Modulated Excitation of Singly Ligated Carboxyhemoglobin

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ABSTRACT We have extended the method of modulated excitation, a small perturbation kinetic method, to study ligand binding and conformational change of hemoglobin tetramers with a single ligand bound. To restrict the excitation to the first ligand, only 1% of the hemes have bound CO, and the remainder are kept unliganded. A detailed theory is presented which agrees well with the experimental observations. This method of observing ligand recombination also provides a novel and simple method for determination of hemoglobin concentration.

Additional relaxation processes are also observed. By fitting independently determined spectra to the spectra associated with the relaxations, these processes are assigned as thermal excitation and thermally driven protonation/deprotonation reactions. These added relaxations arise from the deoxy-Hb portion of the samples, and demonstrate that modulated excitation can be used effectively for temperature perturbation in the absence of photodissociation. The spectra observed are not well described by the spectra of allosteric change, however, and we conclude that there is no significant mixing of quaternary states at the first ligation step. In an appendix we present a derivation of the particular features seen in thermally modulated protonation reactions.

INTRODUCTION

Cooperative ligand binding in hemoglobin represents a difference in affinity between the binding of the last ligand and the binding of the first. Understanding the structural basis of cooperativity requires studying various structural events which accompany ligand binding. Modulated excitation is a kinetic method which has been used to study changes in quaternary and tertiary structure (salt bridges) before the last ligand is bound. (Ferrone, 1991, 1994) The essence of the method is to use periodic excitation and phase tuning to remove the event of ligand binding and release per se so as to investigate the related structural events. This can be viewed as providing a reference frame for the study of the structural events. Thus those events are measured relative to the ligand binding and release process.

In this paper we show that it is also possible to use modulated excitation to investigate events surrounding the first ligand binding process. The idea of the experiment is quite simple. A small quantity of HbCO (about 1% of the hemes) is introduced into a sample of deoxy-Hb. Because the affinity of CO is so high, most of the CO remains bound, albeit reproportioned so that only 1% of the hemes have ligands. Thus the dominant species is deoxy-Hb, with about 1% singly liganded Hb. Photolysis removes a CO molecule, which then will be rebound by one of the many deoxy hemes present. Because the deoxy hemes are in great excess, the rate will be proportional to the unliganded heme concentration. This could prove useful, as will be discussed. We have provided preliminary communications of these methods previously (Ferrone, 1991); this is the first complete study to address modulation of the singly ligated species.

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In addition to the obvious interest in studying the first ligand binding event, there are specific reasons such a study is of interest. In a study of the pH dependence of oxygen binding isotherms, Johnson and Ackers (Johnson et al., 1984) proposed that, rather than being almost pure T structure, a substantial fraction of the tetramers with a single ligand bound would be in the R structure.The measurements performed here directly test that prediction. Moreover, Mozzarelli et al. (Mozzarelli et al., 1991; Rivetti et al., 1993) have observed ligand binding to single crystals of hemoglobin, and found that the affinity is lower than solution measurements, with no evidence of a Bohr effect. The decrease in binding constant for the first ligand is about a factor of 6, for a free energy difference of about 1 kcal/mol. It has been proposed (Rivetti et al., 1993) that Hb might exist in the T state in two conformations which differ in the formation of a number of salt bridges, first identified by Perutz (1970a, 1970b). Two states of similar free energy might interconvert readily. Thus also we sought to use the sensitive method of modulated excitation to look for evidence of multiple T-state conformations.

In this paper we present the detailed theory of modulated excitation with a single ligand. We find that the theory that describes the kinetics of binding the first ligand agrees well with the data. In addition, using this procedure provides a simple method for determination of hemoglobin concentration where path length is not known. Additional relaxation processes are observed beyond ligand binding and release. These processes are identified on the basis of independently determined spectra as thermal excitation, and thermally driven protonation/deprotonation reactions. These added relaxations arise from the deoxy-Hb part of the samples, and demonstrate that modulated excitation can be used effectively for temperature perturbation. The spectra observed are not well described by the spectra of allosteric change, and we conclude that there is no significant mixing of quaternary states at the first ligation step. In the Appendix we present

a derivation of the particular features seen in thermally modulated protonation reactions.

THEORY OF MODULATED EXCITATION WITH ONE LIGAND

This section describes the theoretical foundations of the method. The notation of the MWC model (Monod et al., 1965) will be used, in which singly liganded molecules in the T state have a population denoted T_1, etc. The results are quite general, and are independent of the MWC or other model. This is because the critical constructs of the model, e.g., equal, independent ligand binding within a quaternary structure, are not used in our analysis. The rate constant for binding the first ligand will be denoted k_T. First, we assume that there are no additional species, other than single and zero liganded species, T_0 and T_1. Let a be the rate of removing a ligand from a T_1 molecule. a is proportional to quantum yield q, path length z, intensity I, and extinction coefficient ϵ, and is given by $a = 2.3q\epsilon zI = a \cdot I$. Then,

$$\frac{dT_o}{dt} = -k_T[CO](4T_o + 3T_1) + a(e^{i\omega t} + 1)T_1. \quad (1)$$

Note that a T_0 state will have four deoxy hemes. The excitation term is written as a complex number because phase relationships are simplified in that representation; the 1 is required to keep the excitation ≥ 0. Both T_0 and T_1 can be written as harmonic series, e.g.,

$$T_0 = \sum_{n=0} T_{o,n}e^{in\omega t}, \quad (2)$$

and similarly for T_1. [CO] is present is such small quantities that it will also have an observable oscillatory character, and we can write

$$[CO] = \sum_{n=0} X_n e^{in\omega t}. \quad (3)$$

For $n \geq 1$, $X_n = -T_{1,n}$, i.e., ligand concentration oscillations are due to loss of ligand from T_1. A similar conservation relationship arises for the protein concentration; we can write $c_o = T_0 + T_1$. Since the total concentration c_o is not oscillatory, the harmonic terms of T_0 and T_1 are also negatives of one another. This allows the rewriting of the first harmonic part of Eq. 1 as

$$\begin{aligned} i\omega T_{0,1} &= -k_T(X_0T_{0,1} + X_1T_{0,0}) + a(c_o - T_{0,0} - T_{0,1}) \\ &= (-k_T(X_0 + T_{0,0}) - a)T_{0,1} + a(c_o - T_{0,0}) \end{aligned} \quad (4)$$

In constructing Eq. 4 we have only kept similar harmonic terms. The total concentration of free ligand, X_o, will be $\ll T_{0,0}$. Furthermore, $c_o - T_{0,0}$ is approximately the initial CO-liganded population, $T_{0,1}$. (There is a small difference due to steady state photolysis.) If, as is usual in modulated excitation, we define the tangent of absolute phase as the ratio of imaginary to real parts of $T_{0,1}$, then clearly,

$$\tan \phi = -\omega/(k_T c_o + a'I), \quad (5)$$

where we have also used the approximation that c_o is nearly $T_{0,0}$.

We next consider the case in which T_1 can convert to R_1 spontaneously. In a complete treatment, R_1 could be photolyzed to R_0, and a four level system would be described and subsequently solved. Because this creates cumbersome equations we will describe instead a simpler system which is physically described by rapid conversion of R_0 to T_0, and negligible rates for T_0 to R_0. Such a system is shown in Fig. 1. Because the ground state T_1 is perturbed, the R_1 state is also perturbed. The population in states R_1 and T_1 interchange with rates denoted k_{RT} and k_{TR}. These in general will differ from rates between R and T structures with different numbers of ligands bound. Using notation analogous to that above (denoting excitations from R and T as a_R and a_T), it is straightforward to show that

$$\begin{aligned} i\omega T_{1,1} = &-(a_T + k_{TR})T_{1,1} - k_T c_o T_{0,1} \\ &+ k_{RT}R_{1,1} - a_T T_{1,0} \end{aligned} \quad (6a)$$

and

$$i\omega R_{1,1} = -(a_R + k_{RT})R_{1,1} + k_{TR}T_{1,1} - a_R R_{1,0} \quad (6b)$$

Because there are three oscillatory states we have the conservation condition that

$$R_{1,1} + T_{1,1} + T_{0,1} = 0. \quad (7)$$

We are interested in the fraction of excitations which cause the structure to change, and are out of phase with respect to the ligand binding signal. The last condition is a practical concern, since it maximizes the signal in real time. Thus we want Γ, which is defined as

$$\Gamma = \mathrm{Im}[R_{1,1}/T_{0,1}]. \quad (8)$$

For convenience we define the ratio $\rho = a_T T_{1,0}/a_R R_{1,0}$, and

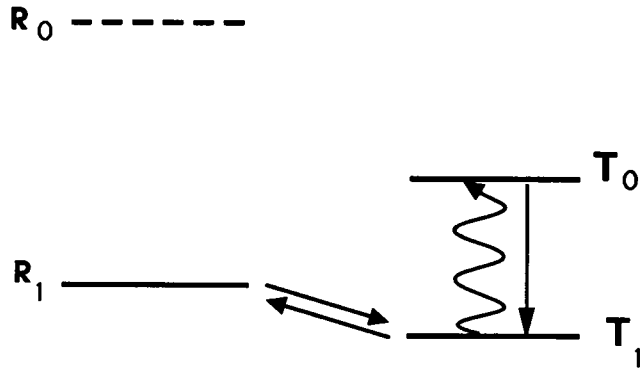

FIGURE 1 Three state model for conformational change with one ligand bound. The vertical axis represents free energy. The oscillatory depletion of the "ground state" T_1 leads to oscillations of the R_1 state in thermal equilibrium with T_1. R_1 is assumed to be sufficiently empty that excitations from R_1 to R_0 can be neglected.

the sum of the allosteric rates as $k_A = k_{RT} + k_{TR}$. Then after some algebra it follows that

$$\Gamma = -\frac{\{k_A + k_T c_o + \rho[k_A + k_T c_o + a_T - a_R] - (1+\rho)^2 k_{TR}\omega\}}{(1+\rho)^2\left[\left(k_A + \frac{a_T + \rho a_R}{1+\rho}\right)^2 + \omega^2\right]} \quad (9)$$

In the limit where $r \gg 1$, as it is in usual MWC parameterizations, then

$$\Gamma = \frac{k_{TR}\omega}{(k_A + a_R)^2 + \omega^2}, \quad (10)$$

which is completely analogous to the expression used in modulated excitation measurements on the three liganded state, namely,

$$\Gamma_3 = \frac{k_{RT,3}\omega}{(k_{A,3} + k_T[CO])^2 + \omega^2}, \quad (11)$$

where analogous quantities whose values differ have been denoted with a subscript 3 (Ferrone, 1991, 1993). From Eq.10 it is clear that the concentration dependence is absent and in Eq. 9, it can be seen the c_o will only enter weakly. Hence a signature of any hypothesized spectral feature assigned to the allosteric change must be its lack of dependence on the concentration of Hb.

MATERIALS AND METHODS

Hemoglobin was prepared according to standard procedures used in this laboratory and stored frozen in liquid nitrogen (Martino and Ferrone, 1989). When thawed, the hemoglobin was concentrated as required by the use of Centricon centrifuge filtration devices. Hemoglobin was used in phosphate buffer, at pH 7.02. Sodium dithionite at a concentration four to five times that of the hemoglobin was added to the solution to fully reduce and deoxygenate samples. A separate aliquot of Hb was flushed with CO, and 1% by volume of the resulting HbCO was added to the original deoxygenated Hb. 5 to 10 μl of the resulting solution were placed on a microscope slide and sealed with sticky wax in a nitrogen-purged cold chamber.

Thermal difference spectra were obtained in the following way. A sample of deoxy-Hb was sealed in a cuvette, in which a thermistor probe was also placed. The cuvette was placed in the thermostatted holder of an HP 8452 diode array spectrophotometer. The temperature setting of the thermostatting bath was then changed. Since the diode array spectrophotometer records a complete spectrum rapidly, we collected spectra as the temperature changed, recording the temperature in the cuvette at the time each spectrum was taken. The thermostat was then changed, and the process repeated, followed by another cycle of temperature decrease and increase. By following the temperature changes in both directions, any time-dependent changes are excluded from the analysis.

The 49 spectra were then reduced by singular value decomposition techniques (Shrager and Hendler, 1982). The data matrix A is decomposed into the product of three matrices, thus $A = USV^T$. The matrix U contains a set of basis spectra in which the first column, U_1, is the best single spectrum description of the data, U_1 and U_2 are the best two spectra description of the data, etc. S is a diagonal matrix whose elements are called the singular values, which describe the relative importance of the spectra to the complete description. The columns of V describe the evolution of the basis spectra as time and temperature change. These mathematical descriptions of the data A are related to physical spectra, D, which are linear combinations of the columns of U. Each physical spectrum corresponds to a physical process (temperature change, drift). A matrix called F describes the *assumed* processes. In our case, these are a constant (background), linear temperature dependence, and linear drift. Thus it is also true that, in matrix notation, $A = DF$. To convert basis spectra U to physical spectra D requires knowledge of the appropriate mixing coefficients. These are determined by fitting the columns of V to the functions contained in F. Fig. 2 *a* shows the three principal spectra obtained in this way; this gives the spectrum shown in Fig. 2 *b* for the thermal difference. Fig. 2 *c* shows the fit to the temperature line. As can be seen, an excellent fit is obtained.

Spectral change due to pH change in the solution was also obtained. This was done by collecting nine spectra of deoxy-Hb at 8.2 μM at different pH values. SVD procedures were again employed, this time using a constant and a Henderson-Hasselbach equation in the fitting matrix F. Fig. 3 *a* shows the principal spectra. Fig. 3 *b* shows the physical spectrum corresponding to the pH difference spectrum. Fig. 3 *c* shows the titration curve of the spectrum shown in 3 *b*, which has a pK of 6.6

Modulated excitation measurements were performed as described elsewhere (Zhao et al., 1993). Because the sample has a 1% maximal photolyzable component, the customary caution of not exciting more than 1% of the sample is not a concern here. Laser power was set by the electrooptic modulator, and was measured by monitoring the output of the electrooptic modulator with an AC and DC digital voltmeter.

RESULTS

From Eq. 5 it is clear that $\omega/\tan\phi$ should be linear in laser intensity, with an intercept which yields the rate constant k_T. Fig. 4 shows such experiments for two different concentrations. As can be seen the linearity is good, and the value of k_T obtained is 112 mM^{-1} s^{-1}, in good agreement with other workers (Parkhurst, 1979).

From Eq. 5, it is evident that $\tan\phi$ should also be proportional to frequency, with a slope which decreases with increasing concentration. Fig. 5 shows $\tan\phi$ vs. ω for 2, 5, and 10 mM (heme) concentrations. The curves are drawn

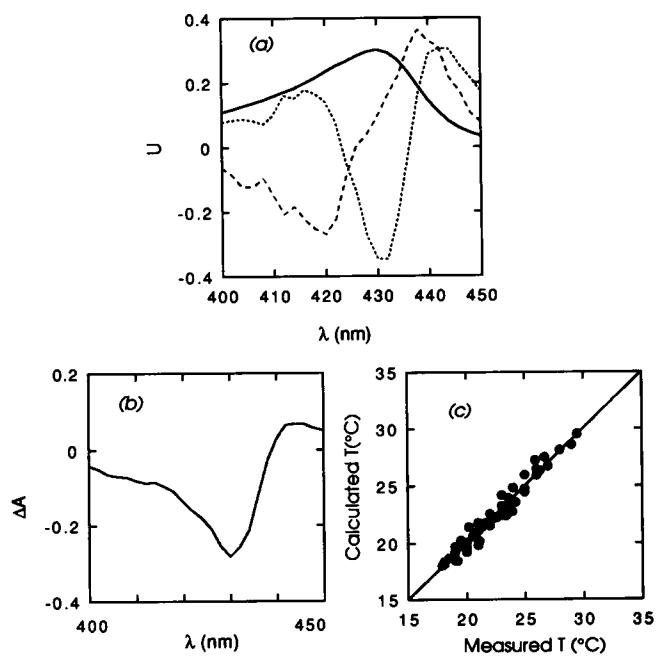

FIGURE 2 Temperature difference spectrum analysis. (*A*) Principal spectra describing the temperature dependence experiment. Singular values were 31, 0.083, and 0.040. (*B*) Spectrum describing physical processes corresponding to temperature change. (*C*) Fit of the spectrum for temperature dependence.

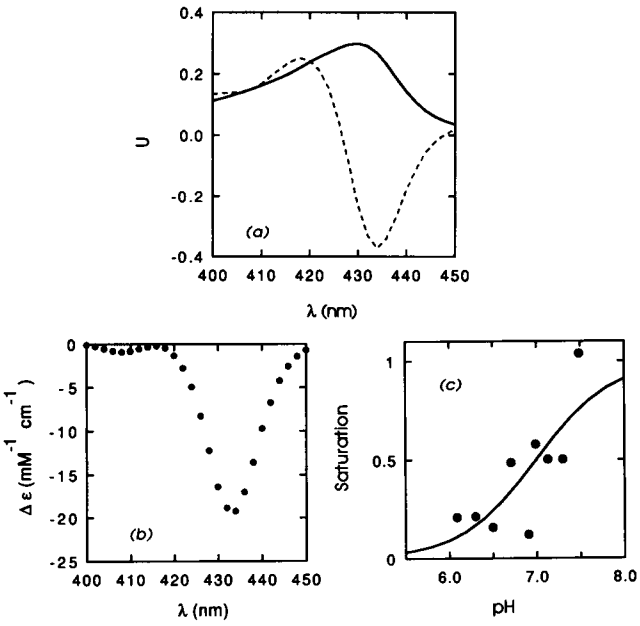

FIGURE 3 pH difference spectrum analysis. (*A*) Principal spectra describing the pH titration experiment. Singular values of the second, and third components were 14%, and 1.4% of the first component. (*B*) Spectral difference of full change between acid, and alkali forms. (*C*) Fit of the spectrum in *B* to a Henderson-Hasselbach equation with pK = 6.63.

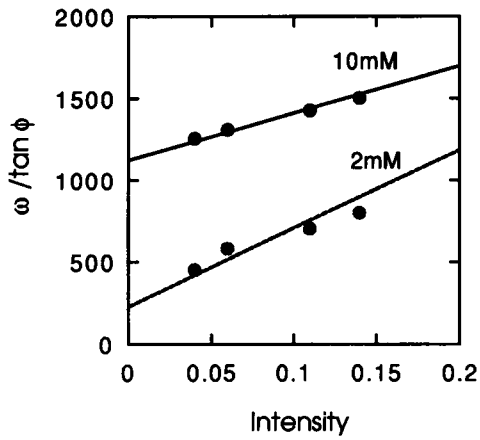

FIGURE 4 $\omega/\tan\phi$ versus laser intensity. The slope gives the constant a' in Eq. 5, while the intercept gives $k_T c_o$. This gives k_T = 112 mM^{-1} s^{-1}.

from the parameters obtained in Fig. 4. The presence of large values of $\tan\phi$ limits the usable frequency range in this experiment, since for $\tan\phi > 1$, the signal level begins to fall off as $1/\tan\phi$.

Modulated excitation spectra were also collected at various frequencies between 40 and 300 Hz for 2, 5, and 10 mM (heme) concentrations. The principal components in and out of phase are shown in Fig. 6. As can be seen, the in-phase component is well described by deoxy-Hb minus HbCO. However, there is also a distinct out-of-phase component. We now turn to the task of identifying that spectrum.

At first sight the out-of-phase spectra resemble the classic difference spectra seen upon conversion of R to T quaternary

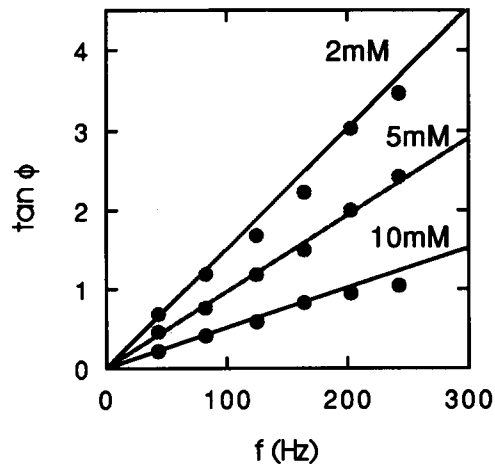

FIGURE 5 tan ϕ versus excitation frequency f for three different protein concentrations. $\omega = 2\pi f$. Lines are drawn from the parameters generated in Fig. 4 and are *not* fits to this data.

structures (Olson, 1976; Perutz et al., 1974). However, an attempt to fit the data shows that there is similarity but not identity. The peak in this data is close to 430 nm, while R-T difference spectra peak near 425 nm. Furthermore, the data between 400 and 410 nm is not at all recovered in such a fit. Inclusion of the deoxy-HbCO difference spectrum to account for mistuning does not rectify the problem either. Fig. 7 shows the fit of the data in the 5 mM sample using the in-phase and R-T difference spectra.

Moreover, from Eqs. 9 and 10, the relative magnitude of the out-of-phase component should not depend on the concentration of hemes. This is also not the case, as can be seen in a rough way without curve fitting by comparison of singular values. For example, the data collected at 10 mM has out-of-phase spectra with singular values that are 13% of the in-phase spectra, while at 2 mM over essentially the same frequency range, the weight of the out-of-phase spectra drops to 7.5%. (Since the data are collected on essentially the same frequency grid, this comparison can be used.)

In this experiment, the laser-induced heating is greater than is usually the case for CO photolysis. This is because the photolysis beam has no way a priori of finding the the 1% liganded hemes, and so a large flux of light is required to photolyze the few hemes containing CO. This suggested that a thermal difference spectrum might be contributing to the out-of-phase signal. The spectrum shown in Fig. 2 *b* clearly is closer to the spectrum observed, having its peak closer to 430 nm, for example.

To confirm the presence of thermal modulation, we prepared a 5 mM sample without injection of the 1% HbCO, and used the same modulation geometry and light flux. This data is shown in Fig. 8. There is still evidence for some CO (about 0.1%) which must represent contamination of the original sample. The in-phase spectra (Fig. 8 *a*) are no longer simple, but show evidence of the out-of-phase contribution. The out-of-phase spectrum is now 51% of the magnitude of the in-phase spectrum, and is almost identical in size with spectra collected for the 5 mM sample with 1% HbCO, although the

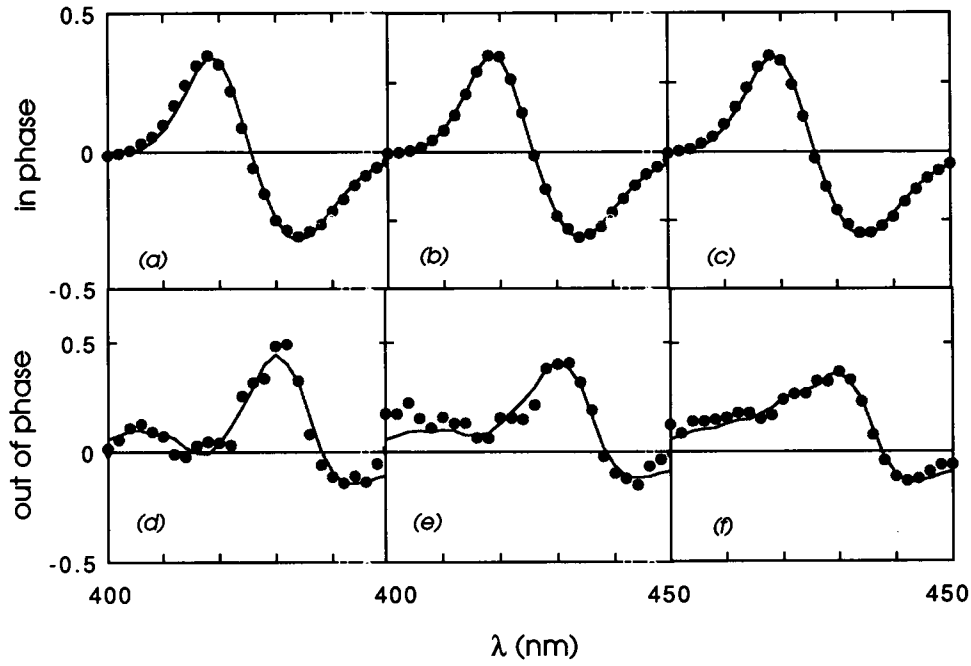

FIGURE 6 Principal components of modulated excitation spectra. (*a–c*) In-phase components for 2, 5, and 10 mM Hb, respectively. (*d–f*) Out-of-phase components for the same set of data. Singular values for *d–f* were 13, 6, and 8% as large as the components in *a–c*. The data have been fit by a combination of three spectra: CO-deoxy difference spectrum, a static temperature difference spectrum (Fig. 2 *b*), and a pH difference spectrum (Fig. 3 *b*).

in-phase spectrum has deceased by about 10-fold. This argues most strongly that the out-of-phase spectrum is not the result of the photolyzed hemes (which have just decreased 10-fold) but of the remainder of the hemes which have been kept constant. Thus the principal contributor is the thermal spectrum. This agrees with the spectra observed. The thermal difference spectrum generated in static experiments (Fig. 2 *b*) provides a very good fit of the out-of-phase spectra in Figs. 6 and 8.

It is interesting, however, that the in-phase part of Fig. 7 is not perfectly fit by the inclusion of the thermal spectrum, but shows a significant difference in the 436–440-nm region. One possible explanation for this difference is that the thermal difference spectrum may have more than one component, and these components may have slightly different relaxation rates. For example, if a change in temperature caused a reaction to change its equilibrium point, as well producing purely spectroscopic changes, the net spectrum would have a component which followed temperature perfectly (being electronic relaxation) and a component which had its own relaxation kinetics due to its reaction rate. In the case of Hb, the well known salt bridges are major enthalpic contributors to oxygenation (Ackers, 1980), and thus a change in temperature ought to change average occupancy of the salt bridges as well as changing the heme spectra. Changes in salt bridges are mimicked by changes in pH, and hence we used the pH difference spectrum (Fig. 3 *b*) as a third fitting component. The fits are excellent as can be seen in Figs. 6 and 8.

The frequency dependence of the tangents of phase angles of the average thermal and the pH spectra is shown in Fig. 9. Two things are of note. First, the tangents (and of course the phase angles) are not much larger than those observed in the ligand rebinding signal (compare with Fig. 5). This indicates that laser heating and cooling modulated the temperature in the sample. The higher concentration samples are thinner, and can cool faster. Therefore, they have smaller phase angles. The second point of interest is that the pH phase angles are smaller than the thermal angles—all the tangents for pH spectral change lie below those for the thermal change. This is discussed below.

DISCUSSION

The results show that the kinetics of the first ligand binding to Hb can be measured accurately by modulated excitation. The spectrum which corresponds to ligand release and rebinding is accompanied by smaller spectra whose magnitude depends on the deoxy heme concentration rather than the photolyzable component. This spectrum is well described by thermal and pH difference spectra, and is not well described by the spectrum of allosteric change.

It has been assumed that α and β subunits have the same properties. This assumption is justified by NMR studies on CO (Ho, 1992), as well as by equilibrium studies on crystals in which the magnitude of thermodynamic inequivalence for O_2 is less than a factor of three difference between the subunits (Rivetti et al., 1993). (In the data we have collected, this limit cannot be made smaller, and allows differences of about a factor of 4.)

Modulated excitation of singly liganded species can be a useful tool for determination of concentration of Hb. For

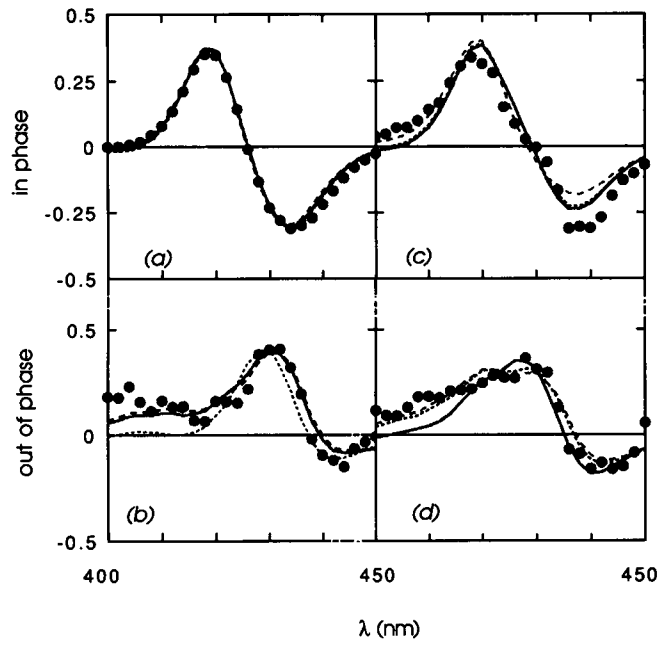

FIGURE 7 5 mM sample data fit by several different spectra. (*a* and *b*) In- and out-of-phase spectra for the 5 mM sample with 1% HbCO. (*c* and *d*) In- and out-of-phase spectra for the 5 mM sample nominally deoxygenated (with a trace HbCO). Solid line shows a fit using HbCO-Hb difference spectra, and R-T difference spectra. Dashed line shows a fit using HbCO-Hb difference spectra, and thermal spectra (from Fig. 3) only. Dotted line shows HbCO-Hb, RT difference spectra, and thermal spectra. None of the three provides a good overall fit. Fits in Figs. 6 and 8 have been executed using the pH difference spectra, and those are clearly superior.

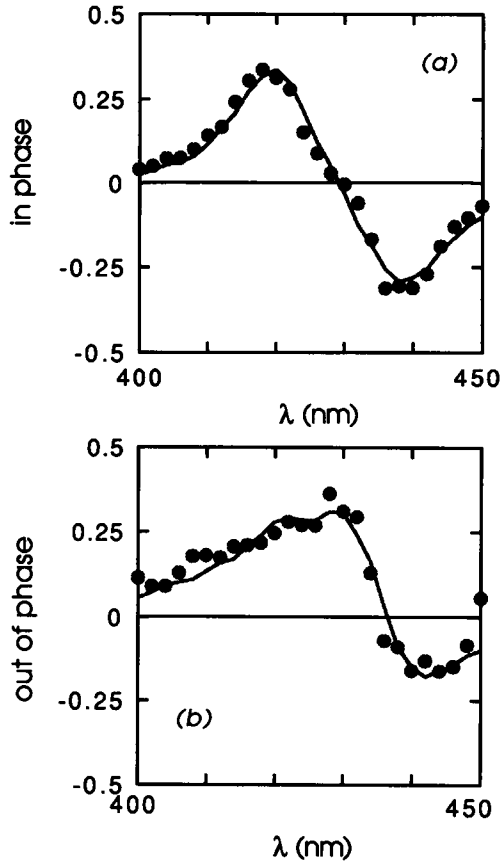

FIGURE 8 Principal components in sample without added CO. (*a*) In-phase spectrum. Singular value of the main component is 1.16; next component (not shown) is 0.11. (*b*) Out-of-phase component. Singular value of its main component is 0.59; the next component (also not shown) is again 0.11. The out-of-phase and in-phase spectra are within a factor of 2 of being the same magnitude. This contrasts with Fig. 6, *b* and *e*, where the out-of-phase component is only 6% of the in phase.

example, the concentration in an erythrocyte could be determined by performing the experiments described here after exposing the red cell to a gas containing a small trace of CO. Using the same procedure as in Fig. 4, i.e., extrapolation to zero laser intensity, the concentration of hemoglobin can be found totally independent of path length.

The absence of an allosteric contribution to the out-of-phase spectrum argues that the singly liganded R state mixes very little with the singly liganded T state. This directly challenges the implication of a thermodynamic model proposed by Johnson and Ackers (1984), namely, that there is a significant population (about 27%) of R states present when a single ligand is bound to a tetramer. From the present data, it appears that <3% of the hemes might belong to switched structures. Although the equilibrium studies were carried out at pH 7.4 in 0.15 M Cl buffer, instead of pH 7 phosphate, the differences are expected to be rather small (compare with the review by Shulman et al. (1975)). As pointed out by Lee et al. (1988), the prediction involves the assumption that the Bohr effect is identical to the R-T switch, and thus its failure can be attributed to that assumption.

Since this is a kinetic method, the question may legitimately be raised whether kinetic effects suppress the expected signal from allosteric conversion between states which could otherwise mix given infinite equilibration time. From information available from the work of others, this must be considered unlikely. Cho and Hopfield (1979) determined the singly liganded RT rate, which they found to be $1.4 \times 10^4\ s^{-1}$. If L_1 ($=T_1/R_1 = k_{RT}/k_{TR}$) ~ 3 as required by Johnson and Ackers (1984) then, from Eq. 9, Γ will have a maximum value of about 0.05. Since there are three deoxy hemes in a singly liganded tetramer which switches structure, the magnitude of the out-of-phase signal is 0.15 times the in-phase signal (i.e., a $\Gamma = 0.05$ for singly liganded hemoglobin is three times as prominent as that of triply liganded hemoglobin), which is a readily observable signal level in these experiments.

We next turn to the thermal and pH effects observed. It is important to recognize that the assignment of the spectral features has been done entirely with spectra generated in static experiments, in which the perturbation is clearly identified. Moreover, while the shapes of the out-of-phase spectra at first appear to be easily described by either RT spectra or thermal spectra, the detailed fitting shown in Fig. 6–8 does not allow this ambiguity. Finally, it is important to note that the spectra must arise from the deoxy hemes since they do not change when the HbCO is drastically reduced.

The oxygen affinity for T-structure crystals of Hb is notably less than the affinity for the first ligand in solution

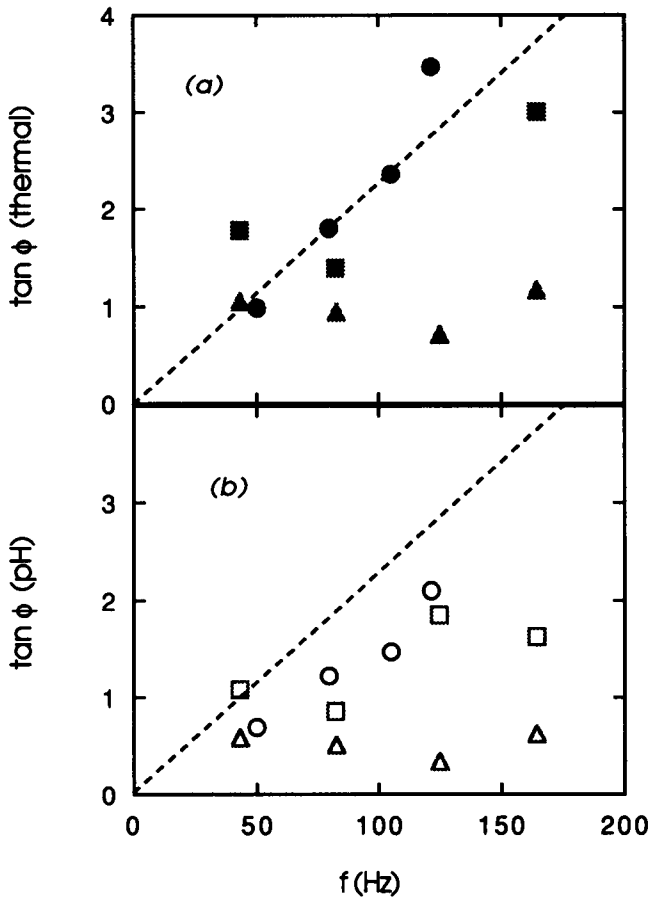

FIGURE 9 Tangent of phase angles relative to the excited population. Tangents were calculated from the ratio of the fit coefficient of the out-of-phase divided by the fit coefficient of the in-phase spectrum.

(Mozzarelli et al., 1991; Rivetti et al., 1993). This has been attributed to the presence of salt bridges in the crystal which are only partially made in solution (Rivetti et al., 1993). Proton reactions are a major source of the enthalpic effects in oxygenation of Hb (Ackers, 1980). Hence, temperature perturbation should produce changes in protonation. This has been seen in conventional temperature jump studies (Okonjo et al., 1989). In our data we see evidence for the thermal modulation driving a pH modulation. Therefore it should be possible in principle to utilize the findings we have presented here to follow the salt bridge formation and release kinetically. A full analysis requires that the thermal difference be decomposed into pH and spectroscopic parts. Such studies are now underway. However, we can examine our kinetic data in a preliminary way, to see whether it is consistent with such a view.

Since the thermal excitation is driving the pH changes, it seems contradictory at first glance that the temperature lags the excitation more than the pH changes lag the excitation. This is actually expected because the thermal change spectrum includes a contribution from the pH effect. That is, a steady state change in temperature changes both the average protonation as well as spectroscopic features unconnected with the pH. When the data is fit by the steady state spectrum, therefore, the pH contribution is overestimated. This is because pH change will, in turn, lag the thermal excitation, and its amplitude will then be smaller and phase greater. Having fit the data with "too much" pH difference thermally, the pH difference spectrum must now compensate. This is presented as a rigorous derivation in the Appendix.

A simple model, also given in the Appendix, shows that the tangent of phase of the pH spectrum relative to its source of excitation (the thermal modulation) is expected to show simple behavior. If the rate constant for protonation and deprotonation are given by k_+ and k_-, respectively, then

$$\tan\theta = -\frac{\omega}{k_+[H^+] + k_-}. \tag{12}$$

The data do not show this linear behavior but instead display almost constant phase shift over the limited frequency range observed. Such behavior could result from heterogeneity in the rates—a more complicated model which cannot be uniquely analyzed from the data here. In Fig. 10 we show one possible double-rate model which fits the data. The salt bridges which have been identified in Hb have different environments, and might be expected to have very different rates. While it is not possible to make a unique decomposition, it is possible to place limits on the data. For example, the sum of the rates (e.g., the denominator in Eq. 12) is near (and must exceed) 5000 s^{-1}. This is the expected order of magnitude. Given protonation rate constants on the scale of 10^{10} M^{-1} s^{-1} (Eigen, 1964), at pH 7 the protonation rate would be of the order 10^3. Temperature jump studies on deoxy-Hb and pH indicators have, in fact, yielded rates with

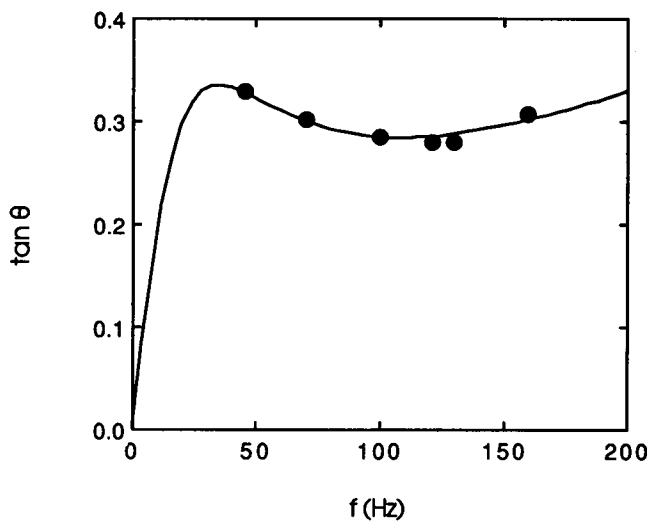

FIGURE 10 Tangent of phase of the pH difference angle minus the thermal angle for the 10 mM sample. The curve through the data has been drawn using Eqs. A10 and A11, assuming two relaxation processes, one with the sum of rates of 5300 s^{-1}, and the other with the sum of the rates of 140 s^{-1}. Both were assumed to have the same activation enthalpy, but had different forward rates of 2300 and 50 s^{-1}, respectively. The latter rates are not a unique requirement, however, and any combination of forward rates, and activation enthalphy (i.e., the parameter α in Eq. A10) which differed by the factor of 46 (=2300/50) would suffice.

a sum of 5500 s^{-1} (Okonjo et al., 1989). Slower rates could be the result of a rate-limiting conformational step. Slow conformational effects also have been observed in flash/flow experiments which mixed about 10% CO with deoxy-Hb (Khaleque and Sawicki, 1986; Zahroon and Sawicki, 1989). Hence, while the data presently cannot be used to determine protonation/deprotonation rate constants for the salt bridges, the magnitude, sign, and spectral shape all suggest that the fundamental assignment is correct. This implies that it should be possible to construct experiments which follow the salt bridge kinetics using intrinsic hemoglobin probes.

APPENDIX: THERMALLY DRIVEN PH CHANGES

Phase relationships

Let the spectral change from the purely spectroscopic part be s_θ, and the spectral change from the pH part be s_H. Thus the calibration of the thermal spectrum gives a combination spectrum, $s_\theta + s_H$. Let the oscillatory population be written by an imaginary number whose magnitude represents the fraction of molecules and whose phase shows the kinetic relationship. Thus there are two populations, P_Q and P_H. The measured signal from these two is

$$V = s_\theta P_\theta + s_H P_H \tag{A1}$$

$$= (s_\theta + s_H)P_\theta + s_H(P_H - P_\theta). \tag{A2}$$

The second equality shows the spectra used to extract coefficients of the populations. That is, in the method of modulated excitation, the spectra $(s_\theta + s_H)$ and s_H are fit to obtain the projections of the coefficients P_θ and $(P_H - P_\theta)$. Projections are obtained because these are complex numbers. Which projection is obtained depends on how the phase has been tuned.

Now, since P_θ drives P_H, we can write

$$P_H = \mathscr{D}P_\theta, \tag{A3}$$

where D represents the dynamic driving terms, which are also represented by a complex number with an amplitude and phase angle. The tangent of that phase is given by $\mathrm{Im}(\mathscr{D})/\mathrm{Re}(\mathscr{D})$. If we only had P_θ and P_H signals, this would give phase relationship between the two. From Eq. A2, however, the difference signal $(P_H - P_\theta)$ will become $\mathscr{D} - 1$, and now we get $\mathrm{Im}(\mathscr{D}-1)/\mathrm{Re}(\mathscr{D}-1) = \mathrm{Im}(\mathscr{D})/\mathrm{Re}(\mathscr{D}-1)$. Since $\mathscr{D}$ will be < 1, the denominator must now be negative, and the tangent will have the opposite sign as $\mathrm{Im}(\mathscr{D})/\mathrm{Re}(\mathscr{D})$ had. That is, where the signal would have lagged before, now it must *lead*.

A simple model

Suppose we have two states, A and B, which interconvert with temperature-dependent rate constants k_{AB} and k_{BA} for A to B and B to A, respectively. These can be written as

$$k_{AB} = k^* e^{-\Delta G/R_G T}, \tag{A4}$$

in which R_G is the gas constant and T is the absolute temperature, and similarly for k_{BA}. (Note that in this part of the Appendix, we are not using allosteric states, so that all T values will represent temperature.)

Let us expand k_{AB} around the temperature T_0 in a Taylor series:

$$k_{AB}(T) = k_{AB}(T_0) + \left(\frac{\partial k}{\partial T}\right)_{T_0}(T - T_0)$$

$$= k_{AB}(T_0)\left(1 - \frac{\Delta H_{AB}}{R_G T_0}\frac{T - T_0}{T_0}\right) \tag{A5}$$

ΔH_{AB} is the enthalpy for the kinetic process which takes A to B. In the language of transition state theory, it is the enthalpic height of the transition state above A. The variation of T from T_0 is assumed to be oscillatory: $T - T_0 = T_1 e^{i\omega t}$. Assume the states oscillate so that the population in state A has the form $\sum A_n e^{in\omega t}$ and similarly for B. Then the rate of conversion of A, $d(\sum A_y e^{in\omega t})/dt$, becomes

$$i\omega A_1 = k_{BA}B_1 - k_{AB}A_1 - \left(k_{BA}B_0\frac{\Delta H_{BA}}{T_0} - k_{AB}A_0\frac{\Delta H_{AB}}{T_0}\right) \tag{A6}$$

where we have cancelled $e^{i\omega t}$ terms, and k_{BA} and k_{AB} are evaluated at T_0. The other equation required to solve Eq. A6 is the conservation equation

$$A_1 + B_1 = 0, \tag{A7}$$

which arises since A can only be made at the expense of B. If we denote by α the quantity in the brackets in Eq. A6, the solution of Eqs. A6 and A7 is simply given by

$$A_1 = \frac{\alpha}{k_{AB} + k_{BA} + i\omega}. \tag{A8}$$

Then the tangent of the phase for this process, $\tan\theta$, is given by $\tan\theta = \mathrm{Im}(A_1)/\mathrm{Re}(A_1)$, which becomes

$$\tan\theta = -\omega/k_{AB} + k_{BA}. \tag{A9}$$

Note that this phase angle is with respect to the thermal excitation, which itself will lag the laser excitation.

If there is more than one such reaction the situation is complicated considerably. Not only must another pair of rates be considered, but the driving amplitudes (α) will likely differ. The latter can differ in steady state populations, enthalpies, or simply the rate constants. The procedure for solving such equations is completely straightforward, however. If a second set of states is denoted by primes, then state A_1' looks like Eq. A8 with primed quantities. We have the composite state A_{tot} where

$$A_{tot} = A_1 + A_2 = \frac{\alpha}{k_{AB} + k_{BA} + i\omega} + \frac{\alpha'}{k'_{AB} + k'_{BA} + i\omega} \tag{A10}$$

and

$$\tan\theta = \mathrm{Im}(A_{tot})/\mathrm{Re}(A_{tot}). \tag{A11}$$

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