Protein Science (1997), *6*:484–489. Cambridge University Press. Printed in the USA.

FOR THE RECORD

Allosteric effectors do not alter the oxygen affinity of hemoglobin crystals

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(RECEIVED October 17, 1996; ACCEPTED November 5, 1996)

Abstract: In solution, the oxygen affinity of hemoglobin in the *T* quaternary structure is decreased in the presence of allosteric effectors such as protons and organic phosphates. To explain these effects, as well as the absence of the Bohr effect and the lower oxygen affinity of *T*-state hemoglobin in the crystal compared to solution, Rivetti C et al. (1993a, *Biochemistry 32*:2888–2906) suggested that there are high- and low-affinity subunit conformations of *T*, associated with broken and unbroken intersubunit salt bridges. In this model, the crystal of *T*-state hemoglobin has the lowest possible oxygen affinity because the salt bridges remain intact upon oxygenation. Binding of allosteric effectors in the crystal should therefore not influence the oxygen affinity. To test this hypothesis, we used polarized absorption spectroscopy to measure oxygen binding curves of single crystals of hemoglobin in the *T* quaternary structure in the presence of the "strong" allosteric effectors, inositol hexaphosphate and bezafibrate. In solution, these effectors reduce the oxygen affinity of the *T* state by 10–30-fold. We find no change in affinity (<10%) of the crystal. The crystal binding curve, moreover, is noncooperative, which is consistent with the essential feature of the two-state allosteric model of Monod J, Wyman J, and Changeux JP (1965, *J Mol Biol 12*:88–118) that cooperative binding requires a change in quaternary structure. Noncooperative binding by the crystal is not caused by cooperative interactions being masked by fortuitous compensation from a difference in the affinity of the α and β subunits. This was shown by calculating the separate α and β subunit binding curves from the two sets of polarized optical spectra using geometric factors from the X-ray structures of deoxygenated and fully oxygenated *T*-state molecules determined by Paoli M et al. (1996, *J Mol Biol 256*:775–792).

Keywords: allosteric models; hemoglobin crystals; microspectrophotometry; oxygen binding

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The role of hemoglobin as the paradigm for understanding cooperative interactions in multisubunit proteins has been questioned in recent years because of confusion in the literature concerning the applicability of the two-state allosteric mechanism of Monod, Wyman, and Changeux (MWC), and the stereochemical mechanism of Perutz (P) (Monod et al., 1965; Perutz, 1970; Edelstein, 1973; Shulman et al., 1975; Perutz et al., 1987). According to the two-state MWC model, cooperativity results from a shift in the population of molecules from the low-affinity (*T*) quaternary structure to the high-affinity (*R*) quaternary structure as successive oxygen molecules bind, and not from subunit interactions within a single quaternary structure. The structural basis of this mechanism was suggested by Perutz (Perutz, 1970; Perutz et al., 1987). Perutz's mechanism describes how oxygen binding causes structural changes that are transmitted to the subunit interfaces and destabilize the *T* quaternary structure relative to the *R* quaternary structure. He also explained why allosteric effectors such as protons and organic phosphates bind preferentially to *T*. Intersubunit salt bridges are central to Perutz's mechanism. They stabilize *T* relative to *R*, lower the oxygen affinity in *T*, and are responsible for the Bohr effect. The statistical mechanical formulation of the Perutz model by Szabo and Karplus (SK) showed that it is consistent with the two-state MWC model under a fixed set of solution conditions, but that the condition dependence of the equilibrium oxygen binding data required a modification of the MWC proposal for how allosteric effectors influenced oxygen binding (Szabo & Karplus, 1972, 1975; Lee et al., 1988).

Overall, this MWC-PSK model has provided an extremely useful framework for synthesizing the results from a vast array of structural, spectroscopic, equilibrium, and kinetic experiments, but, until relatively recently, it has only been tested rigorously for explaining solution oxygen binding curves and equilibrium data on tetramer–dimer dissociation. Neither of these tests has been fruitful. The solution oxygen binding curves contain relatively little information, and numerous models have been proposed over the years that are consistent with such data (Antonini & Brunori, 1971; Imai, 1982; Wyman & Gill, 1990). The investigation of tetramer–dimer dissociation equilibria has focused on artificial intermedi-

ates, particularly those containing the cyanide complex of oxidized hemes (Smith & Ackers, 1985; Perrella et al., 1990a; Doyle & Ackers, 1992). This work has led to the conclusion that the two-state MWC model is incompatible with the experimental results (Ackers & Smith, 1987; Ackers, 1990; Ackers et al., 1992). However, Edelstein has pointed out that these artificial intermediates are poor analogues of oxygenation intermediates because the parameters derived cannot reproduce the oxygen binding curve (Edelstein, 1996; Huang et al., 1996).

Critical tests of the MWC model require new kinds of experiments. Several such experiments have been conducted over the past several years, including determination of the equilibrium ligation state distribution by low-temperature electrophoresis (Perrella et al., 1990a; Perrella & Denisov, 1995), oxygen binding curves of hemoglobin encapsulated in silica gels (Shibayama & Saigo, 1995), and nanosecond–millisecond ligand binding and conformational kinetics (Henry et al., 1996). Perhaps the most incisive "new" experiment is the measurement of oxygen binding to the T quaternary structure in single crystals of hemoglobin (Mozzarelli et al., 1991; Rivetti et al., 1993a, 1993b; Kavanaugh et al., 1995; Bettati et al., 1996). Because the oxygen affinity of the free chains is similar to that of hemoglobin in the R quaternary structure, cooperativity arises from the release of constraints that are present in the fully deoxygenated T quaternary structure. It is for this reason that the issue of the applicability of the MWC model has centered around oxygen binding to the T state. Does cooperativity arise solely from the transition from T to R, or is there significant cooperativity in binding to T? The single crystal binding studies, in which the molecule remains in the T quaternary structure over the entire range of oxygen pressures, have addressed this issue directly. These studies have confirmed the essential point of the MWC model that there is only a very small deviation from perfectly noncooperative binding to the T state (Mozzarelli et al., 1991; Rivetti et al., 1993a; Bettati et al., 1996). They also showed that oxygen binding in the crystal is pH-independent, in keeping with Perutz's proposal that the Bohr effect requires breakage of intersubunit salt bridges (Rivetti et al., 1993a; Bettati et al., 1996).

Another important result from the crystal studies is that the oxygen affinity in the crystal is 4–40-fold lower than the oxygen affinity of the T quaternary structure in solution. To explain the lower affinity in the crystal, Rivetti et al. (1993a) proposed that there are two conformations for subunits in the T quaternary structure, a low-affinity conformation with intact intersubunit salt bridges in both liganded and unliganded states, as found in the crystal, and a high-affinity conformation with broken salt bridges in both ligation states. The binding polynomial for this model is:

$$Q = L\left(\frac{l+1}{l}\right)^4\left[1 + \left(\frac{l}{l+1}K_{Tt} + \frac{1}{l+1}K_{Tr}\right)p\right]^4 + [1 + K_R p]^4,$$

where L is the allosteric equilibrium constant for the quaternary conformational change of the completely unliganded tetramer with all four subunits in the t conformation ($\equiv [Tt_4]/[R]$), l is the equilibrium constant for the tertiary conformational change of the unliganded subunits in the T quaternary structure ($\equiv [Tt]/[Tr]$), K_{Tt} is the binding constant to the low-affinity tertiary conformation in the T quaternary structure with unbroken salt bridges in both unliganded and liganded states, K_{Tr} is the binding constant to the high-affinity tertiary conformation in the T quaternary structure with broken salt bridges in both unliganded and liganded states, K_R is the binding constant to a subunit in the R quaternary structure, and p is the oxygen pressure.[3] The binding polynomial is identical to that for a two-state allosteric model if the composite equilibrium constant for binding to the T state, $(l/l+1)K_{Tt} + (1/l+1)K_{Tr}$, is identified with the MWC K_T, and the prefactor $L[(l+1)/l]^4$ is identified with the MWC L.[4] In this model, the variability in the affinity of the T quaternary structure results from the dependence of l on solution conditions. This proposal represents a generalization of the SK model, which only considered subunit conformations having unbroken salt bridges in the unliganded state, and broken salt bridges in the liganded state, and the extended SK model of Lee et al. (1988), which included conformations with unbroken salt bridges in the liganded state as well. The model of Rivetti et al. (1993a) explains the effect of allosteric effectors on the affinity of the T state in solution as resulting from a change in the relative population of the two tertiary conformations. It makes, moreover, the simple prediction that allosteric effectors will have no influence on the oxygen affinity of the T quaternary structure in the crystal because all of the subunits are in the low-affinity conformation in both the deoxygenated and oxygenated states.

To test this prediction, we have measured the single crystal oxygen binding curves in the presence and absence of two "strong" allosteric effectors, inositol hexaphosphate (IHP) and bezafibrate (Bzf). In solution, IHP lowers the T-state affinity by ~30-fold relative to stripped hemoglobin, whereas the decrease produced by BZF is ~10-fold (Marden et al., 1990). The X-ray structures of hemoglobin crystallized in IHP are now known in both the fully oxygenated and fully deoxygenated states (Paoli et al., 1996). IHP, like 2,3-diphosphoglycerate (DPG), binds in the cleft between the β subunits, but is disordered (Arnone & Perutz, 1974; Fermi & Perutz, 1981; Waller & Liddington, 1990). Bzf binds in the smaller cleft between the α subunits, and therefore has a different structural mechanism for stabilizing the T quaternary structure (Perutz et al., 1986).

Our experiment consists of measuring the complete optical absorption spectrum between 450 and 700 nm as a function of oxygen pressure, with light linearly polarized parallel to either the a or c axes of this orthorhombic crystal. The data for one crystal are presented in Figure 1A. Figure 1B and C show representative fits to the spectra to determine the fractional population of each species (oxy-, deoxy-, and methemes). The fractional saturation of reduced hemes as a function of oxygen pressure and a Hill plot of the fractional saturation are shown in Figure 1D, E, F, and G. Table 1 summarizes the results of the experiments. All the Hill n

[3] The quantity $[(l+1)/l]^4$ in Equation 19 of Rivetti et al. (1993a) was written incorrectly as $[l/(l+1)]^4$.

[4] Kinetic studies suggest that there are high- and low-affinity tertiary conformations in the R quaternary structure (Henry et al., 1996), indicating that a more complete binding polynomial would contain a composite equilibrium constant with the same form as the T state, but with a distinct tertiary equilibrium constant and presumably different affinities, i.e.,

$$Q = L\left(\frac{l_T+1}{l_T}\right)^4\left[1 + \left(\frac{l_T}{l_T+1}K_{Tt} + \frac{1}{l_T+1}K_{Tr}\right)p\right]^4 + (l_R+1)^4\left[1 + \left(\frac{l_R}{l_R+1}K_{Rt} + \frac{1}{l_R+1}K_{Rr}\right)p\right]^4,$$

where $L \equiv [Tt_4]/[Rr_4]$. Notice that, for $l_T \to \infty$ and $l_R \to 0$, this binding polynomial becomes the simple two-state MWC polynomial. There remains the interesting possibility that $K_{Tt} \approx K_{Rt}$ and $K_{Tr} \approx K_{Rr}$, which would constitute a "tertiary two-state" model, in which tertiary–quaternary coupling is incomplete (Herzfeld & Stanley, 1974).

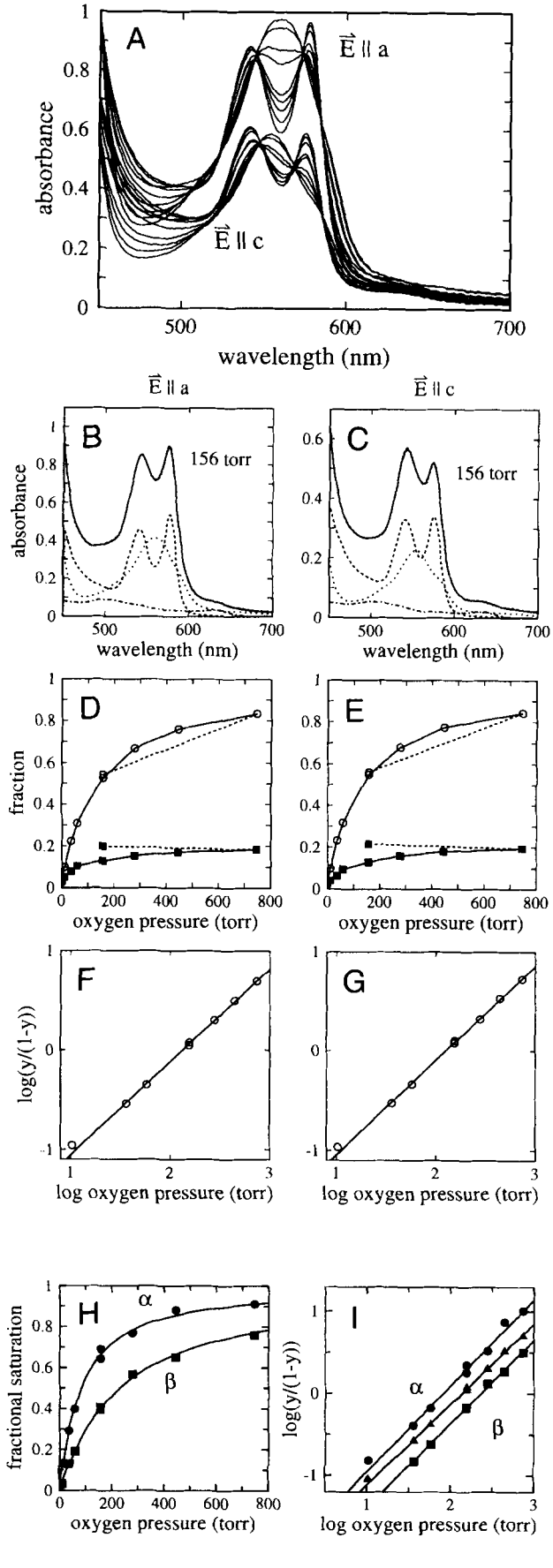

Fig. 1. Single crystal optical spectra and oxygen binding curves. **A:** Polarized absorption spectra at increasing oxygen pressure (15 °C; 0.01 M potassium phosphate, 54% (*w/v*) PEG 1000, 2 mM IHP, 1 mM EDTA, 90 μg/mL catalase). Upper set are for linearly polarized light incident normal to the *ac* (010) crystal face with electric vector parallel to the *a* crystal axis, and the lower set are for light polarized parallel to the *c* crystal axis. Spectral measurements were made with a microspectrophotometer as described in detail by Rivetti et al. (1993a). **B,C:** Representative fits of the observed spectra as linear combinations of oxyHb, deoxyHb, and metHb reference spectra. The reference spectra obtained by Rivetti et al. (1993a) were used for all of the analysis in the present work. In each panel, the measured spectra are the continuous curves and the dashed curves are the sums of the component spectra that result from the least-squares fit to the reference spectra. The individual components are also shown in each panel as a dotted curve for the deoxyHb component, a dashed curve for oxyHb, and a dashed-dotted curve for metHb. **D,E:** Apparent fractional saturation (open circles) and metheme composition (filled squares) as a function of oxygen pressure, derived from fits of spectra measured with light linearly polarized parallel to the *a* and *c* crystal directions. The fractional saturation of reduced hemes with oxygen is shown as open circles, and the fractional population of oxidized hemes is shown as filled squares. The oxygen pressure was increased monotonically during the experiment, except for a single measurement at an intermediate pressure performed at the end of the experiment. This demonstrates the reversibility of the estimated oxygen binding curve and the continuing increase of the estimated metheme population. **F,G:** Hill plots of the apparent fractional saturations. Parameters are given in Table 1. **H,I:** Calculated binding curves for α (filled circles) and β (filled squares) subunits. Saturations were determined using the method described by Rivetti et al. (1993a), with geometric factors derived from the heme plane projections given in Table 2 for IHP-containing crystals. The continuous curves in H were obtained from a least-squares fit using equation 21 of Rivetti et al. (1993a) for the binding curves of α and β subunits in a cooperative dimer, varying the intrinsic affinities of the α and β subunits and the cooperativity parameter δ (see text). Parameters derived from this analysis are given in Table 1. The triangles in I represent the actual binding curve for the tetramer computed from the binding curves of the α and β subunits.

Table 1. *Summary of data*

Effector	n		p50 (torr)		p50(α) (torr)[a]	q	δ
	a-axis	c-axis	a-axis	c-axis			
IHP[b]	0.94	0.95	134	126	88	3.1	1.3
	0.93	0.94	144	138	100	1.9	0.9
Average	0.94 ± 0.01		136 ± 7		94 ± 6	2.5 ± 0.6	1.1 ± 0.2
Bzf	0.96	1.0	140	128			
	0.94	0.97	135	124			
	0.93	0.95	139	131			
Average	0.96 ± 0.02		133 ± 6				
None[c]	0.99	0.99	133	129	76	4.8	1.8
	1.04	1.05	124	123	85	4.9	2.4
	0.99	0.99	152	148	75	5.7	1.6
Average	1.01 ± 0.03		135 ± 11		79 ± 5	5.1 ± 0.4	1.9 ± 0.3

[a]Data in the last three columns are the adjustable parameters obtained from simultaneous fits to the sets of a- and c-polarized spectra using Equation 21 of Rivetti et al. (1993a) for the fractional saturations of α and β subunits of a cooperative dimer.

[b]Geometric factors for the separate determination of α and β subunit saturations are those given in Table 2 for the IHP-containing crystals.

[c]Results are for three data sets originally treated by Rivetti et al. (1993a), specifically those featured in their Figure 15. For the calculation of α and β binding curves shown in Figures 1H and 1I, the heme projections and geometric factors were derived by the procedure of Rivetti et al. (1993a) (described in their Table 1). However, unpublished or unfinalized coordinate sets were replaced by more recently available published coordinate sets. Specific coordinate sets were Protein Data Bank files 1HGA for deoxyhemoglobin and 1HGB for methemoglobin (Liddington et al., 1992), as well as 1NIH for the alpha-nickel beta-iron hybrid (Luisi et al., 1990) and 1HGC for the semioxyhemoglobin (Liddington et al., 1992) structures used to synthesize the T-state oxyhemoglobin structure.

values are close to 1.0, indicating noncooperative binding. The average p50 in the absence of allosteric effectors is 135 torr, in the presence of IHP is 136 torr, and in the presence of Bzf is 133 torr. The striking result, then, is that neither IHP nor Bzf has any effect on the apparent oxygen affinity of the crystal (Table 1), as predicted by the model of Rivetti et al. (1993a).

There are additional results that are unique to binding curves measured on single crystals in polarized light. Rivetti et al. (1993a) pointed out that noncooperative binding to the tetramer could result from fortuitous cancellation of an arbitrarily large amount of cooperativity by a large difference in intrinsic affinity of the α and β subunits. This can be appreciated readily by considering binding to a cooperative dimer with the α subunit having a very much higher affinity than the β subunit. The α subunit will bind the first ligand. If the cooperative interaction between the α and β subunits is of just the right magnitude, the second ligand can bind to the β subunit with an affinity that is exactly the same as the first. Thus, even though there are cooperative interactions, the binding curve for the *dimer* is (apparently) noncooperative. For a tetramer with Hill $n = 1$ that contains two cooperative dimers (the "cooperon" model of Brunori and coworkers), the relation between the ratio of subunit affinities, q, and subunit interaction parameter, δ, defined by the binding polynomial: $Q = [1 + (K_\alpha + K_\beta)p + \delta K_\alpha K_\beta]^2$, is given by $\delta = (q + 1)^2/4q$ (Szabo & Karplus, 1975; Brunori et al., 1986; Gill et al., 1986). The parameter δ defines the increase in the affinity of a subunit due to the presence of a ligand bound to the other subunit of the dimer. Rivetti et al. (1993a) exploited the difference in projection of the α and β hemes onto the crystal face of the measurements to calculate the separate α and β subunit binding curves and to obtain δ and q by fitting these data with the cooperon model. They found $q \sim 5$ ($p50(\alpha) \sim 80$ torr, $p50(\beta) \sim 370$ torr) and $\delta \sim 2$. Bettati et al. (1996) studied the binding of the β subunits separately in a hybrid hemoglobin in which the iron(II) of the α subunits was replaced by the non-oxygen binding nickel(II). They found p50(β) = 110 ± 20 torr, suggesting that Rivetti et al. (1993a), if anything, overestimated q and therefore did not underestimate δ. These results therefore supported the conclusion of Rivetti et al. (1993a) that there is only a small deviation from perfectly noncooperative binding.

The present data, together with the recent X-ray study by Paoli et al. (1996), give us the opportunity to calculate q and δ without the uncertainties that confronted Rivetti et al. (1993a) in computing the geometric factors associated with the heme orientations for the crystals not containing IHP (Table 2). Rivetti et al. (1993a) used the X-ray structure of deoxyhemoglobin crystallized without IHP to calculate geometric factors from the heme plane projections, but the geometric factors used for the oxygenated structure were synthesized from the structure of the incompletely oxygenated α subunit and the carbon monoxide complex of the β subunit (superimposed) from a different crystal lattice. No such uncertainty exists in the present case, because the X-ray structure of the fully oxygenated T-state molecule in the presence of IHP is now known from the work of Paoli et al. (1996).

As observed previously for crystals without IHP (Rivetti et al., 1993a), the apparent p50 for the a-axis binding curve is higher than for the c-axis curve in all of the data sets (Table 1). Figure 1H and I show the binding curves of the tetramer and the α and β subunits separately, calculated as described by Rivetti et al. (1993a). The fits of these curves using the cooperon model with geometric factors given in Table 2 for the IHP-containing crystals are shown in Figure 1H. As was found by Rivetti et al. (1993a) for hemoglobin in the absence of IHP, the α subunit has a slightly higher affinity than the β subunit. The cooperativity parameter in the presence of IHP is smaller (1.1 ± 0.2, Table 1). This value is very small compared to the 75-fold increase in affinity of the tetramer associated with the change from T to R observed in solution in the

Table 2. *Projections of heme planes used in calculations of separate α and β binding curves in IHP-containing crystals*

Subunit	$\sin^2 z_i a$	$\sin^2 z_i c$
Oxy[a]		
α1	0.740	0.711
α2	0.740	0.401
β1	0.844	0.265
β2	0.768	0.598
Deoxy[b]		
α1	0.754	0.716
α2	0.783	0.405
β1	0.881	0.274
β2	0.795	0.539
Met[c]		
α1	0.742	0.689
α2	0.755	0.420
β1	0.857	0.275
β2	0.771	0.546

[a]Paoli et al. (1996).
[b]Unpublished results of J. Tame, G. Dodson, and coworkers.
[c]Protein Data Bank File 1HGB (Liddington et al., 1992). This crystal appears not to have contained IHP.

presence of IHP (Marden et al., 1990). That is, cooperativity within the *T* quaternary structure is insignificant, compared to the cooperativity generated by the quaternary structural change, providing a critical test of the MWC mechanism.

The MWC mechanism has now been subjected to and passed several demanding tests, in addition to the crystal binding experiments. Henry et al. (1996) have shown that a comprehensive set of kinetic data on carbon monoxide binding and conformational kinetics over a wide dynamic range (nanoseconds–milliseconds) can be fit with an MWC two-state model, expanded to include geminate states and tertiary conformational changes. The kinetic parameters from this modeling, moreover, reproduce the equilibrium distribution of ligation states as a function of carbon monoxide saturation obtained in the low-temperature electrophoresis study of Perrella and coworkers (Perrella et al., 1990b; Denisov & Perrella 1995). Finally, Shibayama and Saigo (1995) have performed an experiment that shows ideal MWC behavior—deoxyhemoglobin encapsulated in a silica gel binds oxygen noncooperatively with a low affinity, whereas dissociation of oxygen from oxyhemoglobin encapsulated in the gel is also noncooperative, but with a 500-fold higher affinity. Thus, it would appear that deviations from perfect MWC behavior are small, and it may be extremely difficult to uncover the structural basis of this perturbation, although some attempts have been made (Paoli et al., 1996). The MWC model must, however, be modified to account for the influence of allosteric effectors—so-called heterotropic effects (Szabo & Karplus, 1972, 1975; Rivetti et al., 1993a). The model was formulated before the high-resolution X-ray structure was known, and Monod et al. (1965) anticipated that their mechanism for heterotropic effects, in which allosteric effectors influence only *L* (the equilibrium between quaternary structures at zero ligation), was an oversimplification. This is an area where considerable additional work is required.

Acknowledgments: We are indebted to Jeremy Tame and Guy Dodson for sending us unpublished atomic coordinates of hemoglobin, and to Boehringer-Mannheim for providing bezafibrate. This work was supported in part by a NATO Collaborative Research grant (930826).

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