Changes in the apparent quantum efficiency for photolysis of $Hb(CO)_1$

Ikbal A. Zahroon and Charles A. Sawicki
Physics Department, North Dakota State University, Fargo, North Dakota 58105

ABSTRACT Recent studies suggest that the allosteric state of the protein surrounding the hemes in hemoglobin affects both geminate recombination of CO and the apparent quantum efficiency (AQE) for photolysis (Rohlfs, R. J., J. S. Olson, and Q. H. Gibson. 1988. *J. Biol. Chem.* 263:1803–1813.

We report combined flow/flash experiments in which the AQE for photolysis of $Hb(CO)_1$ was measured as a function of time delay after its formation. Experiments were carried out at 20°C in 0.1 M phosphate buffer at pH 7.0 with CO saturations of 10% or less. The AQE was observed to decrease from a value close to 1.0 at short times to ~0.6 after 2 s. The fundamental photolysis step for carboxyhemoglobin is known to have a quantum efficiency of nearly 1.0, whereas the lower AQE values we observe result from competition between rapid geminate recombination and a rapid reaction step leading to escape of the CO to the solution phase. Changes in AQE values reflect changes in these rapid reaction steps which presumably result from conformational change in $Hb(CO)_1$. The change in AQE is consistent with conversion of one or more hemes to an R-like state but these changes could not be even approximately described in terms of a simple two-state allosteric model.

INTRODUCTION

Several recent experiments suggest that photodissociation of CO, O_2, and NO occurs with a quantum efficiency close to 1.0 and that the lower apparent quantum efficiencies observed in experiments using photolysing pulses ~1 μs long result because a fraction of the photolysed ligand undergoes very rapid geminate recombination before deoxyhemoglobin can be observed (1–3). This general relationship between geminate recombination and quantum efficiency had been recognized in much earlier nanosecond photolysis experiments (4). In brief, for experiments with microsecond resolution, only the fraction of CO molecules escaping to the solution phase contribute to the observed apparent quantum efficiency. Henceforth in this discussion the phrase apparent quantum efficiency will be represented by the acronym AQE.

The simplest model relating geminate recombination and AQE is represented, following reference 5, by Eq. 1. Models similar to this one have been used by a number of investigators to represent the processes taking place after photolysis (5, 6).

$$\mathrm{Hb} + \mathrm{CO} \underset{K_{23}}{\overset{K_{32}}{\rightleftharpoons}} \mathrm{Hb \cdot CO} \underset{K_{12}}{\overset{K_{21}}{\rightleftharpoons}} \mathrm{HbCO} \qquad (1)$$

In Eq. 1 Hb + CO represents a ligand-free heme with the CO molecule in the solution phase. Hb·CO is an intermediate state produced with a quantum efficiency of 1.0 by photolysis of CO-bound heme (HbCO). In this intermediate state the heme is ligand free and the CO is pictured as being weakly bound nearby (5). The rates K_{21} and K_{23} are very much larger than K_{32} or K_{12} times the free CO concentration in our experiments (5, 6). Under these conditions the fraction Φ of the photolyzed CO which undergoes geminate recombination is given by Eq. 2 (5).

$$\Phi = K_{21}/(K_{21} + K_{23}). \qquad (2)$$

Then the fraction of CO escaping to solution gives the apparent quantum efficiency (AQE) for photolysis as expressed in Eq. 3.

$$\mathrm{AQE} = 1 - \Phi = K_{23}/(K_{21} + K_{23}). \qquad (3)$$

The ratio of these rapid rate constants is given by Eq. 4.

$$K_{23}/K_{21} = \mathrm{AQE}/(1 - \mathrm{AQE}). \qquad (4)$$

Recent nanosecond photolysis experiments have shown that the differences in the CO reaction kinetics for R and T state hemoglobin result in large part from a 30–60-fold change in the inward-going geminate recombination rate (6, 7). In terms of the model presented in Eq. 1, this would be a change in K_{21}. This conclusion is consistent with an earlier proposal by Friedman that modulation of geminate recombination by the protein occurs at the innermost potential barrier to CO recombination (1).

It therefore seems reasonable that measurements of the AQE for photolysis could provide a useful probe to monitor conformational changes in the hemoglobin mole-

0006-3495/89/11/947/07 $2.00

cule. Earlier flow/flash experiments carried out by Sawicki and Gibson showed that as the fractional CO saturation of a hemoglobin solution increased from 0 to 1.0 the AQE for photolysis of CO decreased from 0.99 to 0.47 (8). This data could be well fit with a model in which $Hb(CO)_1$ had an AQE for photolysis of 0.99 while all other liganded intermediates had AQEs of 0.47. In the present experiments we use this same combined flow/microsecond photolysis technique with much lower CO concentrations to study changes in the AQE for photolysis of $Hb(CO)_1$ in the first few seconds after its formation. A preliminary account of a part of this work has been given previously (9).

MATERIALS AND METHODS

Human HbA was prepared and purged of residual CO as previously described (10). Deoxygenated stock solutions, typically 5 mM in heme, were stored at 4°C and used within 10 d of preparation. Myoglobin was prepared as described earlier (11) from sperm whale skeletal muscle myoglobin (product No. M 0380, Sigma Chemical Co., St. Louis, MO).

All experiments were carried out at 20°C in 0.1 M pH 7.0 potassium phosphate buffer. Dilute working solutions of deoxyhemoglobin and CO were prepared as discussed previously (11) with 50-μM concentrations of dithionite and dithiothreitol. The experimental geometry and apparatus are similar to those used in a previous study (8). Aside from a brief description, only differences in experimental approach will be discussed here.

A Gibson-Milnes stopped-flow apparatus (12) was used to mix deoxyhemoglobin and CO solutions. The dye laser (model DL2100B, Phase-R Co., New Durham, NH) was operated with a 5×10^{-5} M methanol solution of rhodamine 590 (Exciton Chemical Co., Inc., Dayton, OH). The 1-μs-long photolysing dye laser pulse centered at 585 nm and the observation beam enter the same window of the 1.5-mm-pathlength optical cell. The ends of this 2.0-mm-diameter optical cell were sealed with large windows (6 mm diameter, 1.5 mm thick) to allow a free path for the laser pulse and observation beam.

Monitoring light was supplied by a 75-W xenon arc lamp powered by a current-controlled DC power supply (model XL150 OLIS, Jefferson, GA). A 3-nm band-pass 437-nm interference filter (Spectro Film, Winchester, MA) in series with a blue glass filter (No. B390, Hoya Optics, Fremont, CA) and an infrared blocking filter (No. HA30, Hoya Optics) was inserted in the monitoring beam to prevent significant photolysis of bound CO by the arc lamp light.

Absorption changes produced by photolysis of the carboxyhemoglobin solutions were observed with a 3-nm spectral band pass centered at 437 nm and selected by a monochromator (model 82-410 Jarrell-Ash Div., Fisher Scientific Co., Waltham, MA). These changes in light absorption were detected with a photomultiplier type 4837, RCA, Lancaster PA), converted to voltage changes with operational amplifiers, and digitized with a 10-MHz, 10-bit transient recorder (model 523A, Physical Data, Beaverton, OR). Data in the form of 4,096 10-bit words was transferred to an IBM-PC for averaging and conversion to absorbance.

Electronic time delays started by the stopping of flow were used to fire the laser and initiate data collection. A sketch of the experimental layout used has been presented earlier (8). Both the stopped-flow syringe bath and the optical cell were held at 20 ± 0.4°C using a refrigerated circulating water bath (model RTE-8Z, Neslab Instruments Inc., Portsmouth, NH). A mixing dead time of 1.2 ± 0.2 ms for the stopped-flow was determined as described earlier (8).

To achieve the nearly constant energy per pulse needed for accurate AQE measurements the analogue meter used to read out the laser charge voltage was replaced with a digital meter and the laser was operated so that it was fired once per minute at constant charge voltage during an experiment. The laser was fired at a voltage high above the lasing threshold so that the energy per pulse was about 1.1 J. Laser pulse energies were measured with a digital energy meter (model 365, Scientech, Boulder CO). Neutral density filters (Hoya Optics) were used to reduce the pulse energy to an appropriate level for the partial photolysis experiments. Partial photolysis levels of 20% or less were used so that repeated photolysis of a given CO occurred to a negligible extent.

The constancy of the laser pulse energy delivered to the sample was tested by repeatedly partially photolysing a solution of CO saturated carboxyhemoglobin. With a photolysis level of 20% the variation in pulse energy was typically found to be <1% over 70 pulses. Further details of the methods used to obtain constant photolysis pulse energy at the sample have been discussed earlier (8).

The AQE for photolysis of CO is proportional to the natural logarithm of the ratio of the concentrations of bound CO observed just before and just after the laser pulse (8). The concentrations of bound CO were calculated from observed absorbance changes using the measured absorbance change per micromolar of photolysed CO. AQE values were calibrated against CO-saturated myoglobin, which has an AQE of 0.97 (13). A detailed discussion of the calibration procedure has been given earlier (8).

RESULTS AND DISCUSSION

Fig. 1 presents AQE data plotted against the time delay after the stopping of flow. The vertical arrow indicates the

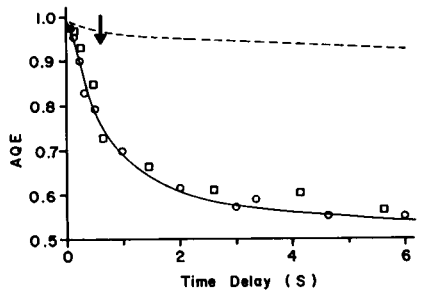

FIGURE 1 Data points giving the apparent quantum efficiency for photolysis of mixtures of hemoglobin and CO are plotted against the time delay in seconds after the stopping of flow. Each data point represents the average of at least six experiments. All experiments were performed at 20°C in 0.1 M pH 7.0 potassium phosphate buffer with a hemoglobin concentration of 40 μM. (o) Experiments carried out with a CO concentration of 4 μM. (□) Experiments carried out with a 1 μM CO concentration. Vertical arrow represents the time delay at which at least 90% of the CO is bound for all experiments. The solid line is drawn through these data points. The AQE immediately after CO binds was taken to be 0.99 as found in reference 8. Dashed curve represents a two-state model simulation discussed in detail in the text in which the apparent quantum efficiencies for photolysis of CO bound to T and R state hemoglobin molecules are respectively taken to be 0.99 and 0.47.

time at which CO binding, for all samples, is >90% complete.

The results of Fig. 1 indicate that the CO-bound heme sites convert to a low-AQE form with a half-time of ~0.6 s. The AQE decreases from nearly 1.0 at short times to ~0.6 after 2 s. Measurements made for times between 6 and 60 s, which are not represented in Fig. 1, showed that the AQE remained nearly constant at 0.55. The time course for conversion to this low AQE material was found to be the same, within the experimental error, for CO concentrations of 1 and 4 μM.

In these experiments singly liganded carboxyhemoglobin is expected to be the dominant species present just after the CO binds. If the binding is assumed to be statistical, a sample with 40 μM Hb and 1 μM CO is expected to initially have 90% of the bound CO on the $Hb(CO)_1$ species whereas samples with 4 μM CO are expected to begin with >70% of the bound CO on the singly liganded species.

Using the simple model of Eq. 1 the observed changes in AQE can be related to large changes in the ratio of the geminate rates K_{23} and K_{21}. At short times the AQE is taken to be 0.99 (8) so Eq. 4 gives $K_{23}/K_{21} = 99$, whereas 2 s after the stopping of flow the AQE is ~0.6, giving $K_{23}/K_{21} = 1.5$. In terms of the model of Eq. 1 the observed AQE changes result from changes of ~60 times in the ratio of rate constants K_{23}/K_{21} in the first few seconds after CO binds. As mentioned earlier, nanosecond experiments show a difference of this same order between the K_{21} values observed for T and R state hemoglobin molecules (6, 7).

Flow/flash experiments were also carried out in which 40 μM myoglobin was mixed with 4 μM CO and partially photolysed at a range of time delays between 0.1 and 20 s. The AQE for photolysis in this case had a constant value independent of time delay.

Nanosecond studies of modified human hemoglobin locked into a *T*-like state by addition of inositol hexaphosphate gave a geminate yield Φ of <0.01 (6). This agrees with our value of $\Phi = 1 - \text{AQE}$ if we take AQE = 0.99, the value for singly liganded hemoglobin just after the CO binds (8).

We next consider some possible types of modifications of the $Hb(CO)_1$ molecule which could produce the AQE changes we observe. These changes might, for example, be produced by rearrangement of CO molecules between the α and β chains after the initial binding or by dissociation of tetrameric hemoglobin to dimers. Another possible origin for AQE changes is conformational change in $Hb(CO)_1$.

In consideration of these possible processes we will assume, for simplicity, that only two species with different AQE values are present in our hemoglobin solutions. The data of Fig. 1 indicates that the $Hb(CO)_1$ species present immediately after CO binds has an AQE of 0.99. After binding, this high AQE form at least partially converts, within a few seconds, to a species with an AQE no greater than 0.55.

In the next paragraph simple calculations of the AQE for photolysis of a mixture of two species with different AQE values are found to yield information which will be useful later in this section to differentiate between possible explanations for the AQE change.

Regardless of the process producing the low AQE material a large fraction of the CO-bound hemoglobin must convert to the low-AQE form to explain the data of Fig. 1. This conclusion is independent of the magnitude of the low AQE value. For example, even if the low-AQE species had an AQE of 0.00, 42% of the CO-bound heme sites must be in this low-AQE form at long times (time > 4 s in Fig. 1) to give our observed AQE value of 0.55. If a more reasonable value of 0.47 (the value for $Hb[CO]_4$ under our conditions) is taken for the lower AQE value, 84% of the CO-bound hemes must have this low AQE to give an observed AQE of 0.55.

For processes involving either CO redistribution or dimerization of CO-bound hemoglobin, the considerations discussed above lead to kinetic and equilibrium properties for these species which are very different from those suggested by previous studies. For this reason we will conclude that a conformational change in $Hb(CO)_1$ provides the most reasonable explanation for the data presented in Fig. 1. The difficulties encountered in trying to explain our data in terms of either dimerization or CO rearrangement are discussed in detail in the following paragraphs.

To simplify discussion of the possibility that CO rearrangement produces the AQE change we will refer to the high-AQE hemes as α hemes and the low-AQE hemes as β hemes without implying any specific relation to the α and β chains of hemoglobin. Initial CO binding must occur almost exclusively to the high-AQE α hemes, which must have an AQE of at least 0.99. In addition, >95% of the CO must initially bind to these high-AQE hemes. Both of these assumptions are necessary to obtain the value of the AQE (0.99) observed just after CO binding begins. To obtain the AQE value of 0.55 observed at long times this CO must then substantially rearrange, within a few seconds, to the low-AQE β hemes. This requires that the α hemes have a much larger on-rate for CO binding than the β hemes (≥20 times larger). To give an AQE of 0.55 at long times, the β hemes must have an equilibrium binding affinity for CO similar to or greater than the α hemes. In any case, rearrangement in this system would be a very slow process (compared to the CO dissociation rate for the α hemes). The rearrangement would be slow because many steps of CO dissociation from α hemes followed by rebinding must take place before a large

fraction of the CO molecules even have a reasonable chance to bind to the low on-rate β hemes. The necessity for a many-step process can be easily understood in terms of the difference in CO on-rates. The probability that a dissociated CO will rebind to a β heme is ~0.05 because it is equal to the ratio of the CO on-rates. Rearrangement of 50% of the bound CO would require, on the average, that each CO undergo ~10 cycles of dissociation.

Following the argument in the above paragraph, if we invoke CO rearrangement to explain our AQE data, the fastest CO off-rate would have to be ~10 times more rapid than the rate of the AQE change seen in Fig. 1, which represents the rate of CO rearrangement. Experimental CO dissociation rate constants measured for hemoglobin are in the range of 0.01–0.1 s^{-1} (14, 15). These rate constants are much too small for a significant fraction of the CO initially bound to high-AQE subunits to rearrange to low-AQE subunits in the first few seconds after the stopping of flow. CO rearrangement therefore seems to be an unlikely explanation for the AQE change seen in Fig. 1.

If the AQE change resulted from dimer formation, a large fraction of the $Hb(CO)_1$ formed by CO binding would have to dissociate to dimers within a few seconds. The rate and extent of dimerization required seem very unlikely in terms of the known properties of hemoglobin. The tetramer-dimer dissociation constant of $Hb(CO)_1$ has not been measured but it is expected, on considerations of free energy, to have a very much smaller value than the dissociation constant of $Hb(CO)_4$ These conclusions are not strongly model dependent (so long as $Hb[CO]_1$ has a low T state–like binding affinity for CO).

It is easiest to calculate the dissociation constant for $Hb(CO)_1$ using the two-state model of Monod et al. (16). This calculation gives 0.001 μM (17) for the tetramer-dimer dissociation constant of $Hb(CO)_1$ under the present conditions. In this case a negligible concentration of dimers would be produced in our experiments from $Hb(CO)_1$ even under equilibrium conditions.

If it can be assumed that the tetramer-dimer dissociation properties of oxy- and carboxyhemoglobin are qualitatively similar, other experimental studies also indicate that the concentration of dimers present in our experiments should be negligible. Measurements carried out by Mills et al. (18) gave a tetramer-dimer dissociation constant for $Hb(O_2)_1$ of ~0.002 μM in reasonable agreement with the approximate value of this quantity for $Hb(CO)_1$ referred to in the discussion above. Measurements using haptoglobin binding of the rate constant describing dissociation of the $Hb(O_2)_1$ tetramers to dimers indicate that it is only ~0.003 per second (19). This rate is very much less than the rate of formation of low-AQE material seen in Fig. 1. If a similar rate constant applied to dimerization of $Hb(CO)_1$ the concentration of dimers present in our solutions would be much lower than the equilibrium value. The rapid dimerization of a large fraction of the CO-bound hemoglobin present in our experimental solutions also seems to be an unlikely explanation for the AQE change.

The most likely origin of the change in AQE seen in Fig. 1 seems to be a conformational change in $Hb(CO)_1$. Although this conformational change (with a half-time of ~0.6 s) would be quite slow compared with other structural changes seen for human hemoglobin, a change with a half-time 100 times longer has been reported for bluefin tuna hemoglobin (20).

Occurrence of a conformational change in $Hb(CO)_1$ as suggested by our data is consistent with several observations made on singly liganded hemoglobin using very different experimental techniques.

Hydrogen exchange experiments carried out on hemoglobin as a function of CO saturation by Hallaway et al. (21) indicated the occurrence of conformational change in $Hb(CO)_1$. In particular, this hydrogen exchange data was described in terms of a conformational change leading to increased solvent accessibility which took place after the binding of the first CO molecule.

Studies carried out by Makino and Sugita (22) showed that changes in the reactivity of β-93 SH groups in hemoglobin led the degree of fractional saturation with O_2. These experiments are also consistent with occurrence of a conformational change in singly liganded hemoglobin.

In the rest of this section the present AQE measurements will be related to earlier studies of the AQE for photolysis of carboxyhemoglobin (8) and to studies of CO rebinding after photolysis of $Hb(CO)_1$ (17). We will then unsuccessfully attempt to simulate the AQE data presented in Fig. 1 in terms of a simple form of the two-state allosteric model (16). In this model the AQE change is produced by a T→R quaternary conformational change which occurs as ligand binds to deoxyhemoglobin. Finally we discuss the possibility that the observed AQE change results from a tertiary conformational change in hemoglobin. Although we discuss only two different schemes for how AQE changes could arise from conformational changes it must be emphasized that other reasonable alternative explanations certainly exist.

In earlier measurements good fits were obtained to the AQE changes taking place during CO binding to hemoglobin using a two-state model by decoupling the change in AQE from the confomational change affecting ligand binding (see dashed curve in Fig. 4 of reference 8). Without this decoupling assumption, reasonable two-state model parameters produced a poor fit to the data (see solid curve in same figure). This decoupling was justified by suggesting that the T state was split into

closely spaced substates with very similar free energies but with AQEs that differed by a factor of 2.1. In this way the conformational change affecting the AQE would have little effect on ligand binding properties. This decoupling assumption no longer seems reasonable because, as discussed earlier, the observed change in AQE seen in Fig. 1 relates to changes in geminate rates which are similar to the differences between these rates seen for R and T state hemoglobin. This similarity suggests that the AQE changes of Fig. 1 may represent a switch of one or more of the heme sites on an $Hb(CO)_1$ molecule to an R-like binding state.

Combined flow/flash CO rebinding experiments carried out under the same experimental conditions as the present AQE experiments have indicated that at low fractional CO saturations about half of the CO-bound heme sites converted with a half-time of 5 s to a quickly reacting R-like form (17). The lack of a CO concentration dependence of these results and arguments similar to those presented earlier in this section suggested that a conformational change was the most reasonable explanation for formation of this R-like material.

In these earlier experiments, formation of R-like material had a half-time eight times longer than that describing the present AQE change. However, it is not possible to conclude that these experiments contradict one another. A direct comparison of these results is difficult because of the very different experimental techniques employed in the two experiments. CO rebinding in the earlier experiments had a half-time of ~0.5 s (17) so that significant conformational relaxation of the deoxyhemoglobin produced by photolysis could have taken place before CO rebound and probed the conformational state. In contrast, in the present AQE experiments the conformational state was probed on a time scale of a fraction of a microsecond after photolysis because of the high rates of the geminate processes which determine the observed value of the AQE. Taken together these two very different results suggest that there may be two different conformational changes occurring in $Hb(CO)_1$ after CO binding.

The dashed curve in Fig. 1 represents an attempt to simulate our results using the simplest form of the allosteric two-state model of Monod et al. (16). Conformational change and CO binding in this model are described by Eqs. 5–8 (23). These equations represent the conformational change and CO binding properties of hemoglobin tetramers. Hb^R and Hb^T represent tetramers in the R and T quaternary conformational states, respectively. In this model all heme sites on a particular hemoglobin molecule have CO binding and geminate recombination properties which depend only upon the quaternary conformational state of that molecule.

$$Hb^R(CO)_n \overset{Lc^n}{\rightleftharpoons} Hb^T(CO)_n \quad 0 \le n \le 4 \tag{5}$$

$$Hb^R(CO)_n + CO \underset{(n+1)1_R}{\overset{(4-n)1'_R}{\rightleftharpoons}} Hb^R(CO)_{n+1} \quad 0 \le n \le 3 \tag{6}$$

$$Hb^T(CO)_n + CO \underset{(n+1)1_T}{\overset{(4-n)1'_T}{\rightleftharpoons}} Hb^T(CO)_{n+1} \quad 0 \le n \le 3 \tag{7}$$

$$c = (1_R \cdot 1'_T)/(1'_R \cdot 1_T). \tag{8}$$

In Eqs. 6–8 $1'_R$ and $1'_T$ are, respectively, the microscopic on-rate constants for R and T state heme sites. Similarly, 1_R and 1_T are the microscopic CO dissociation rate constants for R and T state heme sites. L and c are, respectively, the allosteric parameter and the ratio of the microscopic equilibrium dissociation constants for R and T state heme sites as given by Eq. 8.

We have assumed that conformational changes are rapid compared with the ordinary ligand binding processes taking place after the mixing of the deoxyhemoglobin and CO solutions in the stopped-flow. Thus all hemoglobin intermediates are assumed to be in conformational equilibrium before the laser fires. Eq. 5 expresses this equilibrium. In Eq. 5 Lc^n is the ratio of species concentrations $[Hb^T(CO)_n]/[Hb^R(CO)_n]$. As more CO molecules bind to a particular hemoglobin molecule the conformational equilibrium shifts to favor the R state. We have also assumed that significant conformational change does not occur during the 1-μs duration of the laser pulse. Eqs. 6 and 7 represent, respectively, reactions between CO and R and T state tetramers.

Chain differences and dimers were neglected and the AQE for photolysis of CO bound to T stte tetramers was taken to be 0.99 whereas the AQE for CO bound to R state molecules was taken to be 0.47 (8). Other model parameters used in the simulation were also obtained from experiments reported in the literature. The allosteric parameter $L = 1.4 \times 10^7$ was derived from equilibrium data for the reaction of oxygen with hemoglobin (9). $1_R = 0.01\ s^{-1}$ and $1_T = 0.1\ s^{-1}$ the microscopic dissociation rate constants for the R and T states were obtained by assuming that the dissociation rates observed for $Hb(CO)_4$ and $Hb(CO)_1$ (14) reflected the properties of the R and T states, respectively. The microscopic binding rate constants for the R and T states were taken to be $1'_R = 6.5\ \mu M^{-1}s^{-1}$ (11) and $1'_T = 0.1\ \mu M^{-1}s^{-1}$ (24).

Differential equations derived from Eqs. 5–7 were solved, using the parameter values given above, to produce the dashed curve in Fig. 1. This solution was generated using initial conditions that assumed that at $t = 0$ (just after the stopping of flow) all of the hemoglobin was present in the deoxy T state form.

The poor agreement between the simulation using these parameters (dashed line in Fig. 1) and the data results because the T-to-R switch occurs predominately

after binding of three molecules of CO to a hemoglobin molecule, whereas, the data results primarily from a change in the AQE of $Hb(CO)_1$. As mentioned earlier, 84% of the CO-bound heme sites would have to convert to the R state form with an AQE of 0.47 to give the AQE of 0.55 observed for times >4 s. An acceptable fit to the data presented in Fig. 1 cannot be achieved by a reasonable adjustment of the parameters used in the simple two-state model expressed in Eqs. 5–7. The values used for the CO binding and dissociation rate constants in this model are well determined by experimental measurements so a significant improvement in the fit to the data of Fig. 1 can only be attained through a change in the value of L. A sharp decrease in L which results in a T→R switch after binding of only one molecule of CO can produce good fits. For example, taking $L = 200$ with all other parameters values left unchanged gives a reasonably good fit to our data. However such low L values are inconsistent with earlier experiments. For example, because L only involves the free energy difference between deoxyhemoglobin in the R and T states (25) it will be the same for oxy- and carboxyhemoglobin. Fits to oxygen equilibrium data give large values similar to those used in the simulation presented in Fig. 1 (11).

One possible way to explain our results in terms of a functionally significant conformational change (because this seems to be implied by the size of the AQE change) and still maintain consistency with equilibrium measurements would be to consider tertiary conformational changes in $Hb(CO)_1$. In this case a tertiary conformational change could modify the CO binding properties and AQE of the CO-bound subunit without switching all four heme sites to a high-binding affinity R-like state.

In summary these experiments indicate that the AQE for photolysis of $Hb(CO)_1$ decreases from a value close to 1.0 just after CO binding to ~0.6 after 2 s. The short halftime (0.6 s) of this change suggests that it was not due to rearrangement of CO among the available deoxy heme sites, whereas both the short half-time and magnitude of the change made dimer formation an unlikely explanation. Modification of geminate CO recombination rates by a protein conformational change seems to be the most likely explanation. Because the change in these geminate rate constants implied by the change in AQE is similar to the difference found between R and T state hemoglobin, it may be that one or more heme sites on an $Hb(CO)_1$ molecule converts to an R-like binding state. However the exact nature of this conformational change cannot be inferred from our data.

Received for publication 3 February 1989 and in final form 2 June 1989.

REFERENCES

1. Friedman, J. M. 1985. Structure, dynamics, and reactivity in hemoglobin. *Science (Wash. DC).* 228:1273–1280.
2. Olson, J. S., R. J. Rohlfs, and Q. H. Gibson. 1987. Ligand recombination to the α and β subunits of human hemoglobin. *J. Biol. Chem.* 262:12930–12938.
3. Petrich, J. W., C. Poyart, and J. L Martin. 1988. Photophysics and reactivity of heme proteins: a femtosecond absorption study of hemoglobin, myoglobin, and protoheme. *Biochemistry.* 27:4049–4060.
4. Duddel, D. A., R. J. Morris, N. J. Muttucumaru, and J. T. Richards. 1980. The dependence of the quantum yield of ligand photodissociation from haem proteins on ultrafast recombination. *Photochem. Photobiol.* 33:479–484.
5. Campbell, B. F., D. Magde, and V. S. Sharma. 1984. Geminate recombination in carboxy hemoglobin A and its relation to overall carbon monoxide reactivity. *J. Mol. Biol.* 179:143–150.
6. Murry, L. P., J. Hofrichter, E. R. Henry, M. Ikeda-Sato, K. Kitagishi, T. Yonetani, and W. A. Eaton. 1988. The effect of quaternary structure on the kinetics of conformational changes and nanosecond geminate rebinding of carbon monoxide to hemoglobin. *Proc. Natl. Acad. Sci. USA.* 85:2151–2155.
7. Rohlfs, R. J., J. S. Olson, and Q. H. Gibson. 1988. A comparison of the geminate recombination kinetics of several monomeric heme proteins. *J. Biol. Chem.* 263:1803–1813.
8. Sawicki, C. A., and Q. H. Gibson. 1979. Dependence of the quantum efficiency for photolysis of carboxyhemoglobin on the degree of ligation. *J. Biol. Chem.* 254:4058–4062.
9. Sawicki, C. A., and I. A. Zahroon. 1988. Rapid conversion of singly liganded carboxyhemoglobin to a quickly reacting, low quantum efficiency form. *Biophys. J.* 53:627a. (Abstr.)
10. Sawicki, C. A., and Q. H. Gibson. 1977. Properties of T state of human oxyhemoglobin studied by laser photolysis. *J. Biol. Chem.* 252:7538–7547.
11. Sawicki, C. A., and Q. H. Gibson. 1976. Quaternary conformational changes in human hemoglobin studied by laser photolysis of carboxyhemoglobin. *J. Biol. Chem.* 251:1533–1542.
12. Gibson, Q. H., and L. Milnes. 1964. Apparatus for rapid and sensitive spectrophotometry. *Biochem. J.* 91:161–171.
13. Schuresco, D. D., and W. W. Webb. 1978. Carboxylation kinetics of hemoglobin and myoglobin: linear transient response to step perturbation by laser photolysis. *Biophys. J.* 24:382–383.
14. Sharma, V. A., M. R. Schmidt, and H. M. Ranney. 1976. Dissociation of CO from carboxyhemoglobin. *J. Biol. Chem.* 251:4267–4272.
15. Samaja, M., and E. Rovida. 1987. The dissociation of carbon monoxide from the alpha and beta subunits of human carbonmonoxy hemoglobin. *Biochem. Biophys. Res. Commun.* 148:1196–1201.
16. Monod, J., J. Wyman, and J. P. Changeux. 1965. On the nature of allosteric transitions: a plausible model. *J. Mol. Biol.* 12:88–118.
17. Khaleque, M. A., and C. A. Sawicki. 1986. Conversion of singly liganded carboxyhemoglobin to a rapidly reacting form. *Photobiochem. Photobiophys.* 13:155–164.
18. Mills, F. C., M. L. Johnson, and G. K. Ackers. 1976. Oxygenation-linked subunit interactions in human hemoglobin: experimental studies on the concentration dependence of oxygenation curves. *Biochemistry.* 15:5350–5362.

19. Romeo, P. H., J. Thillet, A. Tsapis, and J. Rosa. 1982. Kinetics of subunit dissociation of partially oxygenated hemoglobin determined by haptoglobin binding. *Biochem. Biophys. Res. Commun.* 105:1354–1360.

20. Morris, R. J., W. S. Neckameyer, and Q. Gibson. 1981. Multiple T state conformations in a fish hemoglobin. *J. Biol. Chem.* 256:4598–4603.

21. Hallaway, P. E., B. E. Hallaway, and A. Rosenberg. 1984. Study of hydrogen exchange in hemoglobin as a function of fractional ligand saturation. *Biochemistry.* 23:266–273.

22. Makino, N., and Y. Sugita. 1982. The structure of partially oxygenated hemoglobin. *J. Biol. Chem.* 257:163–168.

23. Sawicki, C. A., and Q. H. Gibson. 1978. The relation between carbonmonoxide binding and the conformational change of hemoglobin. *Biophys. J.* 24:21–33.

24. Antonini, E., E. Chiancone, and M. Brunori. 1967. Studies on the relations between molecular and functional properties of hemoglobin. *J. Biol. Chem.* 242:4360–4366.

25. Shulman, R. G., J. J. Hopfield, and S. Ogawa. 1975. Allosteric interpretation of haemoglobin properties. *Q. Rev. Biophys.* 8:325–420.