Effectors of hemoglobin

Separation of allosteric and affinity factors

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ABSTRACT The relative contributions of the allosteric and affinity factors toward the change in p50 have been calculated for a series of effectors of hemoglobin (Hb). Shifts in the ligand affinity of deoxy Hb and the values for 50% ligand saturation (p50) were obtained from oxygen equilibrium data. Because the high-affinity parameters (liganded conformation) are poorly determined from the equilibrium curves, they were determined from kinetic measurements of the association and dissociation rates with CO as ligand. The CO on-rates were obtained by flash photolysis measurements. The off-rates were determined from the rate of oxidation of HbCO by ferricyanide, or by replacement of CO with NO. The partition function of fully liganded hemoglobin for oxygen and CO is only slightly changed by the effectors.

Measurements were made in the presence of the effectors 2,3-diphosphoglycerate (DPG), inositol hexakisphosphate (IHP), bezafibrate (Bzf), and two recently synthesized derivatives of Bzf (LR16 and L35). Values of p50 change by over a factor of 60; the on-rates decrease by nearly a factor of 8, with little change in the off-rates for the liganded conformation. The data indicate that both allosteric and affinity parameters are changed by the effectors; the changes in ligand affinity represent the larger contribution toward shifts in p50.

INTRODUCTION

The characterization of hemoglobin (Hb) requires the separation of the different states in equilibrium. Hb can bind four ligands such as oxygen or CO, but does so with at least two different affinities. Two allosteric forms, called R and T in the two-state (MWC) model (Monod et al., 1965), are sufficient for most conditions, although the addition of effectors such as 2,3-diphosphoglycerate (DPG) or inositol hexakisphosphate (IHP) indicate that additional states may be needed to completely describe the system (Minton and Imai, 1974; Imai, 1982; Kister et al., 1987*a* and *b*). Thus one problem is to determine whether these effectors simply shift the equilibrium between the original allosteric states (without effectors) or change the intrinsic ligand affinity, implying the need for additional allosteric states.

The separation of the allosteric and affinity effects requires the determination of the affinities for the two allosteric states, K_R and K_T, and the allosteric parameter L which gives the equilibrium ratio T/R for the deoxy form. The oxygenation curves for various effectors are shown in Fig. 1. If two curves are parallel, they differ by a change in the R- and T-state ligand affinities by the same factor, with no change in L; this would be a pure affinity effect. At the other extreme, if the two affinities remain unchanged and L is different, then only the ligation level for the transition point between the original two forms changes; this would be a pure allosteric effect. As seen in Fig. 1, addition of effectors to Hb results in shifts involving a mixture of these two cases. Furthermore, when the effector is so strong that the substates involved (triply and fully liganded) are not fully in the R state, then the observed upper asymptote no longer gives the true R-state affinity.

The low affinity of the T-state is generally well determined from the equilibrium data. The three-dimensional plot in Fig. 2 shows how sigma, the least squares difference between the data and simulation, depends on K_T and L. The bowl-shaped form shows a well-defined minimum, within narrow limits of the two parameters. Deviations from this minimum in any direction result in a rapidly increasing error. A maximum value for sigma was used which generates the plane cutting the plot.

The same plot for K_R and L, Fig. 3, shows a broad minimum in a valley of many possible solutions; there is a compensation between the R-state affinity and L. This is the most optimistic view, because values for the Hill plot $\log[Y/(1 - Y)]$, rather than Y, were used to calculate the error. This gives a much higher weight to the asymptotes which influence the value for the affinities. The minimum was broader when Y was used.

The situation is worse for the case of strong effectors, as seen in Fig. 4; the valley of equally good solutions is much larger and is not sensitive to the weighting method. There is a family of solutions for K_R and L with no upper limit on these parameters, as reported by DiCera et al. (1987*a*). R-State properties are difficult to determine because the

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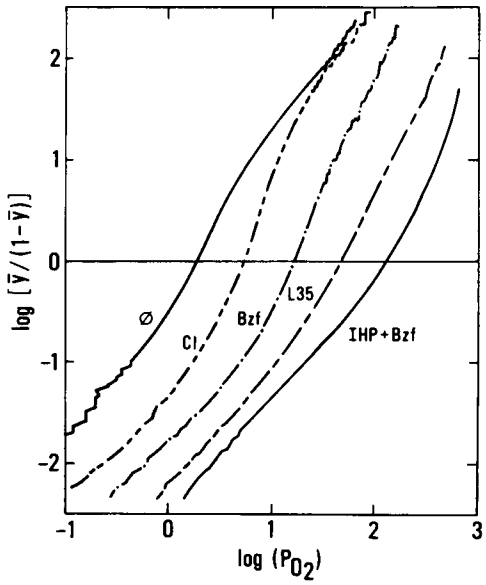


FIGURE 1 Oxygen equilibrium curves of Hb in the presence of the effectors indicated at pH 7.2, 25°C. Other experimental conditions are given in Table 1.

triply and fully liganded forms have a T-state contribution.

There are also experimental problems such as the normalization factor (Marden et al., 1989) and the fact that even under one atmosphere oxygen the sites are not fully saturated. We conclude that the R-state affinity cannot be deduced from the equilibrium curves, even within a factor of 10 for many cases.

Kinetic measurements of the ligand on and off-rates can provide an independent measure of the affinity. This method still has some problems for oxygen due to an incomplete ligand saturation with strong effectors. The

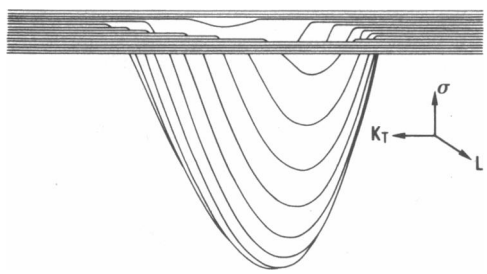


FIGURE 2 Plot of sigma, the least squares error between the data and simulation, as a function of log(K_T) and log(L). Data are the oxygen equilibrium curve for Hb at pH 7.2, 25°C, 100 mM NaCl (second entry in Table 1). Total variations were from 13 to 83 mmHg for K_T (left to right) and from 4.3 to 5.7 for log(L) (back to front) with K_R = 0.3 mmHg. The bowl shape shows a well-defined minimum for the simulations.

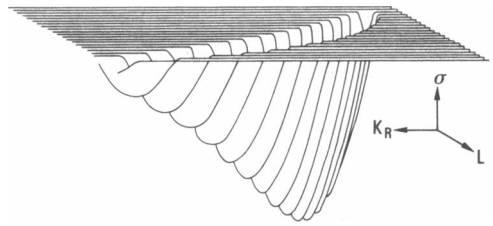


FIGURE 3 Sigma (as in Fig. 2) as a function of log(K_R) and log(L). The broad minimum shows some compensation between the two parameters. Variations were from 0.1 to 1 mmHg for K_R (left to right) and from 3 to 7 for log(L), with K_T = 25 mmHg. Sigma was calculated from the values used to generate the Hill plot (log[Y/(1 − Y)], Fig. 1), which weights the asymptotes more heavily than using Y (fractional saturation) and sharpens the minimum in sigma.

higher affinity of Hb for CO, by a factor of ~250 relative to oxygen, permits preparation of 100% saturated samples. In addition the R- and T-state on-rates are well resolved for CO (factor of 30 as compared to a factor of 3 for oxygen); thus even if there are substantial amounts of T state tetramers present, a clear R-state kinetic signal can be observed. For these reasons, the kinetic parameters were determined for CO.

In addition we made measurements to show that the partition function for the two ligands is little changed by the effectors. This allows the shift in the R-state affinity due to the effector to be considered the same for oxygen and CO. Using the affinity for the first ligand from the equilibrium curves as for the T state, and the kinetically determined affinity (for the fourth ligand) for the R state, we can separate the affinity and allosteric contributions to the shift in p50.

MATERIALS AND METHODS

Hb A was prepared from fresh red blood cell hemolysate by chromatography on a DEAE-Sephadex column. Hb was diluted in a buffer containing 100 mM NaCl and 50 mM bis Tris (Sigma Chemical Co., St. Louis, MO) at pH 7.2, 25°C. Experiments in the absence of chloride were made in 10 mM Hepes buffer adjusted with KOH to pH 7.2. Because the addition of strong effectors may change the solution pH, the

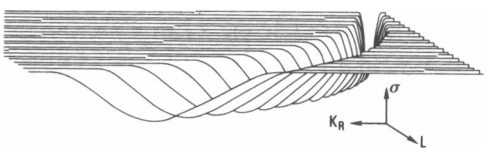


FIGURE 4 Sigma as a function of log(K_R) and log(L) for Hb with both IHP and L35 as effectors. Variations were from 1 to 25 mmHg for K_R and from 5 to 9 for log(L) with K_T = 300 mmHg. No minimum was found. There is a family of solutions of K_R and L.

samples were adjusted to pH 7.2 after addition of the effector. Other chemicals were bezafibrate from Boehringer Mannheim GmbH (Mannheim, FRG), potassium ferricyanide from E. Merck (Darmstadt, FRG), DPG and IHP from Sigma Chemical Co. LR16 and L35[1] were gifts from Dr. Lalezari (Lalezari et al., 1988) of the Montefiore Medical Center (Bronx, NY).

Oxygen equilibrium measurements

Equilibrium curves were measured by a continuous method using a Hemox analyzer (TCS-Medical Products Co., Huntingdon Valley, PA) as previously described (Kister et al., 1987*a*). Simultaneous recordings were made of the P_{O_2} with a Clark electrode and the light transmission at 560 nm in a 1-cm cuvette; typical samples were 60 μM in heme.

Association rates by flash photolysis

The recombination (on) rates were measured for both oxygen and CO after photodissociation by a 10-ns pulse at 532 nm (Quantel YAG laser, France). Samples were 0.1 mM in heme in 1-mm optical cuvettes, with observation at 436 nm (Marden et al., 1988). Measurements were made at 1 and 0.1 atm CO at different dissociation levels. The R-state properties are best observed at low levels of dissociation (<10%) where the main photoproduct is triply liganded tetramer, and at maximum ligand concentration to minimize the extent of the R-to-T transition.

Dissociation rates

The CO off-rates were measured by the oxidation of HbCO by ferricyanide (Antonini et al., 1965; Lanir et al., 1982) or by replacement of CO by NO (Gray and Gibson, 1971; DeYoung et al., 1976). Hb concentration was typically 0.3 mM in heme. The kinetics were recorded using a Cary 219 spectrophotometer (Varian Associates, Inc., Palo Alto, CA).

Oxidation by ferricyanide

Hb samples were first deoxygenated under argon and then equilibrated with 0.1 or 1 atmosphere CO in tonometers attached to a cuvette of 2 mm optical pathlength. Injection of ferricyanide (1–12 mM final concentration) resulted in a change in absorption at 568 nm from ~0.9 OD for the CO form to 0.2 for the ferric Hb. One side effect is the production of ferrocyanide which is known to bind to Hb and may possibly interfere with the measurements (Antonini and Brunori, 1971). Using CO recombination kinetics as a probe, we found that ferrocyanide (formed by addition of Na-dithionite to ferricyanide) acts as an effector like other negative ions (Shimizu and Bucci, 1974; Chiancone et al., 1975). Measurements made in the presence of 100 mM NaCl showed no difference for samples with and without ferrocyanide.

Replacement reaction with nitric oxide

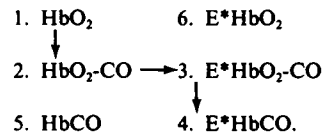
Samples of Hb-CO (equilibrated with 0.1 atm CO) were injected into 1-cm cuvettes containing buffer equilibrated with 1 atm NO. The kinetics were followed at 539 nm.

[1] *Abbreviations used in this paper:* bezafibrate (Bzf), 2-[4-[2[(4-chlorobenzoyl)amino]ethyl]phenoxy]-2-methylproprionic acid; L35, 2-[4[3,5-dichlorophenylureido)phenoxy]-2-methylpropionic acid; LR16, 2-[4-(3,4-dichlorophenylureido)phenoxy]-2-methylpropionic acid.

Oxygen-CO partition coefficient

The change in partition coefficient was observed spectrophotometrically. Samples were prepared in a tonometer attached to an optical cuvette with a 2-mm optical pathlength, and the absorbance spectrum was recorded (Cary 219) between 700 and 500 nm.

A mixture of ~50% oxygen and 50% CO bound Hb was prepared, and effector was then added to see whether the spectrum shifts towards more oxygen or CO form. For each effector (E) the experiment required six spectra:



A series of four spectra (arrows) were recorded on the same sample: (*a*) 1 atm oxygen without effector, (*b*) addition of CO gas which produces a nearly equal mixture of CO and oxy forms, (*c*) addition of effector, and (*d*) reequilibration with 1 atm CO.

Independently, a sample was equilibrated with 1 atm oxygen and then 1 atm CO, so the pure CO spectrum (5) could be deduced from spectrum 1 of each series. In addition, the same amount of effector was added to samples under 1 atm oxygen (1) to obtain spectrum 6 to correct for the minor absorbance changes due to the effectors (typically 0–3% due to a red shift of the alpha peak). Spectra were analyzed at six wavelengths (580, 577, 575, 565, 560, and 520 nm) for the percentage oxygen or CO bound. Spectra 1 and 5 were used as references to calculate the fraction HbO_2 and HbCO for the mixture (2) without effector; the spectra 3, 4, and 6 were used for the case with effectors.

RESULTS

Oxygen equilibrium

The oxygen equilibrium curves for several effectors are shown in Fig. 1. All the effectors showed a shift to the right on the Hill plot, implying a lower oxygen affinity. In addition the transition to the high-affinity state occurs later in the saturation curve, indicating a shift in allosteric equilibrium toward the T state. The stronger effectors show a smaller cooperativity and incomplete saturation, even under one atmosphere of oxygen; in these cases it is difficult to distinguish between an R-state asymptote of lower affinity and an apparent shift due to a T-state contribution in the liganded forms. However, the values for p50 and K_T can be accurately determined from the equilibrium curves and are summarized in Table 1 and Fig. 5.

Association rates

The bimolecular recombination kinetics of CO to Hb after photodissociation were measured for each effector. All the effectors caused a decrease in the on-rate of the rapid (R-state) phase. The R-state kinetics were best observed for low partial photolysis for samples equilibrated with one atmosphere CO. Certain examples have

TABLE 1 Oxygen equilibrium parameters and CO on-rates

Effector	p50	K_T	K_R	log(L)	$k_{R,on}^{CO}$
	mmHg	*mmHg*	*mmHg*		$10^6\ M^{-1}\ s^{-1}$
None	1.9	4.8	0.2	3.8	12.0
Cl	5.4	25.0	0.3	4.9	7.0
DPG	15.0	66.0	0.6	5.5	3.5
Bzf	16.0	60.0	0.9	4.9	2.5
LR 16	19.5	62.0	1.1	4.9	2.0
DPG + Bzf	27.0	133.0	1.1	5.5	2.0
L35	46.0	150.0	1.3	6.0	1.8
IHP	78.0	166.0	2.2	6.0	1.5
IHP + Bzf	130.0	212.0	2.2	6.9	1.5
IHP + L35	138.0	210.0	1.8	7.4	1.8

Solution conditions were 50 mM Bis Tris, pH 7.2, 25°C (Kister et al, 1987). Effector concentrations were 100 mM (Cl), 0.5 mM (LR16 and L35), 1 mM (DPG and IHP), and 5 mM for Bzf. Oxygen equilibrium data were used to determine all values of p50 and K_T, and the first two entries for K_R. The changes in R-state affinity with effectors were calculated from the changes in the CO on- and off-rates.

been published previously (Marden et al., 1988). The weakest effectors were Cl, DPG, and bezafibrate; the strong effectors LR16, L35, and IHP showed two kinetic components of nearly equal amplitude for the R state differing in rate by about a factor of 2. This effect has been reported for IHP (Gray and Gibson, 1971) and was attributed to chain differences.

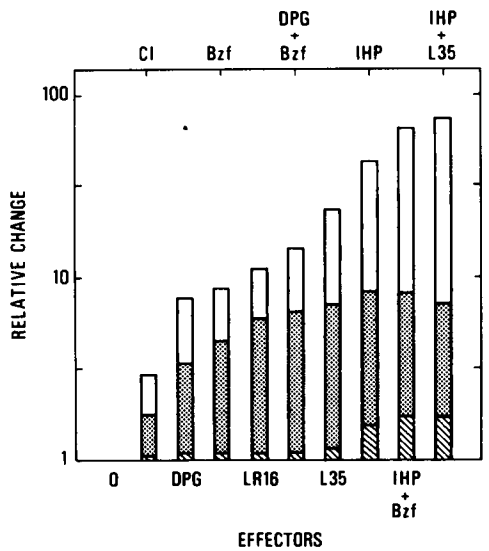


FIGURE 5 Relative change in the ligand-binding parameters (logarithmic scale), with respect to stripped Hb. The total height of the column represents the factor increase in p50 (50% saturation with oxygen). The decrease in the R-state CO on-rate (measured at low partial photolysis, 1 atm CO) is given by the sum of two lower segments. The lowest segment shows the increase in the CO off-rate for the liganded (R) form.

Dissociation rates

As previously observed (DeYoung et al., 1976), the dissociation rates are pH dependent with a transition centered near pH 7. As these off-rates are rather insensitive to the effectors, slight changes in pH could produce comparable changes in rate. Measurements were also made near pH 8 to better observe differences due only to the effectors.

Below pH 7.2 and in the presence of effectors, the ferricyanide data shows kinetics which accelerate. This could be related to the formation of a T-met state (Kilmartin, 1973; Perutz et al., 1974). From the initial rate of the oxidation process, the ferrous R-state rate is involved. Within the experimental errors, the CO off-rate shows little change upon addition of effectors, except for IHP (a factor of 1.6 decrease). The rates calculated from the kinetics of replacement of CO by NO were in agreement with these relative changes.

Oxygen-CO partition coefficient

Effector was added to a sample of Hb with ~50% CO and 50% oxygen bound; a shift toward the oxy or CO reference spectra would indicate that the partition is changed by the effector. Corrections were made for dilution and the small change in absorbance of the pure CO and oxy forms due to the effector. The change in partition coefficient was small as previously reported for IHP (DiCera et al., 1987*b*); IHP caused a 3% increase in the CO form, whereas Bzf favored oxygen as ligand by a few percent. Overall the results indicate that the effectors shift the affinity of the liganded conformation of Hb for oxygen and CO by nearly the same factor.

DISCUSSION

The separation of the allosteric and affinity factors requires values for the R- and T-state affinities. The R-state affinity is difficult to determine from the equilibrium data, especially in the presence of strong effectors which lower the ligand affinity and favor the deoxy allosteric state. Properties of the ligated allosteric state will be masked if the tetramers exist as an appreciable fraction of T state with three or four ligands bound. There cannot be much precision in values of the liganded parameters if the experimental conditions do not achieve full saturation or a full transition to the R state.

Because the affinity for the fourth ligand is poorly determined from oxygen equilibrium data, we have measured the on- and off-rates for CO to estimate changes in this value. The off-rates show little dependence on the effector. The on-rate for CO (and for oxygen) decreases

by as much as a factor of 8. Overall there is a decrease in the R-state affinity for CO by nearly a factor of 10.

The lack of a significant change in the partition coefficient was unexpected, because oxygen and CO show a different dependence on the kinetic parameters. The large difference in affinity for the R and T states (factor of 100 at pH 7.2 with 100 mM NaCl) is due predominantly to the off-rates for oxygen, whereas this factor is mainly accounted for by the on-rates for CO (Gray and Gibson, 1971; Murray et al., 1988). The small changes in the partition coefficient permit use of the CO data to estimate the corresponding shift in affinity for oxygen.

Classification of the effectors

From oxygen equilibrium results, the partial pressure of oxygen to obtain 50% saturation (p50) can be used to estimate the relative strength of the various effectors. This is complicated by the fact that different concentrations of effectors were used: some show a clear plateau of p50 vs. effector concentration (DPG, IHP), whereas others (LR16 and L35) reached the solubility limit before the plateau was obtained. A general classification in order of increasing p50 (Table 1) for oxygen equilibrium data would be: Cl < DPG ≤ Bzf < LR16 < L35 < IHP. Because the binding site for Bzf, LR16, and L35 is different from the DPG (IHP) site (Perutz et al., 1986; Lalezari et al., 1989), effectors can be bound at each site and the observed difference is larger than for either effector alone. The relative change in p50 is shown in Fig. 5; the horizontal axis is a list of effectors in order of increasing p50.

The ligand on-rates provide an additional measure of the relative effector strength and confirm this scheme. The on-rate and p50 do not show the same factor of change for the different effectors. For example the CO on-rate has little additional change beyond the value for LR16, and practically no change for the combination IHP + Bzf relative to IHP alone; however, the p50 value for oxygen equilibrium still shows large changes between LR16 and IHP or between IHP and IHP + Bzf (Fig. 5, values are normalized to that for Hb without effectors).

Substate populations

The allosteric transition in Hb results in a small equilibrium population (<10%) of the partially liganded forms. With a nonallosteric tetramer, 37% of the tetramers would be in the doubly liganded form at 50% ligand saturation. With strong effectors, the transition occurs at a higher ligation level and the doubly liganded population is greatly enhanced (to >30% in the case of IHP + L35), approaching that of a nonallosteric system. However, the sharp transition near full ligand saturation still maintains a low population of triply liganded forms.

The validity of the kinetic method to determine the equilibrium affinity for the R state requires some discussion. One fundamental problem may arise due to differences between the alpha- and beta-chains (Olson and Gibson, 1971; Mansouri and Winterhalter, 1973). If the fourth ligand is more likely to bind to one type of chain, and the quantum yield for the bimolecular phase in flash photolysis experiments is higher for the other type of chain, then it is possible that equilibrium and kinetic studies could be made on two different tetrameric forms. The kinetic results show a single R-state rate for the weak effectors (Cl, DPG, Bzf), indicating that the two chains have the same rate. Strong effectors like IHP show two R-state rates; we have used the faster rate for the calculation of the R-state affinity.

A second question concerns protein relaxation. After photolysis there is a relaxation to a mixture of R and T substates. The R and T on-rates are easily separable for CO recombination. Thus unlike equilibrium measurements which show an averaged asymptote, the kinetic data show resolved R and T signals. Furthermore because the initial state is fully liganded R4, there might be a difference in rebinding to the photoproduct R3* (which is still in the R4 conformation) and to rebinding to stable R3 after relaxation to the R3-T3 equilibrium mixture.

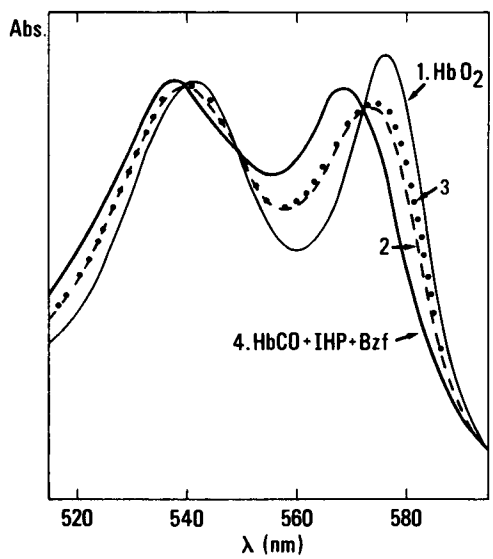


FIGURE 6 Test for the change by the effector in the oxygen, CO partition coefficient at pH 7.1, 25°C. To a sample under 1 atm oxygen (spectrum 1), a volume of CO gas is added to obtain approximately a 50:50 mixture of HbO_2 and HbCO (2). Effectors (IHP and Bzf in this example) are then added (3), before final equilibration under 1 atm CO (4). The small difference between spectra 2 and 3 indicates little change in the partition coefficient due to the effector.

Although no differences have been demonstrated, we emphasize that the kinetically determined affinity refers to binding of the fourth ligand to the fully liganded conformation.

Allosteric vs. affinity factors

From the equilibrium data for the oxygen affinity of the first ligand (T state) and the kinetically determined values for the fourth ligand affinity (R state), the shift in p50 due to the change in affinities can be estimated by recalculating the equilibrium curve using a two-state model. Suppose there is a transition from the curve without effectors (K_R, K_T, L) to (K'_R, K'_T, L') for the case with effector. We can generate an intermediate curve (K'_R, K'_T, L) to determine the shift in p50 relative to (K_R, K_T, L) due to the affinity factors. The remaining shift is attributed to the allosteric parameters. Similarly a curve can be calculated with the original affinities (without effector) and L' (with effector) to estimate the allosteric contribution. The results are represented graphically in Fig. 7, where the allosteric and affinity contributions are separated for the change in p50. Errors in the R-state affinity are the more critical, as values of p50 are nearly linear with K_R.

The results indicate that the changes in the affinities play the larger role in the shift in p50. For example, DPG shifts p50 by a factor of 7.4 relative to Hb without effectors; the contribution due to the change in affinities is approximately a factor of 3.2, or 57% of the total. The

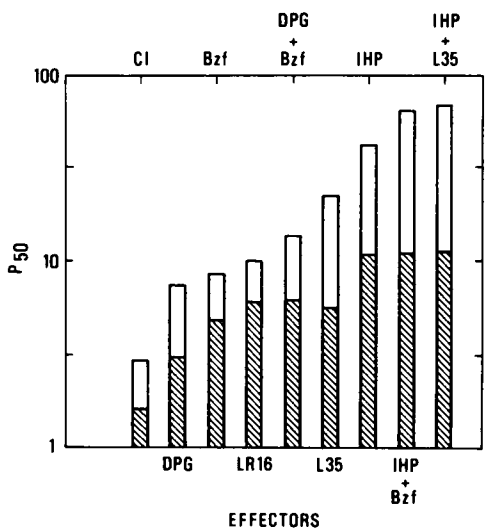


FIGURE 7 Relative contribution of affinity (*hatched region*) and allosteric parameters (*open region*) toward the shift of p50 for the effectors indicated (see Table 1 for experimental conditions). The total height represents the factor change in p50 relative to Hb without effectors (as in Fig. 5).

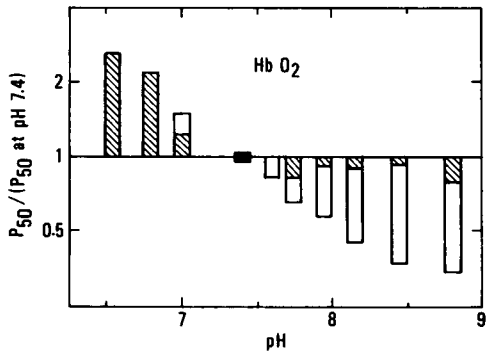


FIGURE 8 Relative contribution of affinity and allosteric factors (as in Fig. 7) for the shift in p50 (total height) due to a change in pH (Lee et al., 1988). Values at pH 7.4 were taken as reference. The transition above pH 7.4 is essentially allosteric in nature, whereas at acid pH the transition is mainly due to the affinity factors.

large affinity contribution is not surprising, because p50 is an average affinity limited between the pure R and T affinities. For a purely allosteric effect, the upper limit for p50 is K_T, which is nearly attained when IHP is present. Based on simulation of the data for stripped Hb, p50 could increase at most by a factor of 5. The observed changes in p50 for the series of effectors are therefore due in large part to the shift in the affinities.

There is a potential problem in calling a state R or T. Both the R- and T-state affinities vary by over a factor of 10 for the different effectors, yet the R-state affinity for Hb with IHP differs from the T-state affinity for Hb without effectors by less than a factor of 2. For each effector, one can discuss a transition from R' to T' which can still be described by a two-state model, yet there is no guarantee that all the "R" states will satisfy other criteria, whether crystallographic or spectroscopic, as clearly being an R state.

The same analysis can be applied to the equilibrium curves vs. pH (Lee et al., 1988). Extracting all parameters from the equilibrium data, we found that there were two regions (Fig. 8). Above pH 7.4 there is little change in K_R and the shift in p50 is mainly allosteric. At low pH the change is due to the affinity factors. The equilibrium results are in agreement with kinetic studies (DeYoung et al., 1976), which show that the rates for the fourth ligand are nearly constant above this critical pH.

CONCLUSIONS

Effectors change the allosteric equilibrium and ligand affinity of both allosteric states. In general a new allosteric state must be considered for each effector and each combination of effectors. Considering p50 as an indicator

of effector strength, the affinity parameters cause a larger shift than the allosteric factors.

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