Allosteric kinetics and equilibria differ for carbon monoxide and oxygen binding to hemoglobin

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ABSTRACT We have measured the forward and reverse rates of the allosteric transition between *R* (relaxed) and *T* (tense) quaternary structures for oxyhemoglobin A from which a single oxygen molecule was removed in pH 7, phosphate buffer, using the method of modulated excitation (Ferrone, F. A., and J. J. Hopfield. 1976. *Proc. Natl. Acad. Sci. USA.* 73:4497–4501 and Ferrone, F. A., A. J. Martino, and S. Basak. 1985. *Biophys. J.* 48:269–282). Despite the low quantum yield, which necessitated large light levels and an associated temperature rise, the data was of superior quality to the equivalent experiment with CO as a ligand, permitting comparison between the allosteric behavior of hemoglobin with different ligands. Qualitatively, the *T* structure is favored more strongly in triligated oxyhemoglobin than triligated carboxyhemoglobin. The rates for the allosteric transition with oxygen bound were essentially temperature independent, whereas for CO both the $R \rightarrow T$ and $T \rightarrow R$ rates increased with temperature, having an activation energy of 2.2 and 2.8 kcal, respectively. The $R \rightarrow T$ rate was higher for O_2 than for CO being $3 \times 10^3\ s^{-1}$ vs. $1.6 \times 10^3\ s^{-1}$ for HbCO at 25°C. The $T \rightarrow R$ rate for HbO_2 was only $2 \times 10^3\ s^{-1}$, vs $4.2 \times 10^3\ s^{-1}$ for HbCO, giving an equilibrium constant between the structures greater than unity ($L_3 = 1.5$). The data suggest that there may be some allosteric inequality between the subunits, but do not require (or rule out) ligand binding heterogeneity. The ligand-dependent differences are compatible with stereochemical studies of HbCO and HbO_2. However, the large population of *T* species with three oxygen molecules bound is much greater than predicted by precision equilibrium studies and a generalized Szabo-Karplus model (Lee, A. W., M. Karplus, C. Poyart, and E. Bursaux. 1988. *Biochemistry.* 27:1285–1301) or by the allosteric model of DiCera (Di Cera, E., C. H. Robert, and S. J. Gill. 1987. *Biochemistry.* 26:4003–4008).

INTRODUCTION

Hemoglobin has long been used as a model for the thermodynamic and kinetic behavior of allosteric proteins. As with most cooperative systems, however, intermediates are difficult to obtain for comparison with theories, and means have been sought to artificially populate these otherwise unmeasurable species. The use of chemical modifications or alternate ligands, however, may alter the very cooperative properties investigated.

Another approach is to study the intermediates by kinetic means, producing transient populations of the intermediates that exceed their equilibrium values. Typical kinetic experiments involve rates in a single direction, so that equilibrium constants are not obtained directly. Modulated excitation, a quasi steady-state technique in which a small periodic perturbation is used, does generate both forward and reverse allosteric rates and hence the equilibrium constant (Ferrone and Hopfield, 1976; Ferrone et al., 1985*b*). Modulated excitation has been previously used with carboxyhemoglobin because of its high photochemical yield (Brunori and Giacometti, 1981), but this again presents the weakness that a model ligand has been used in place of the physiological one. Whereas CO is believed to be a useful model for O_2 binding to hemoglobin, thermodynamic (Di Cera et al., 1987*a*) and structural (Shanaan, 1983) differences have been noted. One positive outcome of the inequivalence of ligands is that it might be possible to relate a change in the protein structure to its function in a way that would be more difficult with larger and more widespread differences.

We report here the successful adaptation of the method of modulated excitation to photolysis of oxyhemoglobin. The low photochemical yield is overcome by using an intense focused laser to generate the <1% photolysis required for the method. The thermal heating attendant on such an experiment is held in check by use of thin samples (Ferrone et al., 1985*a*), though there is still a significant temperature rise. The goal was to compare the data on HbO_2 with that obtained on the more thoroughly investigated HbCO, and to determine the equilibrium constant between triply ligated *R* and *T* structures for comparison with theoretical predictions.

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MATERIALS AND METHODS

Hemoglobin was purified as described elsewhere and stored frozen (Ferrone et al., 1985*b*). When stock solutions were thawed, 2 mM sodium dithionite was added to reduce any methemoglobin present, and the sample was then immediately passed through a small preparative column (PD-10, 9 ml column of Sephadex G-25M; Pharmacia Fine Chemicals, Piscataway, NJ) to remove the dithionite and its products. The column was eluted with pH 7.0, 0.15 M phosphate buffer and the hemoglobin was then concentrated to ~1 mM using Centricon-30 microconcentrators (Amicon Corp., Danvers, MA). Samples so purified were used within 1 wk. Samples were sealed between coverslips in room air. Final Soret absorbance was 1 OD or less. Sample temperature during the experiment was regulated by a thermoelectric controller. For operation below the dew point, a nitrogen flow system was used to eliminate condensation. Absorption spectra were monitored in the Soret during experiments, and no significant changes were seen. A set of HbCO samples were made as previously described (Ferrone et al., 1985*b*) for comparison with the oxyhemoglobin data.

The modulation apparatus is that described by Martino and Ferrone (1989). Modulated absorbance spectra were collected between 400 and 450 nm; excitation was accomplished by a modulated dye laser with emission at 573 nm. Laser power ranged from 8 to 14 mW. The beam was focused to a diameter of 150 μm, and the center of the beam was masked for observation by a field diaphragm. For experiments with CO as a ligand, laser power was reduced by a combination of optical filters and electronic control of the modulation level so that <1% of the hemes were photolyzed. Whereas the excitation level in this type of experiment is purposefully kept small, it is important to note that this does not make the technique prone to small sample impurities unless those impurities will be excited preferentially.

In this method two types of measurement are performed. In the first, a spectrum of the modulated species is measured at a fixed excitation frequency, ω. The monochromators are set to 436.5 nm, an isosbestic of the R and T spectra, and the detection system is phase tuned to bring the entire signal into the in-phase channel. Thus, the in-phase spectrum is contributed equally by R and T state populations, and the detection system is in tune with the *result* of excitation. The out-of-phase spectrum (rotated 90° from the in-phase spectrum) is consequently dominated by the spectrum arising from the change between R and T. The ratio of the out-of-phase signal to the in-phase signal is denoted Γ and is related to the kinetic processes which populate and depopulate the T state. For equivalent subunits,

$$\Gamma = \mathrm{Im}\left[\frac{T}{R+T}\right] = \frac{k_{RT}\omega}{(k_{RT} + k_{TR} + k_T[O_2])^2 + \omega^2}. \quad (1)$$

In this expression, complex notation has been employed to simplify phase relationships (Ferrone and Hopfield, 1976; Ferrone et al., 1985*b*; Martino and Ferrone 1989). The rates constants k_{RT}, k_{TR}, and k_T denote the rate of transition from R to T, T to R, and the rate constant for ligand rebinding in the T state, respectively. No subscripts are used to designate that these rates are assumed to be those which occur with 3 ligands bound. Eq. 1 peaks when ω equals the sum of the rates. The maximum of Γ is directly related to L_3, the allosteric equilibrium constant between R and T with three ligands, in the limit when $k_T[O_2]$ is negligible. This is the case for CO as a ligand, but not for O_2 as discussed in the results. For inequivalent subunits, the expression for Γ is more complicated, but straightforward. If the populations of tetramers in the T conformation having an α or β subunit available for ligand binding are denoted T_α or T_β, and likewise for R, then Γ for inequivalent subunits is given by the expression

$$\Gamma = \mathrm{Im}\left[\frac{T_\alpha + T_\beta}{R_\alpha + T_\alpha + R_\beta + T_\beta}\right]. \quad (2)$$

Because of the many levels of inequality that can exist (in binding rates, quantum yields, and/or allosteric rates), a full exploration is quite involved, but, as will be shown below, it is unnecessary for the issues addressed here.

The second measurement involves setting the detection monochromator to the laser wavelength, 573 nm, and tuning the electronics so that the laser signal is entirely "in phase." In-phase and out-of-phase measurements are then recorded at 436.5 nm. This puts the detection system in phase with the *source* of the excitation (the laser), so that phase shifts show the system response relative to the excitation. The system response is dominated by the ligand rebinding rate in the R state, and is only weakly affected by the kinetics of allosteric change. Then

$$\tan\phi = \frac{\mathrm{Im}(R+T)}{\mathrm{Re}(R+T)}, \quad (3)$$

where again R and T are complex quantities. Asymptotically, as ω becomes large, the tangent of phase is given by $-\omega/k_R[O_2]$. In the case of inequivalent R state binding to the subunits, k_R is the average of the two rate constants.

To determine the effect of heating, a control experiment was performed using a thin slide of cresol red dye of similar thickness to the hemoglobin samples. The dye was dissolved in 1 M, pH 8.0 Tris buffer. Laser energy absorbed by the dye leads to a change in temperature and a pH shift in the buffer. The cresol dye then monitors this pH shift. The actual measurement was by the following null method: the transmission of a sample of dye in Tris buffer was measured at 573 nm. The stage temperature was lowered, and a new transmission was measured, whereupon the laser intensity was adjusted to return the transmission to its first value. At that point, the temperature drop was compensated by the laser intensity. The procedure was then repeated several times to insure against damage to the dye. To obtain the heating for the Hb sample, this temperature rise was then adjusted for laser intensity in the Hb experiment and the difference in sample absorbance.

Whereas the DC thermal effects of the large laser power are noticeable, the AC effects of heating can be neglected. The slow nature of heat transfer suggests that there should be no effect at the frequencies employed here, and this is confirmed in two observations. First, there is no unaccounted spectrum in the data, which ought to appear as a difference spectrum centered on the oxy peak. Second, because of the slow nature of the heating and cooling process, if a significant AC effect were present, it would be largest at low frequencies. In the Γ analysis, the low frequency points decrease toward zero.

RESULTS

Experiments were performed on samples at different temperatures for excitation frequencies between 200 and 3,000 Hz. Excitation ranged between 0.4 and 1%, with accompanying heating of oxyhemoglobin samples between 4 and 15 degrees, and negligible heating of the carboxyhemoglobin sample.

Modulated spectra were collected between 400 and 450 nm. Typical spectra are shown in Fig. 1. The high frequency data had less noise than similar spectra for

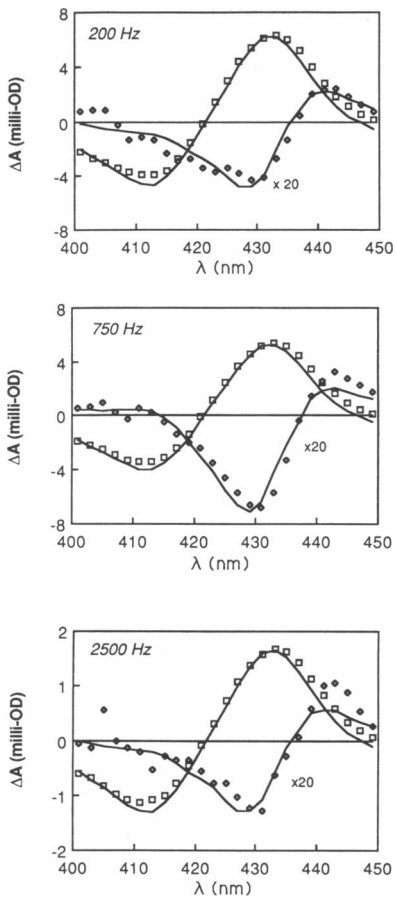

FIGURE 1 Typical spectra in phase (*squares*) and out of phase (*diamonds*) for oxyhemoglobin modulation. Out-of-phase spectra are enlarged 20 times compared with the in-phase spectra, whose magnitude is listed on the *y*-axis. Fits are shown as the solid lines, and were composed from an oxy minus deoxy spectrum and a *T* minus *R* (deoxy) difference spectrum. Sample temperature was 30°C. (Stage temperature was 15°C, and the heating due to the laser was also 15°C.)

HbCO due to the higher rebinding rate for HbO_2. As can be seen, the in-phase spectra were well fit by the two standard difference spectra of oxy minus deoxy and $R - T$ difference. The out-of-phase spectra, also well fit, are quite clearly dominated by the *T* state minus *R* state (deoxy) spectrum. In modulated excitation experiments with CO, an allosterically sensitive liganded spectral difference was found which resembled a small wavelength shift of the COHb peak (Ferrone et al., 1985*b*). In the experiments reported here there is no evidence for an allosterically sensitive spectral difference in oxyhemoglobin, which would have been expected as a perturbation of the HbO_2 Soret band peak position.

The frequency dependence of the normalized out of phase signal (Γ) is shown in Fig. 2 for the experiments on HbO_2. Three temperatures are shown: 7°C, 18°C, and

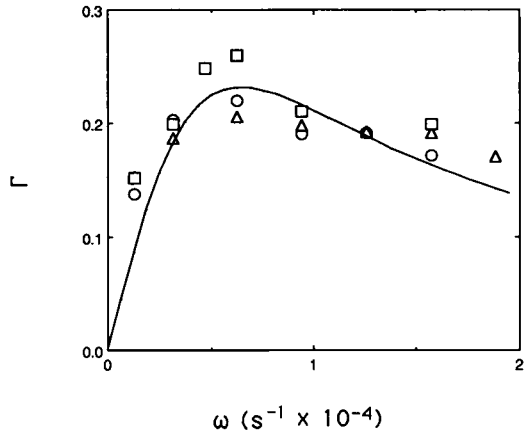

FIGURE 2 The frequency dependence of the normalized out-of-phase signal (Γ) for HbO_2. This signal is a measure of the amount of allosteric conversion as a function of frequency. Three experiments are shown, at 7°C (*circles*), 18°C (*triangles*), and 30°C (*squares*). The curve is drawn using Eq. 1, with $k_{RT} = 3 \times 10^3\ s^{-1}$, $k_{TR} = 2 \times 10^3\ s^{-1}$, and $k_T = 7 \times 10^6\ M^{-1}s^{-1}$ (McCray 1972).

30°C (including laser heating). No temperature dependence to the allosteric kinetics is in evidence.

Fig. 3 shows Γ for two CO experiments at 15 and 35°C. Here, the peak position and height clearly depend on temperature, in contrast to the HbO_2 results. Moreover, it is also evident that the maximum value of Γ for the CO experiments is in all cases less than that for HbO_2.

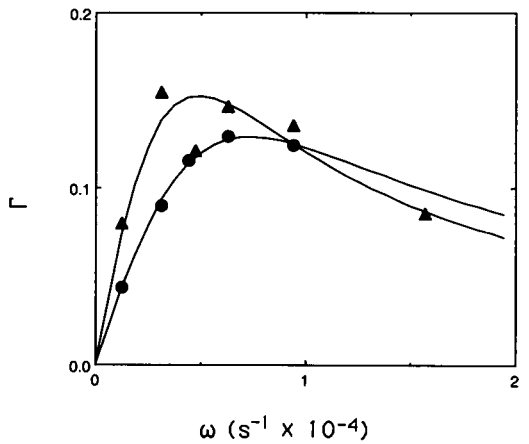

FIGURE 3 The frequency dependence of Γ for two HbCO experiments at 15°C (*triangles*) and 35°C (*circles*). Note that the values for Γ for the HbO_2 data of Fig. 2 is higher for all temperatures, i.e., there is less *R* to *T* switching in the HbCO experiment. Curves are drawn using Eq. 1 with the following parameters: for the 35°C experiment $k_{RT} = 1.9 \times 10^3\ s^{-1}$, $k_{TR} = 5.3 \times 10^3\ s^{-1}$, and for the 15°C experiment $k_{RT} = 1.5 \times 10^3\ s^{-1}$, $k_{TR} = 3.3 \times 10^3\ s^{-1}$; for both experiments, $k_T = 1 \times 10^5\ M^{-1}s^{-1}$.

The frequency dependence of the tangent of absolute phase is shown in Fig. 4 for the 30° experiment on HbO_2 and the 35°C experiment on HbCO. The high frequency tangent for the HbO_2 experiment is lower than the HbCO. This is as expected, given the sensitivity of that asymptote to the *R*-state binding rate. The asymptote of the HbO_2 experiment changes ~10% from 7 to 30°C (not shown), but this is near the reproducibility of the experimental method. For the HbCO experiment, experimental problems in maintaining full solution saturation with CO make comparison between different temperatures presently less reliable.

For analysis, we first turn to the HbCO data of Fig. 3. Taking $k_T[CO] = 100\ s^{-1}$ (Parkhurst, 1979), the forward and reverse rates of conformational change can be determined from Eq. 1. The curves in Fig. 3 show the result of fitting to Eq. 1, and provide an excellent description of the data. Both k_{RT} and k_{TR} show a small but definite temperature dependence, as can be seen from the Arrhenius plot in Fig. 5. The activation energy for k_{RT} is 2.2 ± 1.1 kcal/mol, whereas for k_{TR} it is 2.8 ± 1.2 kcal/mol. Interestingly, this leaves open the possibility that L_3 is essentially temperature independent for HbCO as well as for HbO_2. Taking $k_R[CO] = 6{,}000\ s^{-1}$ (Ferrone et al., 1985*b*) gives the curve shown in Fig. 3 for the tan ϕ without any further adjustment of parameters, which again is in excellent agreement with the data.

Because the rate constant for binding O_2 is large, even in the *T* state, allosteric analysis of the Γ data (Fig. 2) is partially dependent on the choice of k_T. Conceptually, this is because ligand rebinding from the *T* states represents a path to escape from T_3 which is competitive with the conformational change. Once $k_T[O_2]$ is specified, k_{TR} is known. Our goal here is to show what results occur for a reasonable choice for this parameter; a detailed choice of k_T will be discussed in future publications. From Eq. 1 it is clear that there is not much latitude for the allosteric rates. Because the maximum Γ occurs for $\omega = k_{RT} + k_{TR} + k_T[O_2]$, it follows that $k_{RT} = 2\Gamma_{max}\,\omega_{max}$. From the data in Fig. 2, this puts k_{RT} in the range $2.1\text{–}3.4 \times 10^3\ s^{-1}$. Even if $k_T[O_2]$ were negligible, L_3 would be at least 0.72, which is still notably greater than that found for carboxyhemoglobin. Using the parameters of McCray (1972), namely, $k_R = 5 \times 10^7\ M^{-1}s^{-1}$ and $k_T = 7 \times 10^6\ M^{-1}s^{-1}$ a good representation of the data shown in Fig. 2 is obtained with $k_{RT} = 3 \times 10^3\ s^{-1}$ and $k_{TR} = 2 \times 10^3\ s^{-1}$, giving an L_3 of 1.5. Without any variation of parameters, the curve describing tan ϕ is obtained as shown in Fig. 4. Both the predictions for tan ϕ and Γ deviate from the HbO_2 data at low frequencies and this discrepancy cannot be resolved by the simple model described here. Possible reasons for the differences are discussed below.

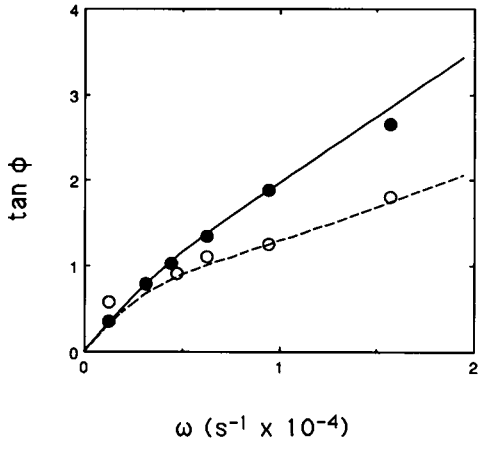

FIGURE 4 The frequency dependence of the tangent of absolute phase is shown for the 30°C experiment on HbO_2 (*open circles, dashed curve*) and the 35°C experiment on HbCO (*solid circles, solid curve*). This measurement is primarily sensitive to the ligand binding kinetics. The high frequency asymptote is given by $\omega/k_R[CO]$ or $\omega/k_R[O_2]$. Curves show the predicted kinetics using Eq. 3. For the HbO_2 experiment, the parameters from Fig. 2 are employed, with $k_R = 5 \times 10^7\ M^{-1}s^{-1}$, (McCray, 1972) whereas for the HbCO experiment, the parameters from Fig. 3 are employed with $k_R = 6 \times 10^6\ s^{-1}$. Note that these curves are predictions, and have not been fit to the tangent data shown.

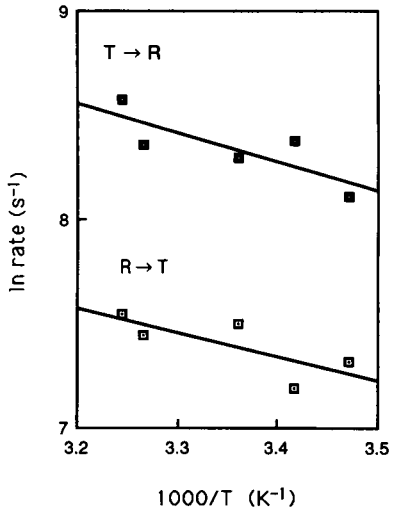

FIGURE 5 Arrhenius plots for the allosteric rates k_{RT} and k_{TR} obtained from the HbCO experiments. Experiments are shown for five temperatures: 15°C, 20°C, 25°C, 33°C, and 35°C. Activation energies were 2.2 ± 1.1 kcal/mol and 2.8 ± 1.2 kcal for the $R \rightarrow T$ and $T \rightarrow R$ rates, respectively.

DISCUSSION

There are two principal aspects of this study. The first is an examination of the effect of the particular ligand on

the allosteric equilibrium and kinetics; the second is a comparison of the value for the equilibrium constant L_3 with detailed models for hemoglobin structure and function. These are examined in turn.

Comparison of ligands

Substituting O_2 for CO as a ligand alters the rates of allosteric change in two respects. (*a*) The equilibrium favors *T* more for HbO_2 than HbCO and (*b*) the allosteric rates in HbO_2 have a lower temperature dependence than HbCO. These can both be rationalized in structural terms. A useful framework has been provided by the description of Gelin et al. (1983), which views ligation in hemoglobin as consisting of changes in an allosteric core which then couple to global quaternary structure changes. Ligation without relaxation of the quaternary structure creates a strain due to the mismatch between the core and its surroundings. Steric hindrance between the proximal histidine and the pyrrole nitrogens requires the into-plane movement to be accompanied by movement of the *F* helix to a position in which the proximal histidine is more normal. Whereas most of the interaction of the core is ligand independent, the known structural differences between HbCO and HbO_2 may play a role. Heidner et al. (1976) and Baldwin (1980) have found, upon x-ray studies of HbCO that the linear CO does not lie normal to the heme plane, but is bent off axis due to the interaction with the His E7 and val E11. Case and Karplus (1978) estimated that ~7 kcal can be taken up in the protein by moving the interfering residues whereas Dedieu et al. (1979) have estimated that 2 kcal are required to bend the ligand from the heme normal. These interactions are for the *R* conformation. The preference for the CO, iron, and proximal histidine to be collinear makes it likely that the strain of the off axis ligand will increase for a ligated *T* structure. This increased strain would rationalize the lessened stability as seen in the value of L_3 for HbCO vs. HbO_2. It also rationalizes the increased activation energy in HbCO as compared with HbO_2 as the increased difficulty of changing structures. This, of course, does not explain the absence of an activation energy in HbO_2. The barrier so envisioned would not necessarily add to the stability of the *R* or *T* states, but would impede their change. This would imply that activation energies for transitions in both directions would be equal as observed.

Sawicki and Gibson (1976, 1977*b*) compared ligand rebinding after full photolysis of CO and O_2 in borate buffer at pH 9. They were able to rationalize their data by assuming that the rate of allosteric change for *R* to *T* decreased with the number of bound ligands and that this decrease was more pronounced for HbO_2 than for HbCO. When combined with experiments on full photolysis, the experiments reported here also demonstrate that the $R - T$ transition rate is decreased with the addition of ligands, but less so for O_2 as a ligand than for CO. Differences in the present results may be because the pH and buffer system differ, or may be due to the fact that the modeling of Sawicki and Gibson's work was not as sensitive to the differences in the ligand as the experiments described here.

The $T \rightarrow R$ transition is affected much more than the $R \rightarrow T$ transition as ligand are added, i.e., from 5×10^4 s^{-1} (Murray et al., 1988) for 0 ligands to 1.6×10^3 s^{-1} for 3 ligands as shown here. One might have expected, therefore, that the greater sensitivity of the $T \rightarrow R$ rate would also be manifest when CO is replaced by O_2. This is not what is seen, implying that a simple transition state picture is insufficient unless the transition state itself is modified by the ligand.

In a simple allosteric description, the difference in equilibrium constants between structures, L_3 can be related to a difference in the allosteric constant *c*, because $L_3 = Lc^3$. From the differences observed, *c* must be smaller for CO than for O_2 by a factor of 1.55. Di Cera et al. (1987*a*) recently observed a difference in ligand binding curves for these two ligands in the presence of the allosteric effector inositol hexaphosphate (IHP). Whereas a difference in cooperativity (i.e., the allosteric constant *c*) between CO and O_2 was seen in that study, it is difficult to exclude the possibility that some of the effect might be due to IHP itself, because IHP has been seen to affect the apparent value of *c* (Shulman et al., 1975). Using the approximation that *c* is the ratio of the first to fourth ligand binding constant, the data of Di Cera imply a difference in *c* between ligands of a factor of 1.49. The close agreement with the results obtained in this study therefore argues that the presence of the allosteric effector does not change the free energy difference due to the change of O_2 to CO.

In previous work on HbCO we have observed an allosteric difference spectrum arising from the ligated hemes as well as the deoxy hemes. Such a difference spectrum is not observed for oxygen as a ligand. This is also plausible as a distal effect on CO. If the CO ligand responds to the the behavior of the allosteric core due to its steric interaction, this could make the CO spectrum allosterically sensitive. Accordingly, the lack of distal interaction of O_2 then renders that spectrum allosterically insensitive.

Given the small, somewhat similar activation energies for both allosteric rates in triligated HbCO, it is possible within the uncertainty of the measurement that their ratio has no temperature dependence, i.e., that there is no enthalpic contribution to the free energy difference between *R* and *T* structures for either ligand. This in turn requires the difference in allosteric equilibrium ($RT \ln \cdot L_3^{O_2} - RT \ln L_3^{CO} = 0.8$ kcal) to be entropic in origin. One

plausible explanation is that, within the liganded T structure, there are fewer possible configurations in which the off axis CO can be accommodated, increasing the entropy.

Comparison with equilibrium models

A central and critical insight into the behavior of hemoglobin was provided by the allosteric model, first proposed by Monod et al., (1965) and subsequently adapted to include kinetics (Hopfield et al., 1972), which postulated that the dominant effect on the affinity is the quaternary structure, not the state of ligation of adjacent subunits. Perutz (1970*a*, *b*) extended those ideas in a stereochemical way by attempting to describe the origin of the cooperativity in terms of the salt bridges that form between subunits. This had the added advantage that the Bohr effect became a natural consequence of the mechanism. Szabo and Karplus (1972) gave this model a complete thermodynamic formulation; however, despite its alluring simplicity the model proved to be unworkable (see Introduction to Lee and Karplus, 1983, and Lee et al., 1988). Lee and Karplus expanded upon the original Perutz-Szabo-Karplus description to allow non-salt-bridge contributions to cooperativity (Lee et al., 1988). This model thus provided a systematic, thermodynamic inclusion of salt bridges into the allosteric model, albeit extended to allow for inequivalence between subunits. By examining the first and last binding constant as a function of pH, the essential pH dependences of the model have been determined, and the molecular parameters deduced (Lee et al., 1988).

In that description, at pH 7 only ~6% of the triligated species are predicted to be in the T state (cf. Fig. 4 of Lee et al., 1988). This could be viewed as an effective L_3 of 0.06. The data obtained in this study show 55% of the triligated species crossed over to the T state. The only weakness in this comparison is that the data presented here is obtained in phosphate buffer, whereas the study of Lee et al. (1988) was done in bis-tris. However, we recently found that the change in buffer system from phosphate to bis tris only decreased L_3 for HbCO by only a factor of ~2 (Martino and Ferrone, 1989). Applying the same correction gives an expected L_3 for HbO_3 of 0.75. This still implies that a large percentage (~43%) of the triligated species would be in the T state.

Because the measurement reported here involves observation of a spectroscopic marker, it is worth asking whether the disagreement might be ascribed to the nature of the probe. The absorbance signals originally used have been augmented in other experiments by fluorescence detection of a DPG analogue which is quenched upon binding, and the agreement of these probes establishes unequivocally that the absorption responds to changes in the protein's quaternary structure (Martino and Ferrone, 1989). Furthermore, pulse-probe studies have demonstrated that the spectral change also corresponds to functional effects (i.e., a change from quickly to slowly reacting form) (Murray et al., 1988). Therefore, we conclude that the spectral measurements employed here are an accurate representation of the protein's ligand affinity.

A natural question then is whether this finding contradicts the extended Szabo-Karplus model. It is difficult to assess the impact of the more than tenfold difference (0.06 vs. 0.75) between predicted and measured L_3 because there are many intricately related parameters in the model, and appropriate "tuning" of some combination may be able to encompass the current result without sacrificing the other successes of the SKL model. However, the current data amply demonstrate the need for such adjustment. This is turn provides precise limitations on the way in which hemoglobin cooperativity can be modeled.

There have also been other attempts to relate the hemoglobin binding affinity to structure. Di Cera et al. (1987*b*) have proposed recently that the triligated T state is not populated in equilibrium measurements due to sterochemical effects which give the beta subunits negligible affinity. Our results here directly contradict that model, in observing a substantial population of triply liganded molecules with the T structure.

Ackers and co-workers have also proposed a number of alternate formulations of the hemoglobin mechanism (Ackers and Smith, 1987; Johnson et al., 1984). Whereas those models in general also find a smaller population of low-affinity triligated species than seen in these experiments, the difference in pH and buffer system make the extrapolation of these results impossible at present.

Recently, Marden et al. (1989) have made the important point that binding curves alone are unlikely to be sufficiently precise to determine the population of the triply ligated state. This is added impetus to utilize experiments such as these in conjunction with equilibrium studies for determining the thermodynamic properties of hemoglobin.

Further improvement

The data have been described in terms of the simplest allosteric description, (Eq. 1–3) neglecting, for example, inequivalence between subunits. Because the model does not fit all the data (most notably, missing the low frequency values), it is appropriate to ask whether elaboration of the allosteric model improves the agreement with data, and how this might affect the conclusions reached above.

Based on a study of oxygen binding kinetics as a function of oxygen saturation, Sawicki and Gibson (1977*a*) concluded that oxygen bound to the T state with different rate constants for the α and β subunits, namely, 2.9 and 11.8 $\times$ 10^6 $M^{-1}s^{-1}$ but without signficant inequality in the R state, which had a single rate constant of 5.9 $\times$ 10^7 $M^{-1}s^{-1}$. The rate constant for R state binding is slightly higher than seen in our data (tan ϕ). To examine the effect of chain inequivalence using the findings of Sawicki and Gibson, the R state rate constant was scaled by 0.84 to equal that found by McCray (1972) (vide supra), and then the T state rate constants were scaled back by the same factor. The resulting curves, which are not shown, are barely distinguishable from those obtained with the parameters described above. Philo and Lary (1990) have found inequivalent binding rates in binding to the R state. When we used their parameters, again, there was no discernible effect on Γ. We therefore conclude that chain inequivalence in ligand binding is not the cause of the missed low frequency points, and that our results are relatively insensitive to ligand binding rate constants.

A possible explanation for the discrepancies is in the heterogeneity of the allosteric rates (which may or may not lead to heterogeneity in the equilibria). Such heterogeneity could simultaneously give the rapid low frequency rise, and the slow drop at high frequencies. Support for different allosteric kinetics for α and β subunits comes from Shaanan's observations on the structure of oxyhemoglobin, in which he noted considerable differences between the subunits (e.g., hydrogen bonding), which did not exist in HbCO (Shanaan, 1983). Because such allosteric inequality creates so many free parameters, distinguishing among possible scenarios becomes almost impossible with the data shown here, and thus has not been explored. This will be investigated in future work.

At atmospheric oxygen pressure, hemoglobin is not fully saturated, and this presents another possible source of the discrepancy between the data and the simple model used here. Because the few percent deoxy will likely be triply liganded species, their excitation will create a doubly ligated species which will switch structures at a different, and presumably more rapid rate. Whereas this will not be a major effect, because the 1% laser excitation does not discriminate between 3 or 4 liganded R state hemes, it could be magnified if the quantum efficiencies are much greater in the T state. A tenfold difference in quantum yield has in fact been reported by Morris and Gibson (1984). Nonetheless, magnification due to T state excitation requires considerable switching to the T state with three ligands bound. A full assessment of such effects will also be dealt with in future work.

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