface of the Hb molecule. This observation certainly hints a new mechanism of action. Another example among mammalian Hbs is given by the dromedary Hb [27] in which two oxygen-linked binding sites either for 2,3-diphosphoglycerate or IHP with high affinity for the oxy form have been reported to be present. Perhaps a similar effect might take place and be physiologically relevant to the Hb from related camelids from South America adapted to high altitude.

Several articles previously published by others have already indicated the binding of chloride [28] and organic phosphates, such as 2,3-diphosphoglycerate or IHP, to Hb in the R state [29–34]. Some even suggested that this binding was functionally significant and could not be neglected [32]. In all, minimal attention was then paid,

maybe because: (1) the results reflected the existence of weaker non-specific binding sites in the R state; (2) the results were sometimes conflicting because of differences in solution conditions; or (3) simply this kind of interaction violated the core principle of the MWC model. Others, on the other hand, pointed out the physiological significance of the invariance of K_4 as an optimal condition for the role of 2,3-diphosphoglycerate on oxygen transport [35,36]. However, as demonstrated recently, this does not always hold true [37]. Upon a change in pH from 9 to 6.6, K_R values of Hb in the presence of 2,3-diphosphoglycerate decreased about one order of magnitude.

If we concurred in that the pay of the energy toll required for a transformation in the oxygenation characteristics in Hb is greatly defrayed by the interactions between this molecule and its allosteric effectors, then the role of the allosteric transition as known to date should be reformulated, as these interactions become important and consequently should deserve attention. As shown in the present study, large functional changes driven by the energy of interaction between heterotropic effectors and the fully ligated Hb do take place and constitute the other aspect of allostery that have been long overlooked.

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