Quaternary structure and geminate recombination in hemoglobin: flow-flash studies on $\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$

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ABSTRACT The kinetics of geminate recombination for the diliganded species $\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$ of human hemoglobin were studied using flash photolysis. The unstable diliganded species were generated just before photolysis by chemical reduction in a continuous flow reactor from the more stable valency hybrids $\alpha_2^{CO}\beta_2^+$ and $\alpha_2^+\beta_2^{CO}$, which could be prepared by high pressure liquid chromatography. Before the flash photolysis studies, the hybrids had been characterized by double-mixing stopped-flow kinetics experiments. At pH 6.0 in the presence of inositol hexaphosphate (IHP) both of the diliganded species show second order kinetics for overall addition of a third CO that is clearly characteristic of the T state ($l' = 1\text{–}2 \times 10^5\ M^{-1}\ s^{-1}$), whereas at higher pH and in the absence of IHP they show combination rates characteristic of an R state. The kinetics of geminate recombination following photolysis of a bound CO, however, showed little dependence on pH and IHP concentration. This surprising observation is explained on the basis that the kinetics of geminate recombination of CO primarily depends on the tertiary structure of the ligand binding site, which apparently does not differ much between the R state and the liganded T state formed on adding IHP in this system. Since this explanation requires distinguishing different tertiary structures within a particular quaternary structure, it amounts to a contradiction to the two-state allosteric model.

INTRODUCTION

Hemoglobin has been a popular subject for investigating cooperative, allosteric behavior in proteins. The two-state allosteric model of Monod et al. (1965), which distinguishes R and T quaternary structures of the tetramer, has accounted well for many aspects of cooperativity.

Adult human hemoglobin, Hb A, in the R state binds CO roughly a thousand times as well as it does in the T state. This change in the equilibrium binding constant is the result of a 15-fold change in CO dissociation rate l and a 60-fold change in the overall CO combination rate l' (Samaja et al., 1987; Sharma, 1983). The reaction of a small ligand like CO with an iron binding site buried within a compact protein involves a sequence of steps: initial entry into the protein matrix, some movement of distal amino acids as the ligand approaches the iron binding site, and finally bond formation itself, which is influenced by a proximal histidine. Insight into these complexities can be gained by photodissociating the iron-ligand bond and observing the kinetics of geminate recombination, that is, how some fraction ϕ of the ligands rebind before they escape from the protein. Geminate rebinding following nanosecond laser photolysis of COHb at room temperature was first identified by Alpert et al. (1979) and by Duddell et al. (1979). They analyzed their results in terms of a single intermediate species, involving CO trapped within the protein. Austin et al. (1975), in low temperature studies of the related protein carboxymyoglobin, distinguished four sequential steps. Based on studies of reactions of O_2, NO, and alkylisocyanides with myoglobin at room temperature, we proposed a model involving two intermediate states (Jongeward et al., 1988). It is possible that reality may be more accurately described as a continuum than by any small number of discrete intermediates. At least for COHb, however, it has been common to represent room temperature kinetics of geminate recombination as approximately a single exponential process.

The question arises whether R to T differences are localized or distributed. Does the difference involve changes in entering the protein, in forming the bond, in some intermediate event, or all of the above? General belief is that bond formation is most changed. From this it follows that geminate recombination after flash photolysis should show a much different ϕ for the R and T states, but not too different a rate of escape from the protein.

Earlier we studied geminate recombination of CO with several hemoglobins and observed only one relaxation, which took place in the nanosecond time domain (Campbell et al., 1985). One of the hemoglobins was from opossum, in the α-chains of which the usual His at position E7 and Val at E11 are replaced by Gln and Ile, respectively. At pH 6.0 in the presence of inositol hexaphosphate, IHP, COHb opossum behaved by several criteria as if it were in the T state, whereas at pH 9.2 in the absence of IHP, it was clearly in the R state. Yet only small differences were observed in the yields and rates of geminate recombination between pH 9.2 and pH 6.0 + IHP. Although small, the changes were in the direction expected: at pH 6.0 + IHP, ϕ was reduced and escape from the protein was somewhat faster. Results of a more recent investigation of carp hemoglobin with methylisocyanide as ligand (Bandyopadhyay et al., 1990), followed the same pattern: quaternary structural change in fully liganded hemoglobin was accompanied by surprisingly modest changes in the yields and rates of geminate rebinding.

The above observations are in sharp contrast both to the general expectation that ϕ should change and to par-

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0006-3495/92/09/673/09 $2.00

ticular results obtained by others. Murray et al. (1988*a*) studied the geminate rebinding of CO to hemoglobin in which cobalt was substituted for iron in the α chains. Such metal hybrids switch to the T state in the presence of allosteric effectors such as IHP and bezafibrate, BZF. It was estimated that ϕ in the T state was near 1%, which was approximately 50 times less than for the R state species that existed in the absence of allosteric effectors. The results from double-flash experiments performed by Marden et al. (1987) also led to an estimate that ϕ in the T state was less than 3%. Consistent with these observations, it has been observed that the carboxy derivatives of the six-coordinate synthetic hemes proposed as model compounds of the T state hemoglobin show no observable geminate recombination (Traylor et al., 1990).

Since the source of these discrepancies is not obvious, we must examine exactly what is measured in each system. Our studies employed fully liganded hemoglobins from opossum and carp, which are switched to the T state by allosteric effectors and low pH. In the absence of crystal structures, the assignment of the T state is made on the basis of some characteristic physico-chemical parameter. The T state assignment of fully liganded Hb opossum was made based on the CO combination rate constant l'_4 of the triliganded species that exists some time after flash photolysis, whereas the geminate recombination studies characterized the species present immediately after photolysis. It was conceivable that the nanosecond geminate recombination actually pertained to the properties of the quaternary R state, and the T state was formed unusually rapidly a few microseconds after photolysis of even a single ligand, before the time required for overall combination reactions. A better criterion was available for COHb carp. In that case, the rate of CO dissociation from the fully liganded molecule had been measured both in the presence and absence of IHP at pH 6.0, and there was little doubt that fully liganded COHb carp at pH 6.0 + IHP exists in the T state. Unfortunately, COHb carp shows very little geminate recombination at any pH either in the presence or absence of IHP, so it was impossible for us to measure whether ϕ changes or not. Instead, we characterized the methylisocyanide derivative of Hb carp. Lin, et al. (1988) had reported that this species at pH 6.0 + IHP also exists in the T state. Since no individual rate or equilibrium constants for the T and R states were determined, however, the conclusions of Lin et al. (1988), and our geminate yield studies based on them, although probably correct, should be considered tentative. In the face of contradictory results on various systems, the metal hybrid studies are also subject to lingering doubt about the effects of substituting another metal for iron. As an alternative to relying on allosteric effectors, Marden et al. (1987) used partial photolysis to produce partially liganded hemoglobin, which can change its quaternary structure over time. A second flash probed the behavior of whatever mixture was present some time after the first photolysis. Since both bimolecular combination and geminate recombination could be measured, they had a criterion for deciding what the fractions of R and T states were at the time of the second flash. However, at excitation levels that remove most of the ligands and thereby maximize the amount of T state, the kinetic signals decrease relative to noise. It was necessary to extrapolate to a limiting condition. If one knows in advance that two widely different species are all that exist, one can probably deduce parameters fairly well; but recognizing intermediate behavior would be difficult.

Ideally we need a hemoglobin for which a functional parameter that defines the quaternary T state of the molecule can be determined without changing the degree of ligation and which can be prepared in near 100% homogeneity. In particular, we want a good T state, because the R state is already well known. In all of the studies mentioned above, it is the T state that is the more problematic. Invariably, in those studies some residual geminate recombination was observed, but was attributed to a persistent fraction of R states and "subtracted away" to support the conclusion that the T state showed very little recombination. The diliganded species of hemoglobin $\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$ seemed to us to offer the best hope yet for preparing liganded T states in a homogeneous preparation without chemical changes to the molecule. Of course, we would have to overcome the severe problem of disproportionation in those species; but if we could do that, as described below, they would offer three advantages: first, being of intermediate ligation, they should switch between R and T forms with relatively mild treatment. Second, the study on HbA would complement our previous work with hemoglobin from more exotic species. Third and most important, the quaternary state of the molecule could be assigned on the basis of the CO combination rate constant of the unliganded subunits without affecting the degree of ligation of the liganded subunits, which can be used for studying the geminate process. One might raise the objection, how do we know that the liganded subunits are in the same state as the unliganded subunits? That, however, is precisely the assumption of the two-state allosteric model that the experiment is designed to test.

EXPERIMENTAL

Preparation of valency hybrids by HPLC

HPLC separation of the two hybrids ($\alpha_2^{CO}\beta_2^+$ and $\alpha_2^+\beta_2^{CO}$) was carried out as described previously (Sharma, 1988), except for the following changes needed to generate larger volumes of more concentrated product: (*a*) A preparative synchropack column CM 300 (250 × 22 mm inner diameter) was used. On this column we were able to load 1.75–2.0 mL of 10–12 gm/100 mL hemolysate, 60% oxidized by ferricyanide, and obtain 1.0–1.5 mL of each hybrid at a concentration of more than 1 mM in heme. (*b*) The following gradient was used: 10 min, 100% buffer A (0.03 M potassium phosphate buffer, pH 6.8), flow rate 4 mL/min; at 20 min 60% of A, 40% of B (0.015 M potassium phos-

phate buffer, pH 7.5), flow rate 4 mL/min; at 80 min 20% A, 80% B and the flow rate was gradually changed from 4 mL/min at 20 mins to 8 mL/min at 80 mins; at 85 min 0% A, 100% B. The hybrid $\alpha_2^{CO}\beta_2^{+}$ eluted in 47 min and $\alpha_2^{+}\beta_2^{CO}$ in 82 min. The hybrid solutions were first concentrated at 4°C by an Amicon stirring ultrafiltration unit with minimum stirring to yield 10–20 mL of sample volume, and finally by centricell 60 (Polyscience, Inc., Warrington, PA) at 4°C to give 1–2 mL of 1–2 mM hybrid samples. The percentage of ferricHb in each concentrated hybrid sample was calculated by the method of Bannerjee and Cassoly (1969) and in most cases was 50 ± 2%. For carboxy derivatives of the hybrids the percentage of ferric hemes did not change significantly over a period of 72 h.

Double mixing kinetic experiments

The methodology of these experiments has been described previously (Bergis et al., 1990).

Geminate recombination experiments

Instrumental procedures for nanosecond laser flash photolysis were described previously (Jongeward et al., 1988). Transmittance was measured at 440 nm before, during, and after a 540 nm laser photolysis pulse of 10 ns duration. Each reaction time course consisted of 1,024 data points digitized electronically. One hundred such traces were summed to yield the final reaction time course used for evaluating kinetic parameters. Extent of photolysis was less than 10%, so that only one ligand was removed from each (diliganded) tetramer.

The novel aspect of the measurements involved reducing the stable valency hybrids $\alpha_2^{CO}\beta_2^{+}$ or $\alpha_2^{+}\beta_2^{CO}$, to the desired ferrous form just before laser photolysis. The flow system consisted of two 10 mL gas-tight syringes mounted on a motor-driven syringe drive. One of the syringes contained a solution of $\alpha_2^{CO}\beta_2^{+}$ or $\alpha_2^{+}\beta_2^{CO}$ hybrid at a heme concentration of 150 μM in 0.1 M bistris, and the other syringe 0.5% sodium dithionite in the same buffer. The distance between the point of mixing and the laser beam was adjusted so that the travel time was 1.8–2.0 s. Independent double-mixing stopped-flow experiments determined that the time required for complete reduction of the ferric subunits was less than 1 s (Sharma, 1988). The total volume of the microcuvet was 32 μL and the rate of flow of solutions through the cuvet was 33 μL s^{-1}. The laser pulse rate was 40–50 pulses/min. These conditions ensured that the hybrid solutions were completely reduced to the ferrous form before photolysis, and that fresh sample was subjected to photolysis by each laser pulse. The remaining concern was whether the incubation time might be long enough for some disproportionation to occur, so that some tetramers would end up triliganded, while others became monoliganded. This issue is discussed again below; but the direct experimental test was that the flow rate of solutions was varied over the range 1–3 mL/min without any significant effect on the results reported.

RESULTS

Double-mixing kinetic studies were carried out and reported previously to ascertain the quaternary structure of diliganded hemoglobin as a function of buffer conditions. At pH 6.0 in the presence of IHP, bimolecular CO combination reactions exhibited a single, slow kinetic phase characteristic of the T state species, $l_1' \approx 1\text{–}2 \times 10^5$ M^{-1} s^{-1} (Sharma, 1988). This is the species of primary interest, which we want to characterize and compare with accepted properties of R states and with past studies on systems purported to be T states. For completeness, however, we also attempted to switch the two species to the R state. At higher pH and in the absence of IHP, overall combination kinetics consisted predominately of a fast phase exhibiting rates expected for an R state species, $l_4' \approx 6\text{–}10 \times 10^6$ M^{-1} s^{-1} (Bergis et al., 1990). However, at those conditions, the combination reaction kinetics is persistently biphasic, showing slightly less than 30% of slow reacting T state species. There may even be a small admixture of fast reacting dimers. All of this was discussed in detail (Bergis et al., 1990). The fractional amount of the slow phase was independent of protein concentration, which rules out any large amount of dimers.

Results similar to the above were reported also by Cassoly and Gibson (1972). They observed monophasic, slow combination with CO at pH 6.6 in the presence of IHP, but biphasic combination in the same relative proportions as we did at higher pH in the absence of IHP. Noteworthy is the fact that Cassoly and Gibson (1972) prepared their valency hybrids by a very different method than we used. In their method, the cyanomet derivative of α or β chains was combined with complementary ferro-oxy chains. The affinity of cyanide ion for ferric hemes is very high; but for ferrous hemes it is very low. Therefore, there is very little chance of any redistribution of the cyanide ligand during preparation of the hybrids. The similarity of results for the two different preparations indicates that the observed biphasicity in the combination reaction kinetics cannot be dismissed as an artifact of the preparation of valency hybrids or the result of disproportionation during the aging time in double mixing experiments. Very recently, Philo (1992) also reported data on cyanomet valency hybrids, which were interpreted as indicating the presence of some fraction of subunits in the T state with very slow rates of converting to the R state.

Laser photolysis of solutions of carboxy derivatives of myoglobin and hemoglobin in the picosecond time domain reveal small amplitude absorbance changes relaxing with a characteristic decay time of a few picoseconds. This relaxation has been assigned (Petrich, 1988) to relaxation of excited states of deliganded heme, partly because very similar spectra are observed in the photolysis of five-coordinated deliganded samples. We, therefore, start with the simplifying assumption that there is no significant CO geminate rebinding in the picosecond time range. Such interpretation of transient absorption in the visible spectrum is supported by recent picosecond infrared studies of Anfinrud et al. (1989), which indicate that 4.6 ± 1.7% of dissociated CO rebinds to Mb over 1 ns, but there is no few-picosecond recombination.

There has been some controversy regarding the use of an exponential form of equation or a power law for representing the geminate rebinding. Early studies of the geminate process indicated that the binding process at low temperatures was not exponential in time (Austin et al., 1975; Frauenfelder and Wolynes, 1985). This was explained by postulating that each protein molecule can assume large numbers of slightly different conforma-

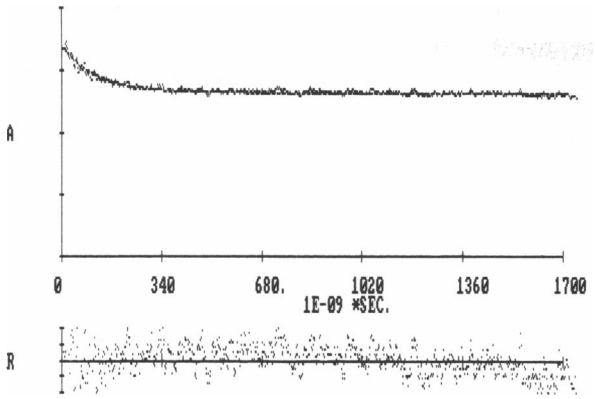


FIGURE 1 Observed and calculated reaction time course of CO recombination for $\alpha_2^{CO}\beta_2$ in 0.1 M bistris pH 6.0 plus eight-fold excess IHP; $[\alpha_2^{CO}\beta_2]$ = 75 μM; [dithionite] = 0.25%; A = absorbance in arbitrary units; R = magnified residuals for data in figure A; line, R = 0. Each small division represents 0.5% deviation from zero residual.

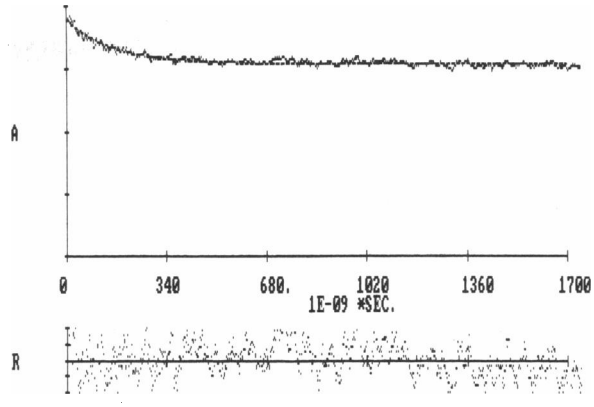


FIGURE 2 Same as Fig. 1 for $\alpha_2\beta_2^{CO}$.

tional substates (Dlott et al., 1983). At ambient temperatures, however, the binding process was adequately represented as a single or double exponential relaxation, implying that under these conditions the differences between the various conformational substates were indistinguishable. The exponential function still could be a simplifying assumption recommended primarily by its simplicity and ability to represent the data adequately. Marshall (1989) concluded that unless the signal to noise ratio was 400 or better, it is difficult to distinguish among possibilities. All our data, irrespective of pH and the presence or absence of IHP, were adequately represented by single exponentials and the relaxation times were calculated by making a least squares fit of data to the equation:

$$A_t = A_\infty + A_g e^{-k_g t}, \tag{1}$$

where A_t and A_∞ are absorbances at time t and at the end of the geminate reaction, and k_g is the first order geminate rate constant. The zero-time amplitude of the geminate process is represented by A_g, so the fractional yield of geminate recombination is $\phi = A_g/(A_g + A_\infty)$. The fits and the observed relaxations for the two hybrids are shown in Figs. 1 and 2, and the numerical values of k_g and ϕ are listed in Table 1.

Observation of a single relaxation indicates the formation of at least one intermediate. Therefore, a reaction model with three discrete species is the minimum required for data analysis:

$$A \underset{2}{\overset{1}{\rightleftarrows}} B \underset{4}{\overset{3}{\rightleftarrows}} C + CO \tag{2}$$

A represents unphotolysed species $\alpha_2^{CO}\beta_2$ or $\alpha_2\beta_2^{CO}$; B the geminate intermediate in which the ligand is still in the protein but without a bond to the heme iron; C represents protein that has lost the photolysed ligand to the surrounding solvent. The physical significance of k_2 and k_3 will depend on the details of the reaction mechanism. In a "one-intermediate" model (Eq. 2), k_2 represents loss of B by bond formation to produce A and k_3 represents loss of B by ligand escape from protein to solvent.

The observed geminate recombination rate constant k_g was further resolved into k_2 and k_3:

$$k_g = k_2 + k_3 \tag{3}$$

$$\phi = \frac{k_2}{k_2 + k_3}. \tag{4}$$

For both hybrids, the observed values of ϕ and the calculated values of k_2 and k_3 are listed in Table 1 for various conditions.

Summary observations

(a) In the absence of IHP the geminate yields and the rates of geminate rebinding k_g are almost identical for the two hybrids, one having α chains liganded and the other have β chains liganded. (b) The effect of pH on geminate yields and rates of recombination is small and similar for both hybrids. (c) In the presence of IHP at both pH values, the geminate yields are only slightly less than in the absence of IHP. (d) At pH 7.0 in the absence of IHP, the overall combination reaction is biphasic and, correspondingly, the geminate recombination is slightly

TABLE 1 **Kinetic data for geminate recombination for CO with $\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$ at 25°C in 0.1 M bistris**

Conditions	Sample	ϕ	$k_g \times 10^{-7}$	$k_2 \times 10^{-6}$	$k_3 \times 10^{-6}$
			s^{-1}	s^{-1}	s^{-1}
pH 6 + IHP	$\alpha_2^{CO}\beta_2$	0.21	0.81 ± 0.03	1.70 ± 0.06	6.40 ± 0.06
pH 6 + IHP	$\alpha_2\beta_2^{CO}$	0.20	0.67 ± 0.02	1.34 ± 0.04	5.36 ± 0.04
pH 6 − IHP	$\alpha_2^{CO}\beta_2$	0.24	1.10 ± 0.03	2.64 ± 0.07	8.36 ± 0.07
pH 6 − IHP	$\alpha_2\beta_2^{CO}$	0.24	1.10 ± 0.04	2.64 ± 0.09	8.36 ± 0.09
pH 7 − IHP	$\alpha_2^{CO}\beta_2$	0.27	1.45 ± 0.02	3.92 ± 0.05	10.58 ± 0.05
pH 7 − IHP	$\alpha_2\beta_2^{CO}$	0.28	1.47 ± 0.03	4.12 ± 0.08	10.58 ± 0.08
pH 7 + IHP	$\alpha_2\beta_2^{CO}$	0.25	1.09 ± 0.04	2.73 ± 0.10	8.17 ± 0.10

reduced, compared to the fully liganded species. (*e*) The combined effect of low pH and IHP is to produce single phase, slow bimolecular combination, with only a modest decrease in geminate recombination. These observations are consistent with our earlier observations on fully liganded COHb A, COHb rabbit, COHb opossum, and COHb Rothschild (Campbell et al., 1985).

DISCUSSION

The first important finding of this study concerns the equivalence of α and β chains. Geminate recombination with CO is quite similar regardless of whether the α or the β chains are liganded. This is consistent with other studies, but more direct. Hofrichter et al. (1985), in their study of iron-cobalt hybrid hemoglobins, observed that the geminate rebinding kinetics of $\alpha(Co)_2\beta(Fe\text{-}CO)_2$ were very similar to those of unsubstituted hemoglobin, $\alpha(Fe\text{-}CO)_2\beta(Fe\text{-}CO)_2$, which implies near equivalence of α and β subunits within the R state tetramer. Recent studies of second order CO combination with site specific Hb mutants also show no difference in the reactivities of α and β chains (Mathews et al., 1991). Similarly, the kinetics of CO dissociation from the two isomers of monoliganded species, $\alpha_2^{(CO)_1}\beta_2$ and $\alpha_2\beta_2^{(CO)_1}$, yield almost identical values of CO dissociation rate constant l_1, namely, $0.16 \pm 0.01\ s^{-1}$ (Sharma et al., 1991). In marked contrast, Morris et al. (1984) measured chain-related differences for the geminate recombination of oxygen with iron-cobalt hybrids of hemoglobin. The difference between this and other studies is possibly related to the use of different ligands (O_2 vs CO).

A more surprising finding of the present study is the quite modest change in geminate recombination kinetics between pH 6.0 + IHP, conditions at which both of the hybrids display T state-like overall kinetics, and pH 7.0 − IHP, conditions at which the hybrids exhibit predominately R-like behavior.

At pH6 + IHP the present study reports $\phi = 0.20$ or 0.21 despite the fact that at those conditions the bimolecular combination is shifted entirely to the slow phase. This is a surprising result. It does not depend on quantification of subtle effects, or extrapolation to limiting conditions. Either the liganded subunits are not well described as T state, even when the unliganded subunits are nearly 100% in the T state; or liganded subunits that are well described as T state are exhibiting substantial geminate recombination. In the former case, the two-state allosteric model has failed; in the latter case, generally expected properties of the T state (namely, that ϕ should be close to zero) must be reexamined and significantly modified.

Consider next the results at higher pH without IHP. At pH 7 − IHP the present study reports $\phi = 0.27$ or 0.28. This recombination yield, and the characteristic rate constant, are roughly what would be expected for an R state preparation, as anticipated. However, this value of ϕ is actually somewhat less than we reported previously for fully liganded R state tetrameric Hb A ($\phi = 0.34$; Campbell et al., 1985). The bimolecular combination of the hybrids under the same conditions of pH 7.0 − IHP showed two phases: 70% fast recombination as expected for R-state combination together with 30% slow recombination that might be assigned to a T-state. It is reasonable to conclude that diliganded tetrameric Hb can exist in both R and T forms and use the fractional amounts of the two bimolecular phases as a measure of the amounts of R and T state species present. (Other possible explanations for the two phases were evaluated and ruled unlikely [Bergis et al., 1990].) Then, one might well conclude that T states show greatly reduced geminate rebinding and the R-state diliganded species behaves just like the fully liganded R state. This accords with expectation and is persuasive. The only difficulty is that our T state preparation, which exhibited 100% slow bimolecular combination, should then show no geminate recombination, which is not what we observed.

One question that might be raised is whether ϕ, as measured here, accounts for all geminate recombination, and whether missing amplitude would affect our conclusions. In fact, there may be a small component missing. The recombination yield ϕ should be equal to $1 - Q$, where Q is the photodissociation quantum yield, a quantity that has been reported previously for COHb A. For conditions similar to those used here, an early measurement obtained $Q = 0.53$ for fully liganded COHb A referenced to COMb, assumed to have a value of unity (Noble et al., 1967). Since we now believe that MbCO shows a few per cent geminate recombination, we can correct that value to about 0.50. This is similar to results ($Q = 0.47 \pm 0.02$ at 20°C in phosphate buffer) of two later studies (Saffran and Gibson, 1977; Sawicki and Gibson, 1979) and to a recent redetermination in this laboratory ($Q = 0.55 \pm 0.05$ at 26°C in bis-tris buffer). We conclude that ϕ should be close to 0.50, or perhaps a little less, for R state COHb A. Our previous investigation of geminate recombination in COHb A obtained $\phi = 0.34$ at 20°C (Campbell, 1985). We were probably missing a component that contributed ~0.1 to 0.15 to the initial dissociation amplitude; and the most likely source for the discrepancy is a small amount of extra geminate recombination taking place over the time range 0.1–10 ns. It was not possible to carry out a precision determination of Q on the T state hybrids, but from the size of the nanosecond signals any missing contribution to ϕ could not have been more than about 0.1 to 0.15 and did not differ significantly between R and T states. The surprising conclusion in this study is that ϕ is as large as it is for the T state species (pH 6.0 + IHP), and any small amount of missing amplitude in either R or T states does not affect that conclusion.

Before proceeding to possible explanations of our results, let us review pertinent previous findings.

Sawicki and Gibson (1979) studied the photodissociation yield Q as a function of degree of ligation for COHb A. At low levels of ligation (for which they expect the Hb to be largely in the T state), they measured somewhat less dissociation than would be predicted by the simple two-state allosteric model. We can interpret this as too much geminate recombination in the T state, in agreement with our present and previous results. At the time, more than ten years ago, a number of different explanations could be entertained. Even then, however, the authors suggested that the evidence was pointing to "two states with different quantum efficiencies within what is normally considered the T state."

Cobau et al. (1985) investigated COHb carp, which can be switched between R and T states by adjusting pH and adding IHP. As mentioned above, this is not a useful model system at room temperature, because neither form exhibits much geminate recombination. They were able to characterize geminate recombination below 200 K, but found that a straightforward extrapolation of their results to room temperature failed to agree with known overall combination rates. While one might point to many difficulties in the extrapolation, their own conclusion was that "outer processes remote from the iron group may be controlling cooperativity." We infer that such processes might not be reflected in ϕ.

Using a flow-flash method, Khaleque and Sawicki (1986) studied bimolecular combination kinetics of CO in monoliganded hemoglobin. They concluded that they could not explain their results within the constraints of the two-state allosteric model.

A double flash experiment by Marden, et al. (1987) that showed evidence for a small ϕ for T state Hb was described in the introduction. A year later, Marden et al. (1988) again investigated liganded T-state Hb A, this time concentrating on the effect of allosteric effectors IHP and BZF, separately and together. The only geminate recombination reported pertained to a situation in which both effectors were used and a significant reduction occurred in ϕ. From the data presented, it is difficult to draw conclusions about the effect of using only one effector at a time. It was clear that there was more slow bimolecular combination when effectors were present; but it was unclear whether that was because the T state was present "immediately" after photolysis or because some transition from R to T took place more quickly in the presence of the effectors. One major conclusion stated in that paper, however, was that the simple two state allosteric model is incomplete. T states forced by allosteric effectors showed a variety of intermediate behaviors.

Keeping in mind these repeated suggestions that allosteric effectors may have more complicated effects that are usually considered, let us consider again the study that offers, perhaps, the most direct evidence for a very low ϕ in the T state. As discussed above in the introduction, Hofricher, et al. (1985) and Murray, et al. (1988*a*) have characterized geminate recombination in metal hybrids of Hb A (α_2(Co)β_2(Fe) and α_2(Fe)β_2(Co)). In the absence of allosteric effectors IHP and BZF, α_2(Fe-CO)β_2(Co) behaved like R state hemoglobin ($\phi \approx$ 40%). The simultaneous addition of both allosteric effectors, which bind at different sites, produced marked reduction in the amount of geminate recombination. At the same time the fraction of slow bimolecular combination increased to 90%. They concluded that the small remaining amplitude of the fast bimolecular form could account for all remaining geminate recombination, and inferred that the geminate recombination of T-state Hb is $< \sim 1\%$. They were not explicit about what was observed using only one allosteric effector—in particular, whether there were ever circumstances in which the slow bimolecular phase was large while the geminate recombination was also large. Their work indicates that it is possible to make a preparation that shows the expected low value of ϕ, but doing so required extreme measures: replacement of two irons by cobalt, presence of only two ligands, and use of two, not one, steric effectors. Even then they did not achieve the clean 100% T-like behavior that we have at pH 6.0 + IHP, but had to argue about the simultaneous presence of two phases, as we have at pH 7.0 − IHP.

Mention of inadequacies in the two-state allosteric model brings to mind recent evidence for a third quaternary species of Hb (Ackers and Smith, 1987). While there may be some ultimate connection between a third quaternary form and the discrepancies being discussed here, the third quaternary form is assigned exclusively to specific diliganded species that are not the ones used in the present investigation.

One final observation seems pertinent, because it bears on the issue of tertiary structures distinct from quaternary structure. Murray et al. (1988*a, b*) reported spectral evolution following flash photolysis that was interpreted as changes in tertiary structure on two time scales: one near 50 ns, coincident with ligand escape from the protein, and another at 0.5–1 μs. Both of these are much faster than the R to T transition itself, which was seen at longer times in the same experiments. More recently, evidence from picosecond circular dichroism measurements has suggested tertiary changes on the subnanosecond time scale (Xie and Simon, 1991).

The qualitative discussion above can be made more quantitative. If we assume the simplest one-intermediate model (Eq. 2), then the overall bimolecular combination rate is related to the fundamental parameters of the mechanism as

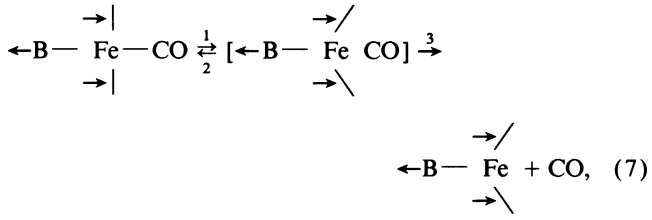
$$l' = k_4\phi, \tag{5}$$

and the thermal dissociation rate is given by

$$l = k_1(1 - \phi), \tag{6}$$

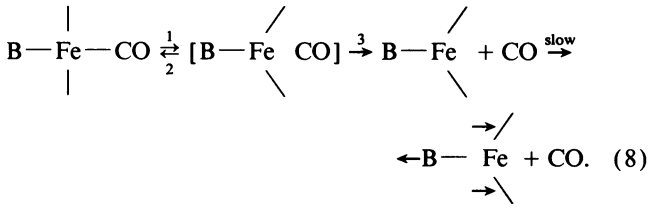
where $\phi = k_2/(k_2 + k_3)$. If ϕ changes little between R and T states, any difference in l must be assigned primarily to

bond disruption k_1 and any difference in l' must be attributed primarily to a change in the rate of ligand entry into the protein k_4. The former is acceptable. The latter conclusion is not attractive. It may certainly be possible that k_4 is somewhat different between R and T and it is likely that such a difference could involve tertiary changes that take place during or after ligand escape, so that the liganded T state would not share this aspect of the unliganded T state; however, it is unlikely that k_4 accounts for all the difference. There are good reasons for thinking k_2 is reduced in the T state. The difference between R and T is generally attributed primarily to increased tension between the proximal histidine and the iron. In that model, photodissociation of a liganded T state should be represented as



(7)

whereas, dissociation from the usual liganded R state should be represented as



(8)

Note that in both equations, the iron remains in plane when a ligand is bound, but moves out of plane "instantaneously" after ligand loss. The difference is that in Eq. 7 extra strain is present even before ligand loss that acts to keep the iron out of plane, while in Eq. 8 such extra strain evolves slowly after ligand loss as protein conformational changes take place on time scales from nanoseconds to (many) microseconds. Quite elaborate schemes have been proposed explaining how movement of the protein can modulate tension in the bond (Friedman, 1985). One effect of strain should be to increase rates for thermal bond rupture k_1. Another likely effect is an increase in the transition state barrier associated with k_2. Model compounds for T-state Hb, in which bulky 2-methyl-imidazole replaces 1-methyl-imidazole as the proximal base, mimic T-state equilibria and overall kinetics quite accurately. They were developed without any concern for geminate recombination (White et al., 1979). Nevertheless, a recent study (Traylor et al., 1990) showed about 50% geminate return of isocyanides to the R-state model and an unmeasureable amount (<5%) to the T-state model on a picosecond time scale. Since diffusion properties should be unaffected by the slight change in the proximal base, the entire difference had to be assigned to a change in k_2. Consequently, we, like Murray et al. (1988*a*) and Marden et al. (1987) expected that ϕ would certainly change if we could prepare a good liganded T-state Hb. Yet, that is not the result of the present study. The problem cannot be circumvented by introducing additional steps to change the one-intermediate mechanism of Eq. 2 into a more complicated sequential mechanism, as we have proposed in the past for other reasons (Jongeward et al., 1988). As long as ϕ represents all the geminate recombination, Eqs. 5 and 6 must apply to k_1 and k_4. All the differences lie in reinterpretations of the intermediate steps.

In our recent study of methylisocyanide binding to Hb carp in R and T states (Bandyopadhyay et al., 1990), where we also found little difference in geminate recombination, there was a hint of the problem that we face here. However, in that case, the binding affinity changes only by a factor of 34 between R and T states, rather than the factor of 500 to 1,000 that we have in the present system. Consequently, it was possible to explain the data by making a modest change in each of the steps of the mechanism, changing each of the rate constants by a factor of two or three, without abandoning the two state allosteric model. In the present case, such a strategy cannot be made to work. In Table 1, k_2 is shown to change by less than a factor of 3 in the direction expected, while k_3 actually changes slightly (less than a factor of 2) in the wrong direction to reduce ϕ. Consequently, if the two state allosteric model is to be retained, k_4 in Eq. 2 must change dramatically and account for almost all the variation in l'.

The only apparent resolution to the clearcut finding of the present report—that the diliganded hybrids show T-state behavior when combining with a third ligand but almost R-state behavior in their geminate recombination—that does not require attributing all the R-T difference in l' to k_4 is to propose that the tertiary structure of the so-called liganded T state brought about by IHP is not the same as the tertiary structure of the deliganded T state. This would be consistent with past observations of tertiary changes occurring during and after ligand escape. This means abandoning the two-state allosteric model and keeping track of the tertiary conformation of each chain. The symbols R and T will then refer explicitly to some aspect of quaternary structure; and lower case letters will be used to denote tertiary structures of each chain, a notation used previously by others. Fully liganded Hb A exists as Rrrrr at normal conditions. Fully deliganded Hb A exists as Ttttt at normal conditions. Initially liganded Hb A would exist transiently as Rtttt a few microseconds after complete photolysis, at a time when the tertiary relaxation described by Murray et al. (1987*b*) is complete but before the quaternary change occurs. Our diliganded hybrids, then, exist at low pH + IHP as either Trrtt or Rrrtt. Then, one could argue that our diliganded hybrids at pH 7.0 − IHP do involve a

mixture of Rrrrr and Ttttt forms in which the intermediate geminate yields observed can be interpreted as a superposition of normal recombination yield for the R fraction and a vanishing yield for the T fraction, with the amount of each fraction measurable by the fractional amplitudes of the bimolecular combination. For both hybrids at pH 7.0 – IHP, we found geminate recombination consistent with as an equilibrium mixture of 70% R state and 30% T state. In the new notation, this would be 70% Rrrrr and 30% Ttttt. This would be entirely consistent with the results of Marden et al. (1987). Overall, our results would mean, somewhat surprisingly, that the effect of IHP is not so much to influence the quaternary structure as to force all the unliganded chains to the t form without affecting much the r form of the liganded chains. It appears, however, that simultaneous use of both IHP and BZF is able to force diliganded metal hybrid Hbs into the Ttttt state. This would explain the results of Murray et al. (1988*a*).

A strong form of the two-state allosteric model would insist that all equilibrium and kinetic properties can be explained with only two quaternary states and that any treatment can only shift the Hb between those states. We believe that it is impossible to maintain the strong statement. A weaker form may still be possible. It is still possible to conjecture that two states are all that exist under "normal" circumstances, while conceding that use of exotic allosteric effectors and other conditions generate unusual forms exhibiting anomolous behavior.

CONCLUSIONS

Preparation of purified valency hybrids by HPLC and reduction to ferrous Hb using simple flow methods can be combined with flash photolysis on all time scales from millisecond to subpicosecond to permit complete kinetic characterization of partially liganded forms. This permits the direct study of intermediates postulated by various theoretical models. Furthermore, it permits testing assumptions about the influence of allosteric effectors or other sample conditions, which are now in question.

The diliganded hybrids that have α chains liganded behave very similar to those that have β chains liganded.

Lack of any significant effect of quaternary structure on the kinetics of nanosecond geminate rebinding in $\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$ is very surprising and disagrees with some previous work, even though it agrees with our own previous results on other systems. At pH 6.0 + IHP, the hybrids can be switched completely to a T-like structure, as indicated by the overall bimolecular association rates. Nevertheless, only a modest reduction (less than a factor of two) in the geminate recombination yield is measured. This means either that most of the difference in l' must be attributed to the step involving ligand entry into the protein (which is unlikely) or that geminate recombination following laser photolysis is occurring within a tertiary structure that is really r-like, which will subsequently relax to the true t-structure that would be encountered by a ligand entering from solvent. Put another way, IHP at pH 6.0 (without BZF) generates an unusual tetrameric structure in which the unliganded subunits have tertiary t-state structures, while the liganded subunits have tertiary r-state structures. Such a structure is unusual in the sense that it contradicts the two-state allosteric model. Whether such structures are rare or common remains to be determined. They may help explain puzzles that appeared in a number of past experiments that observed anomolies in the overall kinetics of various systems but were not able to identify precisely where the problem lay.

This work was supported by grants HL13581, HL31159, and RR02353-1 from the National Institutes of Health and grant CHE8715561 from the National Science Foundation.

Received for publication 15 October 1991 and in final form 2 March 1992.

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