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STABILIZATION OF THE T-STATE OF HEMOGLOBIN

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The effect of inositol hexaphosphate and bezafibrate on binding of O_2 and CO to HbA_0 at high concentrations (1 mM) has been evaluated using thin layer optical techniques. Data analysis shows 1) the occurrence of greatly reduced ligand dependent cooperativity (Hill slope of 2.23 for CO and 1.51 for O_2), and 2) the presence of significant triply ligated species. The data fits a nested allosteric two-state MWC model in which the T state consists of two allosteric substates, T_t and T_r, where T_t binds only to the α chains and T_r binds to both α and β chains. The model indicates that the triply ligated species consists of a predominant amount of T form, agreeing with kinetic observations of CO ligated hemoglobin. The maximum amount of triply ligated R molecules (CO or O_2) implicated is less than 1%, a result similar to that found previously for binding studies made in the absence of BZF and IHP.

Recent equilibrium studies in our laboratory of oxygen and carbon monoxide binding to human hemoglobin A_0 have brought to light an important clue to understanding the cooperative mechanism of HbA_0-- the equilibrium population of all triple ligated states of O_2 and CO intermediates contribute negligibly to the reaction process (1). This key thermodynamic constraint combined with X-ray structural information (2, 3) has formed the basis of a proposed thermodynamic model of Hb cooperativity (4). This model, which might be called the truncated two-state allosteric (TTA) model, has thus far succeeded in fitting our hemoglobin ligand binding data measured over a wide range of solution conditions, namely pH, temperature, and in the presence of various amounts of CO_2, DPG, and IHP(5-8). A principal feature of the TTA model is that it provides a rational explanation for the observation of negligible triple species population. The model assumes two states in equilibrium, the well known T and R end states, where additional cooperative effects are allowed in binding to the α chains within the T state, and where binding to the β chains in the T form is negligible. Thus binding the third and fourth ligands requires

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Abbreviations: BZF, Bezafibrate; EDTA, ethylenediaminetetraacetic acid; HbA_0, adult human hemoglobin; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; IHP, inositol hexaphosphate; DPG, 2,3-diphosphoglycerate; MWC, Monod-Wyman-Changeux two state model (21); MWChet model with heterogeneous binding constants $\kappa_{T\alpha}$ and $\kappa_{T\beta}$ in the T state; MWC(Tnest) model with allosteric substates T_t and T_r in the T form; TTA model, truncated two-state allosteric model (4); T, is the tense, or low ligand affinity, state; R, is the relaxed, or high ligand affinity, state; L is allosteric equilibrium constant for R→T.

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conversion to the R form. In all the previous studies we have made, under a wide variety of solution conditions for concentrated hemoglobin, the TTA model was found capable of fitting the binding data; this suggested that under these solution conditions the Gibbs energy state of T-state triply-ligated species (involving β chains) is significantly higher than that for the triply ligated R form. Further exploration of this picture would be achieved by examining a situation in which the T state is stabilized to such a high degree that triply ligated T forms, involving binding to both the α and β chains, would be found. Such a case has been implicated by recent kinetic studies of CO binding to HbA_0 in the presence of a synergistic combination of T-state effector molecules (IHP) and bezafibrate (BZF) (9). A preliminary oxygen binding study (10) has also demonstrated a significant reduction of oxygen affinity and cooperativity in the presence of BZF and DPG, thereby implicating stabilization of the T form.

Here we wish to report initial results on the equilibrium binding properties of O_2 and CO binding to HbA_0 in the presence of IHP and BZF.

MATERIALS AND METHODS

Sample preparation

Hemoglobin A_0 was prepared by the standard procedures of Williams and Tsay (11), dialyzed against deionized water, and stored in liquid nitrogen until use. The final solution conditions of 0.15 M HEPES, pH 7.0, 0.15 M NaCl, 1.5 mM Na_2EDTA, 15 mM IHP, 5 mM BZF, and 1 mM heme HbA_0 were obtained by volumetric dilution of the stock HbA_0 into the appropriate buffer. The amount of met Hb prior to the experiment was found to be about 1%. Formation of met Hb during sample preparation was minimized by use of an anaerobic glove box (Vacuum Atmospheric model VAC HE-43). Experiments were performed at 11 and 20° C for oxygen in order to reduce met formation during the binding curves, and, based on an unchanging fully liganded spectrum at the start and finish of each experiment, judged to contain only 1% met Hb at the end of each experiment. Due to the high affinity of carbon monoxide, it was necessary to use the higher temperatures of 20 and 30°C. Dissociation of Hb into dimers was considered negligible due to the increased stability of tetrameric Hb in the presence of IHP (12-14). The IHP and HEPES were products of Sigma Corporation. The BZF was a gift from Claude Poyart.

Data Analysis

Oxygen and carbon monoxide binding curves were measured by the thin layer derivative method (15). The technique measures changes in fractional saturation upon logarithmic dilutions in ligand partial pressure. The data are given by the change in absorbance $\Delta A(\xi_i)$ corresponding to a change in the degree of saturation $\theta(x_i)$ and $\theta(x_{i-1})$ between partial pressures x_i and x_{i-1}.

$$\Delta A(\xi_i) = \Delta A_T\left[\theta_{(x_i)} - \theta_{(x_{i-1})}\right] \qquad [1]$$

Here ΔA_T is the absorbance change for complete ligation, and $\Delta A(\xi_i)$ is the measured absorbance change corresponding to the geometric mean (ξ_i) of partial pressures x_i and x_{i-1}, i.e.$\xi_i = \sqrt{x_i x_{i-1}}$. In the thin-layer technique the partial pressures are created by the use of a precision dilution valve which has a dilution factor given by D (In the 20°C experiments to be described in detail, the starting partial pressure and D for O_2 was 607.50 torr and 0.6989, and for CO was 15.193 torr and 0.6997.). The partial pressure at the end of step i is given by $x_i = x_{i-1}$ D. A Taylor expansion of equation [1] in terms of $(\ln x_i - \ln x_{i-1}) = \ln D$, gives, to the first order, the result (16):

$$\Delta A(\xi_i) = \Delta A_T\left(\frac{d\ln\theta}{d\ln x}\right)_{x=\xi_i} \qquad [2]$$

The fractional saturation function at a partial pressure ξ_i, $\theta(\xi_i)$, is written explicitly for a four site macromolecule as

$$\theta(\xi_i) = \frac{1}{4}\left(\frac{d\ln P}{d\ln x}\right)_{x=\xi_i} \qquad [3]$$

where P is the binding polynomial. Each term in the binding polynomial represents the population of the species depicted by the term.

The Adair form of P is cast in terms of the overall stoichiometric equilibrium constants, β_i, called the Adair constants (17), and each term is then proportional to the relative populations of the i^{th} liganded species. The simplest two-state allosteric model (MWC) is based on a set of ligand equilibria between two forms (R and T) of a macromolecule: for the unligated allosteric transition, R→T, the equilibrium constant is L and the intrinsic binding constants to the R and T forms are κ_R and κ_T. Introduction of chain heterogeneity into the allosteric picture requires designation of the binding constants to the α and β forms, such as $\kappa_{T\alpha}$ and $\kappa_{T\beta}$ for ligand binding the β chains in the T form. The TTA model (4) assumes binding to the T form occurs essentially to the α chains, contains a site interaction constant γ between the α chains, and assumes uniform binding to the R form with a binding constant κ_R. Inclusion of a nested allosteric transition within the T state will be designated as the MWC(Tnest) model: one substate (T_t) is a tight form where binding occurs only to the pair of cooperatively interacting α chains, and the other substate (T_r) is a relaxed form where all chains are equally accessible with affinity κ_{Tr}. The equilibrium constant for $T_r \rightarrow T_t$ is given by ℓ. The binding polynomials for these cases are given as follows:

Model	Binding polynomial P
Adair stoichiometric species	$1+\beta_1 x+\beta_2 x^2+\beta_3 x^3+\beta_4 x^4$
MWC	$\frac{L}{1+L}\{1+\kappa_T x\}^4+\frac{1}{1+L}\{1+\kappa_R x\}^4$
MWC(het)	$\frac{L}{1+L}\{1+\kappa_{T\alpha} x\}^2\{1+\kappa_{T\beta} x\}^2+\frac{1}{1+L}\{1+\kappa_R x\}^4$
TTA	$\frac{L}{1+L}\left\{1+2\kappa_{T\alpha} x+\gamma\kappa_{T\alpha}{}^2 x^2\right\}+\frac{1}{1+L}\{1+\kappa_R x\}^4$
MWC(Tnest)	$\frac{L}{1+L}\left[\frac{\ell}{1+\ell}\left\{\left(1+\kappa_{Tt}{}^2 x^2\right)+\frac{1}{1+\ell}(1+\kappa_{Tr} x)^4\right\}\right]+\frac{1}{1+L}\{1+\kappa_R x\}^4$

The familiar measure of cooperativity is the Hill slope, which is determined by the ratio of slope of the observed binding curve to the slope of the binding curve for a molecule with identical independent sites. The Hill slope n_H is given then as follows:

$$n_H = \left(\frac{d\ln\theta}{d\ln x}\right)_{x=\xi_i} \Big/ [\theta(1-\theta)] \qquad [4]$$

Curve fitting to the various models outlined above was performed according to the Gauss-Newton algorithm of Marquardt (18), giving equal weight to all data points. The adequacy of the fit to a particular model with P parameters is indicated by the estimate of the variance σ^2 given as the square root by

$$\sigma = \left[\frac{\sum[\Delta A(\xi_i)^{obs} - \Delta A(\xi_i)^{theo}]^2}{N-P}\right]^{1/2} \qquad [5]$$

where the summation is taken over N data point and the observed absorbance change values are compared with the theoretically calculated values using equations [2] and [3]. A Hewlett-Packard model 9122 computer was used for the data analysis. Confidence limits of the fitted parameters were determined by F-testing each parameter independently (18).

RESULTS

Oxygen and carbon monoxide binding data for HbA_0 in the presence of IHP and BZF for typical thin layer experiments at 20°C are shown in Figure 1. The results are given as the slope of

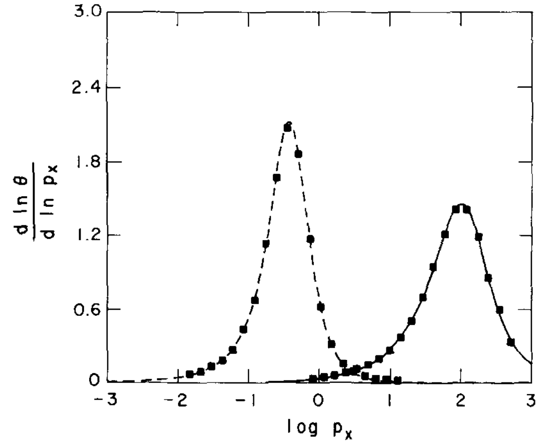

Figure 1: Oxygen and carbon monoxide derivative binding isotherms, $\frac{d\ln\theta}{d\ln p_X}$ where θ is the degree of saturation and p_X is the partial pressure of ligand X (CO or O_2) versus the logarithm of the partial pressure for HbA_0. The experimental points (□) are shown along with the theoretical curves (--- CO; — O_2) drawn from the best fit values of the Adair parameters. Solution conditions for CO were: 0.15 M HEPES, pH 7.0, 5 mM BZF, 15 mM IHP, 600 μM heme; and for O_2 were: 0.13M HEPES, pH 7.0, 4mM BZF, 13mM IHP, 1mM heme. Both sets of data were collected in the Soret region (CO at 419nm and O_2 at 415nm) at 20°C.

binding curve (see equation [2]), computed from the optical absorbance change for stepwise changes in the partial pressure. The data in Figure 1 was first fit to the four site Adair formulation yielding the best fit parameters reported in Table 1. Also summarized in this table are values for the square root of the variance, σ. The magnitude of the Adair model σ values agrees with the estimated noise level of the absorbance measurements, indicating that the Adair model provides as satisfactory a representation of the observed data as one could expect.

TABLE 1

Resolution of Equilibrium Parameters for CO and O_2 Binding to HbA_0

Model	Parameters	Oxygen		Carbon Monoxide	
Adair	β_1 torr^{-1} [b]	0.0314	(0.0275, 0.0357)[c]	4.0	(3.3, 4.9)[c]
	β_2 torr^{-2}	3.74×10^{-4}	(3.30×10^{-4}, 4.19×10^{-4})	11.7	(9.7, 13.7)
	β_3 torr^{-3}	2.67×10^{-6}	(2.18×10^{-6}, 3.18×10^{-6})	8.2	(2.5, 14.0)
	β_4 torr^{-4}	2.32×10^{-8}	(2.03×10^{-8}, 2.63×10^{-8})	109	(99, 120)
	ΔA_T[d]	0.2585	(0.2545, 0.2626)	0.148	(0.146, 0.150)
	σ[e]	2.9×10^{-4}		1.9×10^{-4}	
TTA	σ	10.9×10^{-4}		2.3×10^{-4}	
MWC	σ	4.1×10^{-4}		2.7×10^{-4}	
MWC(het)	σ	4.1×10^{-4}		2.8×10^{-4}	
MWC(Tnest)	σ	3.2×10^{-4}		1.9×10^{-4}	

[a] Solution conditions given in Figure 1 legend.

[b] The equilibrium constants, β_i, are for the reaction: $Hb + iX(g) \rightleftharpoons Hb(X)_i$.

[c] Upper and lower limits are for 67% confidence.

[d] ΔA_T in the total absorbance change for complete ligation.

[e] σ is the square root of the variance of the fit.

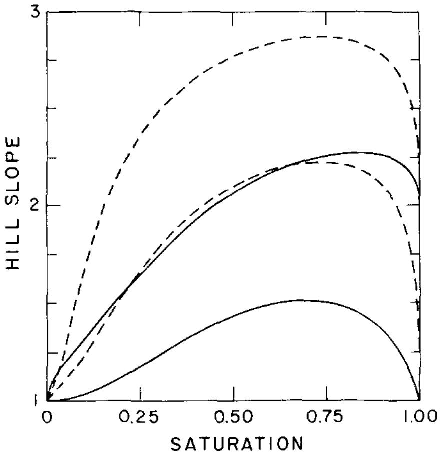

Figure 2: Hill coefficients for oxygen and carbon monoxide binding to HbA$_0$ as a function of degree of saturation θ. Carbon monoxide curves (---) and oxygen curves (—) under two solution conditions: IHP & BZF (bottom) and IHP alone (top) (see (11)).

The derivative, or slope, of the binding curve is proportional to the binding capacity (19) which is a fundamental measure of the cooperativity of binding. However, the Hill slope is particularly convenient for judging the degree of cooperativity and may be calculated from equation [4] as a function of the degree of saturation. The resulting Hill slope values are shown plotted in Figure 2 along with values for conditions where only IHP is present (20). The cooperativity is greatly diminished for either ligand from the normal values of approximately 3 in the absence of the allosteric effectors. The maximum Hill slope in the presence of IHP and BZF is 2.23 for CO and 1.51 for O_2. The low values of the Hill slope along with the approach to unit values at high saturations immediately suggests the presence of significant populations of triply ligated species.

The populations of the different stoichiometric ligated hemoglobin species are determined from the appropriate terms in the binding polynomial. Figure 3 shows the species fractions for the

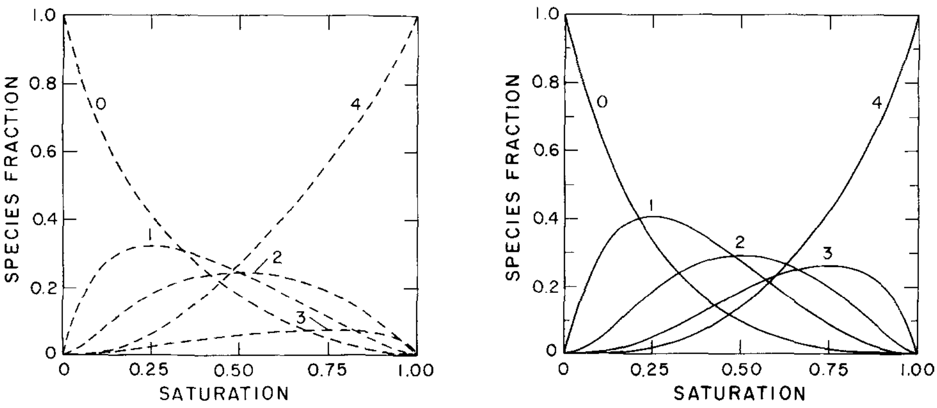

Figure 3: Stoichiometric species fractions of ligated hemoglobin HbA$_0$ for CO (left) and O_2 (right). The curves are specified by the number of ligands bound and were calculated from the best fit Adair parameters listed in Table 1.

various ligated forms for CO and O_2 as a function of degree of saturation. There is a substantial population of triply liganded species for both CO, and especially for O_2 when both IHP and BZF are present. These observations represent the first unequivocal occurrence where we have been able to delineate the presence of significant quantities of triply ligated species for reactions with O_2 and CO in concentrated solutions of HbA_o. As such, they have a direct bearing on mechanistic ideas concerning the functional basis of ligand binding cooperativity in hemoglobin.

DISCUSSION

As noted, previous studies in our laboratory of ligand binding to HbA_0 by means of a thin-layer derivative technique (15) have shown that triply ligated (either O_2 or CO) intermediates do not contribute appreciably to the binding process (1, 20). This striking feature, which imposes stringent constraints on possible models of Hb cooperativity, was reconciled by the formulation of the truncated two-state allosteric model (TTA)(4) described above.

The present study provides a direct measure of the functional properties where the T form is greatly stabilized by combined use of the potent allosteric effectors, BZF and IHP. We fit the data to four two-state allosteric models: 1) the TTA model, 2) the simple MWC model (21), 3) the MWC(het) model in which the T state allows for differences in binding to the α and β chains, and 4) the MWC(Tnest) model which extends the TTA model by inclusion of a second allosteric T form in which ligand binding is identical to both α and β chains. The concept of nested allosteric models is described elsewhere (22) and has also been invoked in formulating the idea of cooperative pairs of sites termed cooperons (23). The results are summarized in Table 1. For CO, the TTA model still fits the data nearly as well as a four site Adair equation, while the MWC and the MWC(het) models fit less well. Inclusion of the two nested allosteric forms in the T state allows one to fit the data as well as the Adair formulation. For O_2, the situation is even more revealing: the TTA, MWC, and MWC(het) models are totally inadequate. On the other hand, the MWC(Tnest) model again fits the data as well as the Adair model. These analyses show first of all that BZF and IHP together promote accessibility to the β chains in the T state. Similar conclusions were also reached in experiments at other temperatures, which are not discussed in detail here.

The simple two-state allosteric models are inadequate in representing oxygen and carbon monoxide binding to hemoglobin in the combined presence of the strong allosteric effectors IHP and BZF. An acceptable model for both CO and O_2 is found by invoking the presence of two allosteric substates within the T form. Structural evidence for such T forms has been given by Perutz (24). Perutz's and our designatiion of the two allosteric T substates correspond to t_β for T_t and t_β' for T_r. Observed non-cooperative oxygen binding to the β chains of the T state $(\alpha\text{-}Ni^{II})_2(\beta\text{-}Fe^{II})_2$ molecule (25) also is consistent with the functional properties we ascribe to the relaxed T_r allosteric sub-state. In the T_t form, binding occurs only to the α chains in a cooperative manner and is presumably the same form as employed in the TTA model. The relaxed form (T_r) is assumed to be open to binding to both the α and β chains. Binding is assumed to be the same for each chain. The allosteric equilibria between the two forms is sensitive to the combined presence of BZF and IHP, such that a significant amount of the T_r form is present.

As seen in Figure 3, the population of triply ligated species, for both O_2 and CO, reaches significant levels. Marden et al (9) concluded from the analysis of their CO kinetic results based on the simple MWC model that the ratio $[T_3]/[R_3]$ was approximately equal to 20. Using the parameters we found by fitting our equilibrium data to the regular MWC model, we obtain a ratio of 10 for CO and 100 for O_2. This agreement of the kinetic results with the equilibrium analysis supports the general ideas of a two state allosteric model invoked in both studies. These values also show that the triply ligated R species remains well below 1% under all ligand conditions. Thus determination of the equilibrium constant between R_3 and R_4 becomes extremely difficult if not impossible. We found this to be the case for the more elaborate allosteric models as well.

The accurate representation of the equilibrium data (IHP and BZF) by the MWC(Tnest) model supports the previous suggestion that the T-state exhibits cooperative interactions in binding to the α chains. Also one finds agreement with the previous studies without BZF (1, 5-8) through the indication that the population of the triply ligated R species is less than 1%. In the presence of IHP and BZF binding to the T-state involves both α and β chains, as though these allosteric effectors act in concert to promote a unique allosteric T-state, designated as T_r. The MWC(Tnest) model explicitly reflects these features.

The present information does not rule out other possible allosteric models, such as the more complex nested allosteric model (26) used to describe features of the cooperative energetics of the ten methemoglobin intermediates (27). Such models contain even more parameters, which at the current level of experimental information cannot be resolved with reasonable confidence. An extended exploration of O_2 and CO with extensive IHP and BZF concentrations should allow more critical evaluation of the mechanistic features of the linkage properties oxygen and carbon monoxide binding in terms of structural features available from crystallographic information (28).

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