# LASER PHOTOLYSIS STUDY OF CONFORMATIONAL CHANGE RATES FOR HEMOGLOBIN IN VISCOUS SOLUTIONS

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ABSTRACT Rates for the R → T conformational change of deoxyhemoglobin formed by laser photolysis of carboxyhemoglobin were determined from CO rebinding observed in three solution systems with viscosities between 1 and 6 cP. Experiments were carried out at 20°C and pH 8.3 in solutions consisting of borate buffer containing various amounts of sucrose, glycerol, or ethylene glycol. As in the case of earlier experiments in borate buffer (Sawicki and Gibson, 1976, *J. Biol. Chem.* , 251:1533–1542), a simple two-state allosteric model which takes into account tetramer-dimer dissociation was found to give a good description of all experimental results. Using measured values for the R- and T-state CO-binding rate constants and the tetramer-dimer dissociation constant, values for the conformational change rate were determined by fitting this model to the experimental data. These rates were compared with Gavish's transient strain model (Gavish, 1978, *Biophys. Struct. Mech.*, 4:37–52), which predicts an inverse dependence of conformational change rate on viscosity. Although fair agreement is found for hemoglobin in sucrose/borate solutions, in glycerol/borate and ethylene glycol/borate solutions, conformational change rate falls off much more rapidly with increasing viscosity than predicted by the model.

## INTRODUCTION

Although conformational changes play a central role in the function of many interesting protein macromolecules, very little is known about the influence of the surrounding solvent on the rates of these changes. Recent experimental (1) and theoretical (2) work by Gavish suggests that solution viscosity is an important influence that has not been given adequate consideration.

Gavish has adapted the theory of Kramers (3) to the dynamics of macromolecules. In this model, interactions between protein and solvent are divided into two distinct types. First, random collisions between solvent molecules and the protein provide the driving force for structural fluctuations that can lead to conformational change. The fluid also exerts a systematic Stokes-like viscous damping force on the protein, which acts to reduce the frequency of these fluctuations. Conformational change can only occur when fluctuations in a relevant structural parameter become of sufficient amplitude that a potential barrier is exceeded. Increased viscosity reduces the frequency of structural fluctuations and thus decreases the rate of conformational change. The expression obtained for the rate $K$ of conformational change is given in Eq. 1:

$$K = (A/\eta) \exp(-\Delta/RT). \quad (1)$$

Here $R$ is the gas constant, $T$ the absolute temperature and $\eta$ the solution viscosity. $A$ is a function of structural parameters that characterize the potential energy barrier profile. The height $\Delta$ of this barrier is assumed to be large compared with the average thermal energy.

For simpler systems consisting of polymers in solution, the inverse first-power viscosity dependence shown in Eq. 1 agrees well with experimental data (4). Investigation of the effect of solution viscosity on the rates of conformational changes in protein macromolecules has proven to be considerably more difficult. In Gavish's studies of the effect of solution viscosity on the rate of hydrolysis of a peptide bond by carboxypeptidase A (1) a conformational change was assumed to be the rate limiting step. For mixed solutions, his results agreed fairly well with Eq. 1, however, the catalysis rate was varied by a factor of <2.5 (for fixed temperature) and results for water were not consistent with the other data. Experiments by Beece et al. with myoglobin (5) and bacteriorhodopsin (6) also suggest that solution viscosity is an important parameter to be considered in reactions of macromolecules. In these experiments, the rates of four processes observed for bacteriorhodopsin were found to vary with viscosity to the inverse 0.1–0.8 power. Similarly, for reactions of myoglobin with $O_2$ and CO, a number of reaction steps were observed with rates that varied with viscosity to the inverse 0.4–0.8 power.

In this paper, we present results from studies on the rate of conformational change of hemoglobin in several viscous solvent systems. A preliminary account of a part of this work has been given previously (7). Ideally, the experimental system used in this type of investigation should meet two stringent requirements. First, the macromolecule

chosen should have a clearly defined conformational change whose rate can be easily determined. Second, changes in solution properties, other than viscosity, such as dielectric constant and pH should have minimal effect on the rate of the conformational change. In the case of enzymes, both of these requirements are difficult to meet. For these proteins, no clearly defined conformational change is available for study, although it may often be assumed that a conformational change leading to product release is the rate limiting step in catalysis reactions (8, 9). Enzymes are also quite sensitive to changes in solution conditions. As the proportions of components used in a mixed solvent system are varied to change the viscosity, changes in dielectric constant and other properties also occur. Corrections must be made for dielectric constant changes since electrostatic interactions between enzyme and substrate are usually important in catalysis. Unfortunately, these corrections are at best semiquantitative (1). Catalysis rates can also be changed by a number of less well defined solvent effects (10).

Hemoglobin was chosen for study because there is evidence suggesting that both of the conditions listed above can be satisfied. Following the classic work of Gibson (11), a number of experiments (12–14) have indicated that rates for the R → T conformational change of deoxyhemoglobin can be extracted from ligand-rebinding kinetics observed following laser photolysis. Spectroscopic and kinetic data presented in these experiments suggest that sudden removal of bound ligand with a light pulse produces deoxyhemoglobin with the R-state conformation of fully liganded hemoglobin. In photolysis experiments carried out at pH 8 and above, the half-life for the relaxation of R deoxyhemoglobin to the T state is ~100 $\mu$s at 20°C (13). Under these conditions, ligand can be removed with a 1 $\mu$s laser pulse before significant conformational change occurs. Because photolysis involves electronic excitation of a heme group, there is some concern that in these experiments some of the observed properties may be those of electronically excited molecules. A wide range of experiments indicate that this is not a problem, because the excited electronic state of hemoglobin produced by absorption of visible light undergoes very rapid nonradiative relaxation to the ground state. Depending upon the experimental evidence used, this relaxation requires as little as 100 fs (15) or possibly as much as several tens of picoseconds (16). Because this relaxation is many orders of magnitude faster than the conformational change, electronic excited states should not be present to interfere with observation of conformational changes. In the present experiments, we can neglect geminate recombination that occurs because part of the photolyzed CO is trapped by the protein and rebinds rather than diffusing into the solvent. This process, with a half time of <100 ns (17, 18), doesn't affect our results because our one-microsecond long laser pulse rephotolyses bound CO molecules until essentially all of the CO is removed to the solvent phase (17). In regard to the effect of changes in solution properties other than viscosity on the rate of the R → T change of hemoglobin, experiments have shown that this rate is essentially independent of pH between pH 8 and 9.5 (13). In the experiments presented here, only small changes in dielectric constant occurred as the proportions of the mixed solvent systems were varied to change viscosities by a factor of ~5. For example, as the weight fraction of ethylene glycol in water is increased to 0.5 the dielectric constant decreases by <20% (see reference 10, page 32). It seems reasonable to expect that these small changes in dielectric constant will have a much smaller effect on conformational change rates of hemoglobin than the accompanying changes in viscosity.

## MATERIALS AND METHODS

The experimental techniques and apparatus used in this study were quite similar to those used earlier to study hemoglobin in water-buffer solutions (13, 14). Aside from a brief description, only differences in experimental approach will be discussed here. Hemoglobin was prepared from pooled hospital blood samples and handled as previously described (13). Sperm whale skeletal-muscle myoglobin was purchased from Sigma Chemical Co. (St. Louis, MO). Viscous solvent solutions were prepared with reagent grade, anhydrous ethylene glycol and glycerol (J. T. Baker Chemical Co., Phillipsburg, NJ) and sucrose (grade 1, Sigma Chemical Co.) dissolved in 0.1 M sodium borate (reagent grade, Mallinckrodt, Inc., St. Louis, MO) water solutions. Viscous solutions were kept in air-tight bottles at 4°C until they were to be used. The exposure of anhydrous glycerol, ethylene glycol, or their solutions to air needed to be minimized to prevent changes in water content. For example, at 21°C and 70% relative humidity an ethylene glycol solution in equilibrium with the air contains 47.5% water by weight (19). Borate buffer was chosen for these experiments both because of its use in previous studies (13, 14) and because of its antibacterial action. The pH of all solutions was adjusted to 8.3 by addition of concentrated NaOH or HCl. This pH was chosen to minimize dimerization of hemoglobin while maintaining the simple recombination kinetics previously observed above pH 8.0 (13).

Small, accurately measured portions of deoxygenated hemoglobin solution and other reagents were added, by injection, to 50 ml serum stopper sealed syringes containing deoxygenated viscous solutions. Enough sodium dithionite (recrystallized grade, Virginia Chemicals, Inc., Portsmouth, VA) and dithiothreitol (Sigma Chemical Co.) were injected in the form of 100 mM solutions to give 50 $\mu$M concentrations in the experimental solutions. These solutions were then injected into serum stopper sealed cells that had been rinsed with nitrogen gas, for use in photolysis experiments that were carried out at 20.0 ± 0.3°C. Hemoglobin appeared to be quite stable in these viscous solutions since no changes in CO recombination kinetics were observed over a 3-h period when the samples were held at 20°C. The viscosities of all solutions were determined using a falling ball viscosimeter (Gilmont Industries Inc., Great Neck, NY, model V-2100, with a stainless steel ball). Operation of the viscosimeter in the recommended vertical orientation was found to give results that scattered by as much as 5%. Tilting the device at a 10° angle reduced the scatter to <1% between measurements. In operation, the viscosimeter was maintained at 20.0 ± 0.2°C and at least 10 measurements were made with each solution. All solutions were run through a 0.45 $\mu$m pore membrane filter to remove particles. To calculate viscosities, the densities of solutions were determined with a digital density meter (model DMA46, Mettler Instrument Corp., Hightstown, NJ). The viscosimeter was calibrated with distilled deionized water, and the calibration was checked in the range used in these experiments (1–6 cP) with solutions of sucrose and ethylene glycol in water, prepared by weighing the components to 0.1%. In all cases, the measured and tabulated viscosities (20) agreed to within 0.3%.

Absorption changes produced by photolysis of the carboxyhemoglobin solutions were observed with a 3-nm spectral band pass that was centered at 437 nm and selected by a monochromator (Jarrell-Ash Div., Fisher Scientific Co., Waltham, MA, model 82–410). Both the laser pulse and the monitoring beam entered the same face of the sample cell in the previously described nearly coincident geometry (13). The 540 nm photolysing light pulse was provided by a flash-lamp pumped dye laser (Phase R Corp., New Durham, NH, model 2100A) operated with a $10^{-4}$ M solution of Pilot 495 laser dye (Pilot Div., New England Nuclear, Boston, MA). Monitoring light was supplied by a 75 W xenon arc lamp, powered by a current-controlled DC supply (OLIS, Jefferson, Georgia, XL 150). Voltage changes produced by the photomultiplier (RCA Electro-Optics & Devices, Lancaster, PA, 4836) were amplified and stored in the 4,096 word memory of a 10 MHz transient recorder with 10-bit amplitude resolution (Physical Data, Inc., Beaverton, OR, model 523A). Kinetic data were plotted out on graph paper using an *XY* recorder (Houston Instrument Div. Bausch & Lomb, Inc., Austin, TX, model 164) interfaced to the transient recorder. For each kinetic trace, a piece of prephotolysis baseline was recorded by using a time delay to start data recording at an appropriate time before the laser was fired.

CO solubilities were determined for all of the viscous solutions. To carry out these measurements, samples of the solutions were bubbled with CO at 20.0 ± 0.5°C under conditions of known barometric pressure. Accurately measured portions of these CO-saturated solutions were then added to deoxymyoglobin solutions of known concentration (typically 6 μM in heme). Added portions were small enough so that the available heme sites were only partially saturated. These samples were injected into 1-cm cells for determination of fractional CO saturation by photolysis. Saturation was determined by comparing the absorbance changes observed for these partially saturated samples with the full absorbance changes found following CO saturation of the samples. Full saturation was produced by adding a bubble of CO to each cell and shaking. With the assumption that all CO is bound, the known myoglobin concentration then allows calculation of the CO solubility. The accuracy of this method was checked by determining the solubility of CO in distilled water. These values were within ± 3% of published data (21). In these experiments, the monitoring beam was attenuated by a factor of more than 40 with a 10-nm band-pass, 437-nm interference filter and neutral density filters to prevent unwanted photolysis of bound CO. CO solubilities are given in Table I.

## RESULTS AND DISCUSSION

Eqs. 2–5 present the form of two-state model used in the interpretation of our data. This model, which has proven to be useful in the study of reactions of CO and $O_2$ with hemoglobin (13, 14, 22) includes dimerization, and assumes that the subunits of hemoglobin have identical CO binding properties. In these equations, $L$ and $c$ are, respectively, the allosteric parameter and the ratio of the microscopic equilibrium dissociation constants for the R and T states. $Hb^R(CO)_n$ and $Hb^T(CO)_n$ represent the R and T conformations of hemoglobin with $n$ bound CO molecules. Eqs. 2 and 3 give the reactions assumed to occur between hemoglobin and CO. $l'_R$ and $l'_T$ are, respectively, the microscopic rate constants for CO combination with R- and T-state hemoglobin. Conformational relaxation and CO binding at the R-state rate by dimers are represented by Eqs. 4 and 5, respectively. $D(CO)_n$ represents the dimer species with $n$ bound CO molecules.

In our experiments, competition between CO binding and conformational change for R-state hemoglobin provides the basis for determination of conformational change rate. Therefore we cannot, for example, assume that all conformational changes are rapid compared with CO binding, as is often done in analysis of stopped-flow data (23). It is also impractical to determine all 10 of the R → T conformational change rates by varying them independently and obtaining a best fit to our data. In the present model, the use of the parameter $d$ which gives the effect of bound CO on conformational change rate, allowed us to

TABLE I
PARAMETERS FOR HEMOGLOBIN IN VISCOUS SOLUTIONS*

| Solution | Solute weight fraction | $\eta$ | CO solubility‡ | $K_{4,2}$ | $l'_R$ | $l'_T$ | $K_o$ |
|---|---|---|---|---|---|---|---|
| | | *cP* | *μM* | *μM* | $\mu M^{-1}S^{-1}$ | $\mu M^{-1}S^{-1}$ | $S^{-1}$ |
| Borate | --- | 1.08 | 1,030 | 0.11 ± 0.01 | 9.8 ± 0.3 | 0.30 ± 0.02 | 6,400 ± 300 |
| Sucrose/borate | 0.126 | 1.54 | 820 | 0.11 ± 0.01 | 11.6 ± 0.6 | 0.39 ± 0.02 | 3,800 ± 200 |
| | 0.226 | 2.65 | 690 | 0.16 ± 0.01 | 12.9 ± 0.3 | 0.46 ± 0.03 | 2,700 ± 200 |
| | 0.299 | 3.84 | 590 | 0.18 ± 0.02 | 14.5 ± 0.5 | 0.51 ± 0.03 | 2,100 ± 100 |
| | 0.363 | 5.94 | 530 | 0.23 ± 0.02 | 15.9 ± 0.4 | 0.51 ± 0.04 | 1,500 ± 100 |
| Glycerol/borate | 0.125 | 1.58 | 820 | 0.08 ± 0.01 | 12.1 ± 0.7 | 0.30 ± 0.01 | 3,300 ± 200 |
| | 0.232 | 2.25 | 700 | 0.11 ± 0.01 | 13.2 ± 0.4 | 0.33 ± 0.01 | 1,900 ± 100 |
| | 0.332 | 3.27 | 610 | 0.20 ± 0.01 | 14.7 ± 0.6 | 0.38 ± 0.01 | 1,100 ± 100 |
| | 0.422 | 4.78 | 530 | 0.42 ± 0.02 | 16.6 ± 0.3 | 0.42 ± 0.01 | 700 ± 50 |
| Ethylene glycol/borate | 0.162 | 1.65 | 870 | 0.14 ± 0.01 | 10.8 ± 0.2 | 0.29 ± 0.01 | 2,000 ± 100 |
| | 0.314 | 2.55 | 750 | 0.37 ± 0.04 | 11.8 ± 0.1 | 0.31 ± 0.02 | 770 ± 50 |
| | 0.486 | 4.04 | 690 | 2.6 ± 0.4 | 11.5 ± 0.4 | 0.45 ± 0.02 | 320 ± 50 |
| | 0.631 | --- | --- | 8 ± 6 | 10.2 ± 0.3 | 0.6 ± 0.2 | --- |

*All parameters are for 20°C and a solution pH of 8.3
‡CO solubilities are for 760 mmHg.

avoid these difficulties. A discussion and test of the reasonableness of the use of this parameter are given in the discussion associated with Figs. 4 and 5 in reference 13. Dissociation of CO from hemoglobin was neglected, since it is too slow to contribute to the observed kinetics in the present experiments (24). Dimer recombination following photolysis was also neglected, since it is several orders of magnitude slower (25, 26) than CO recombination. Detailed discussions of the other assumptions made in the use of this model have been given previously (13, 14).

$$\mathrm{Hb^R(CO)}_n + \mathrm{CO} \xrightarrow{(4-n)\cdot l'_R} \mathrm{Hb^R(CO)}_{n+1}. \quad 0 \le n \le 3 \quad (2)$$

$$\mathrm{Hb^T(CO)}_n + \mathrm{CO} \xrightarrow{(4-n)\cdot l'_T} \mathrm{Hb^T(CO)}_{n+1}. \quad 0 \le n \le 3 \quad (3)$$

$$\mathrm{Hb^R(CO)}_n \underset{K_{o/[L(cd)^n]}}{\overset{K_{o/d^n}}{\rightleftharpoons}} \mathrm{Hb^T(CO)}_n. \quad 0 \le n \le 4 \quad (4)$$

$$\mathrm{D(CO)}_n + \mathrm{CO} \xrightarrow{(2-n)\cdot l'_R} \mathrm{D(CO)}_{n+1}. \quad 0 \le n \le 1 \quad (5)$$

Differential equations derived from Eqs. 2–5 with appropriate parameter values were solved numerically with a digital computer using a second-order Runge-Kutta algorithm (27). The program keeps track of 10 R- and T-state species and 3 dimer species. The initial conditions used to produce solutions to the differential equations were these: immediately following full photolysis all hemoglobin was present in the form of R-state deoxytetramers and dimers. Reactions of these species were treated independently because, as previously discussed, interconversion was negligible on the time scale of these experiments. $K_o$ was varied to obtain a best fit of the model calculations to the experimental data collected for each solution.

Before discussing the methods used for determination of parameters, it is useful to develop a qualitative understanding of the form of CO recombination kinetics expected for this model. The microscopic ON rate constant for CO reacting with the R-state hemoglobin and dimers present after photolysis is more than 30 times larger than that for T-state hemoglobin. CO recombination is therefore expected to exhibit quite distinct rapid and slow phases. The R-state deoxyhemoglobin present following photolysis can either bind CO or relax to the slowly reacting T state. For the low levels of dimerization present in these experiments, the division of the recombination kinetics between rapid and slow phases is thus largely determined by a competition between rapid CO binding and conformational relaxation. A decrease in the R → T relaxation rate produced by, for example, an increase in viscosity would result in an increase in the fraction of rapidly reacting hemoglobin observed following photolysis.

In the next few paragraphs, the derivation of parameter values used in simulations of kinetic data will be discussed. The parameters $L$ and $c$ have the same definitions originally given by Monod et al. (28). Because the allosteric parameter $L$ is related to the free-energy difference between the R and T states of deoxyhemoglobin, changes in $L$ with changes in the weight fraction of solute might be expected to alter the rates of conformational change independent of any effect of viscosity. $L$ was taken to be 3,000 (14). We also assumed that $L$ was independent of the weight fraction of added solute. Justification of this important assumption is given in the Appendix. Similarly, it was assumed that $c$, the ratio of the microscopic equilibrium CO dissociation constants for the R and T states, was independent of solute concentration. As shown in Table I the effects of solute concentration on $l'_R$ and $l'_T$ were in the same direction and partially cancel each other in calculation of $c$. While we have no data related to the changes in R- and T-state OFF rates resulting from changes in solute concentration, it has been observed that OFF rates for myoglobin are independent of glycerol concentration (29). Experiments by Sharma et al. (24) give microscopic CO-dissociation rate constants of 0.09 $s^{-1}$ and 0.009 $s^{-1}$ for the T and R states, respectively. The present experiments in pH 8.3 borate buffer gave 9.8 $\mu M^{-1}\,s^{-1}$ and 0.30 $\mu M^{-1}\,s^{-1}$ for the microscopic association rate constants for the R and T states, respectively. We therefore assigned $c$ the value 0.003 in our calculations. The parameter $d$ was set to the value 2.3 found in earlier studies (13). Fits of the model to our data carried out by varying both $K_o$ and $d$ indicated that this was a reasonable choice for $d$. The best fit value obtained for $K_o$ depended somewhat upon the value chosen for $d$. For example, increasing $d$ from 2.3 to 4.6 results in about a 9% increase in the best-fit value obtained for $K_o$ in fitting our data for hemoglobin in borate buffer.

Tetramer-dimer dissociation constants, defined by Eq. 6, were determined for all samples, using photolysis of dilute carboxyhemoglobin solutions as has been previously described (30). In Eq. 6, $D$ and $T$ are the concentrations of dimeric and tetrameric species, respectively.

$$K_{4,2} = D^2/T. \quad (6)$$

In these measurements the use of low concentrations of CO made rebinding of CO to R-state hemoglobin much slower than the R → T conformational change of deoxyhemoglobin. Under these conditions, conformational relaxation occurs before CO rebinding takes place so that the fraction of heme sites in the form of dimers is just the fraction of rapidly rebinding hemoglobin observed following full photolysis (30, 31). Due to the high affinity of hemoglobin for dioxygen at pH 8.3, dithionite was added to all samples to eliminate possible artifacts. Addition of dithionite caused a large increase in $K_{4,2}$ values; however, this increase was prevented as described earlier by addition of dithiothreitol (30). If $\alpha$ is defined as the fraction of rapidly reacting hemoglobin observed following photolysis, $K_{4,2}$ can be written in the useful form given in Eq. 7 (32). Here Hb is the total hemoglobin concentration in solution.

$$K_{4,2} = \mathrm{Hb}[\alpha^2/(1-\alpha)]. \quad (7)$$

The value of $\alpha$ for use in Eq. 7 was determined for each solution by least-squares fitting of an exponential function to the slow phase of the recombination kinetics. The slow absorbance excursion projected back to the time the laser was fired divided by the full excursion at this time then gives $1 - \alpha$. At least three determinations of $K_{4,2}$ made using Eq. 7 were averaged to produce the values presented in Table I. Our results, $K_{4,2} = 0.11 \pm 0.01$ $\mu$M for carboxyhemoglobin at pH 8.3, were consistent with the gel chromatography measurements of Atha and Riggs (33). The curve through their data on the effect of pH on $K_{4,2}$ (Fig. 1 of their paper) and the errors given yield a value for $K_{4,2}$ between 0.2 and 0.02 $\mu$M at pH 8.3. In our experimental solutions (typically 25 $\mu$M in heme), conditions were usually favorable for the study of conformational change in tetrameric hemoglobin, since dimers made up <12% of the total heme, except in the case of the two solutions with the highest weight fraction of ethylene glycol. In the most concentrated ethylene glycol solution, the large and rather uncertain value of $K_{4,2}$ prevented determination of the conformational change rate $K_o$.

The increase in $K_{4,2}$ with increasing fraction of solute seen in Table I was consistent with a simple model presented by Blank (34) in which electrostatic forces play a significant part in the tetramer-dimer equilibrium of hemoglobin. With this simple model, Blank (34) is able to give qualitative explanations for a number of properties of this equilibrium, including the increase in dissociation observed at both acidic and alkaline pH. Applying Blank's model to our results, we see that as solution pH moved away from the isoelectric point of carboxyhemoglobin, charge built up on each hemoglobin molecule. When a molecule dissociated these charges were divided between dimers that were far apart. Repulsion of like charges thus resulted in a lowering of the free energy of the dimer with respect to that of the tetramer. In terms of this picture, an increase in the fraction of solute, which lowers the solution dielectric constant (10), increased repulsive forces and thus led to the increased dissociation shown in Table I.

$l'_R$, the rate constant for CO binding to R-state hemoglobin, was determined as previously described (13) from CO recombination kinetics observed following low levels of partial photolysis carried out on fully CO-saturated samples that were typically 25 $\mu$M in heme. In our experiments, the photolysis level was 5% or less, so that essentially all of the deoxyheme sites produced were present on the species $Hb(CO)_3$, which remains in the R state and gives rise to a single rapid phase of ligand rebinding described by $l'_R$. $l'_R$ values were determined from least-squares fits of an exponential function to the experimental data. $l'_T$, the rate constant for CO binding to T-state hemoglobin, was determined in a manner similar to that discussed previously for dioxygen (35). In the present studies, experimental solutions contained deoxyhemoglobin (typically 25 $\mu$M) with enough CO to give <1.5% saturation. Because the fractional CO saturation was low,

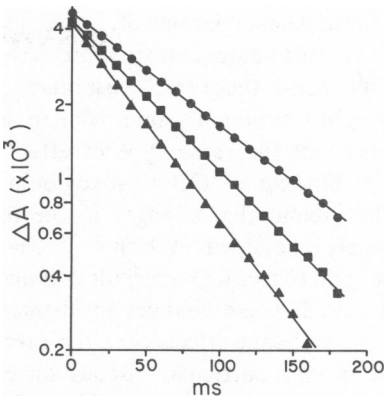


FIGURE 1 Semilogarithmic plots of the time-dependent absorbance changes observed at 437 nm with a 3-nm band pass, following photolysis of partially CO-saturated 25-$\mu$M hemoglobin samples. Solutions were 0.1 M borate buffer containing viscous solutes. The laser was fired at zero on the time scale. All experiments were performed at 20 ± 0.3°C and pH 8.3 in 2 mm cells. •, 0.422 weight fraction glycerol and 1.2% CO saturation. ■, 0.363 weight fraction sucrose and 1.2% CO saturation. ▲, 0.631 weight fraction ethylene glycol and 1.1% CO saturation. The straight lines were drawn through the points.

rebinding following full photolysis occurred at a rate determined by the properties of the T state. In contrast to the biphasic T-state binding observed for dioxygen, where chain differences are important, CO rebinding could be well represented by a single exponential. Values of $l'_T$ were obtained by least-squares fitting of a single exponential to these kinetic data. Fig. 1 presents a semilogarithmic plot of some typical kinetic data used to determine $l'_T$. The single exponential character of T-state rebinding is consistent with our use of a two-state model that neglects differences in the CO binding properties of hemoglobin subunits.

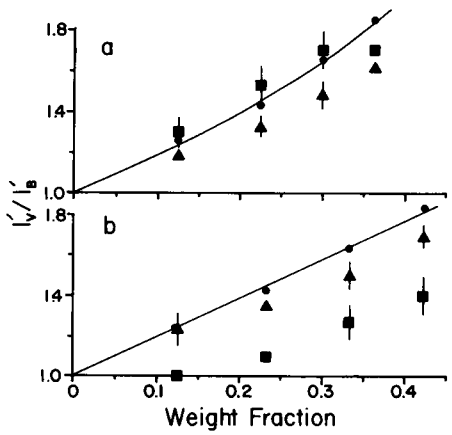


FIGURE 2 The ratio of the CO-binding rate constant in viscous solution to the rate constant in borate buffer is plotted against weight fraction solute. ▲, the ratio of R state on rate constants. ■, the ratio of T state on rate constants. *a*, results for sucrose/borate solutions. *b*, results for glycerol/borate solutions. In each part of the figure the solid circles (•) give the CO solubility in borate buffer divided by the CO solubility in viscous solvent. The solid lines (—) were drawn through these circles. See text for a discussion of the determination of rate constants and CO solubilities. The error bars were determined from the standard errors for the parameters in Table I.

At least three determinations of $l'_R$ and $l'_T$ were used to calculate the average values and standard errors presented in Table I. In general, these rate constants increased with increasing weight fraction of solute. McKinnie and Olson (29) have observed the same type of effect in detailed studies of the binding of CO to myoglobin in glycerol solutions. They found that changes in the rate constant could be largely explained in terms of changes in the chemical potential of free CO molecules related to changes in CO solubility. If these changes in chemical potential were the only significant effect, then the rate constant $l'_V$ for CO binding in a particular viscous solvent could be derived from the rate constant $l'_B$ for CO binding in borate buffer, according to Eq. 8.

$$l'_V/l'_B = S_B/S_V. \quad (8)$$

$S_B$ and $S_V$ are the CO solubilities in borate buffer and the viscous solvent, respectively. Fig. 2 compares the solute concentration dependences of the ratio of rate constants on the left side of Eq. 8 with that of the ratio of CO solubilities on the right side. At best, only fair agreement was seen between these quantities. If CO binding to hemoglobin were a diffusion-limited process, we would expect a decrease in binding rate constants as weight fraction of solute, and thus viscosity, were increased. Because no such effect was seen in the data of Fig. 2 it seems likely that, as suggested for myoglobin (29, 36), some process internal to the protein provides the rate-limiting step in the CO binding reaction. The change in CO solubility with fraction solute at least gives a qualitative explanation for the increase in binding rate constants shown in Fig. 2. Differences seen between the solute concentration dependences of the R- and T-state rate constants in this figure are outside the experimental errors in some cases and presumably reflect differences in the properties of the R and T states.

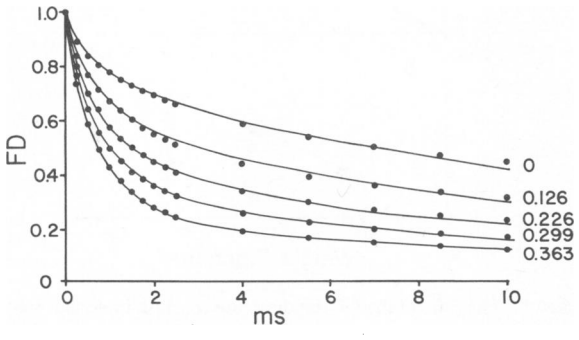


FIGURE 3 CO recombination kinetics observed following photolysis of carboxyhemoglobin in sucrose/borate solutions. The fraction of deoxyhemoglobin is given by the data points. The weight fraction of sucrose is given to the *right* of each curve. Experimental conditions were as given in Fig. 1, except that the total CO concentration was 91 $\mu$M. The solid curves (—), represent fits of a two-state model (discussed in the text) to the data points. $K_o$, the rate of the R → T conformational change, was varied to obtain these fits. Best fit values of $K_o$ are given in Table I.

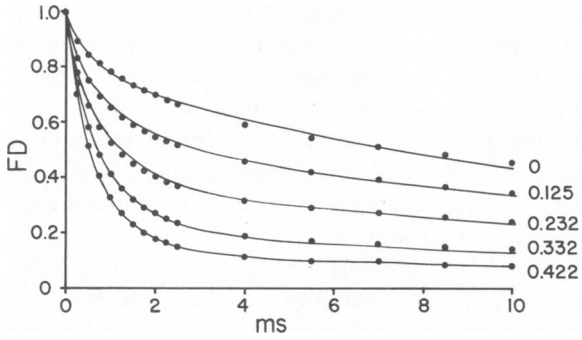


FIGURE 4 CO recombination kinetics observed following photolysis of carboxyhemoglobin in glycerol/borate solutions. The fraction of deoxyhemoglobin is given by the data points. Experimental conditions were as given in Fig. 3. The weight fraction of glycerol is given to the *right* of each curve. The solid curves represent fits of a two-state model (discussed in the text) to the data points. Best fit values of $K_o$ are given in Table I.

Figs. 3–5 show that, for fixed CO concentration, the fraction of rapidly reacting hemoglobin increased with increasing weight fraction of solute for the three solution systems studied. This increase in rapid phase did not result from an increase in hemoglobin dimerization, since the $K_{4,2}$ values in Table I show that, except in the case of the most concentrated ethylene glycol solution of Fig. 5, dimers make up <12% of the hemoglobin in solution. Reasonably good fits to the experimental data points are obtained by varying the value of $K_o$ in the previously described two-state model. The best fit values obtained for $K_o$, corresponding to the solid curves in Figs. 3–5, are listed in Table I. In all cases, $K_o$ decreased with increasing viscosity.

If the two-state model provides a reasonable representation of the behavior of hemoglobin in viscous solvents, the best fit values obtained for $K_o$ should be independent of CO concentration. However, we expected that an increase in CO concentration would result in an increase in the fraction of fast rebinding hemoglobin, since more of the R-state hemoglobin present after photolysis binds CO,

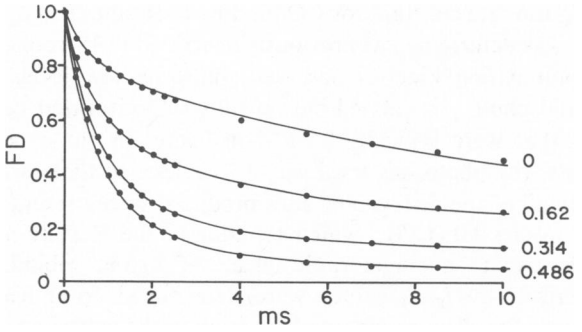


FIGURE 5 CO recombination kinetics observed following photolysis of carboxyhemoglobin in ethylene glycol/borate solutions. The fraction of deoxyhemoglobin is given by the data points. The weight fraction of ethylene glycol is given to the *right* of each curve. Other experimental conditions were as given in Fig. 3. The solid curves represent fits of a two state model discussed in the text to the data points. Best fit values of $K_o$ are given in Table I.

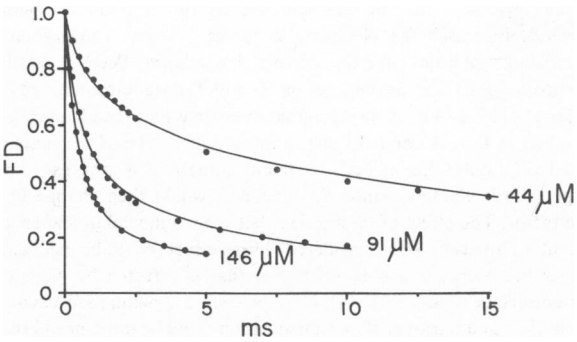


FIGURE 6 Effect of CO concentration on the recombination kinetics observed following photolysis of carboxyhemoglobin in solutions containing 0.299 weight fraction of sucrose. The fraction of deoxyhemoglobin is given by the data points. Total CO concentrations are given to the *right* of each curve. All other experimental conditions were as given in Fig. 1. The solid curves (—) represent fits of a two-state model (described in the text) to the data. The best fit values obtained for $K_o$ were $K_o$ = 2,200 $s^{-1}$ for 44 μM CO; $K_o$ = 2,100 $s^{-1}$ for 91 μM CO; and $K_o$ = 2,300 $s^{-1}$ for 146 μM CO.

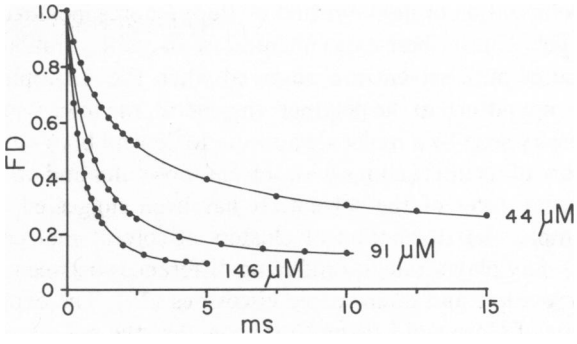


FIGURE 8 Effect of CO concentration on the recombination kinetics observed following photolysis of carboxyhemoglobin in solutions containing 0.314 weight fraction of ethylene glycol. The fraction of deoxyhemoglobin is given by the data points. Total CO concentrations are given to the right of each curve. All other experimental conditions were as given in Fig. 1. The solid curves (—) represent fits of a two-state model (described in the text) to the data. The best fit values for $K_o$ were $K_o$ = 750 $s^{-1}$ for 44 μM CO; $K_o$ = 770 $s^{-1}$ for 91 μM CO; $K_o$ = 780 $s^{-1}$ for 146 μM CO.

rather than switching to the slowly reacting T state. Figs. 6–8 present data collected to test this idea. The data in each of these figures represent the results of an experiment in which CO concentration was varied while the fraction of solute was held constant. Again, reasonably good fits to the data were obtained by varying $K_o$. As expected, increased CO concentration led to an increased fraction of fast-reacting hemoglobin. Values obtained for $K_o$ are given in the legends of Figs. 6–8. As predicted, conformational change rate was essentially independent of CO concentration.

Fig. 9 plots conformation change rates taken from Table I vs. viscosity for each of the three solution systems studied. The inverse viscosity dependence predicted by Gavish's adaptation of Kramers's theory is represented by the dashed curve. Although we found that conformational change rate decreased with increasing viscosity, agreement with Gavish's model was at best moderately satisfactory in the case of sucrose solutions. The data of Fig. 9 show that the chemical nature of the solute also had a significant effect on conformational change rate. Very different rates were observed for different solutions having the same macroscopic viscosity. One possible explanation for this effect might lie in differences between the bulk or macroscopic viscosity, which we have used, and the local or microscopic viscosity of solvent in the vicinity of a hemoglobin molecule. The microscopic viscosity is the appropriate quantity to use in Eq. 1.

The simplest instance of differences between these two types of viscosity relates to experiments in polymer gel-forming systems that have very high macroscopic viscosities. These gels contain many small cavities filled with essentially pure solvent. Measurements of the microscopic viscosities of these systems have been made using the

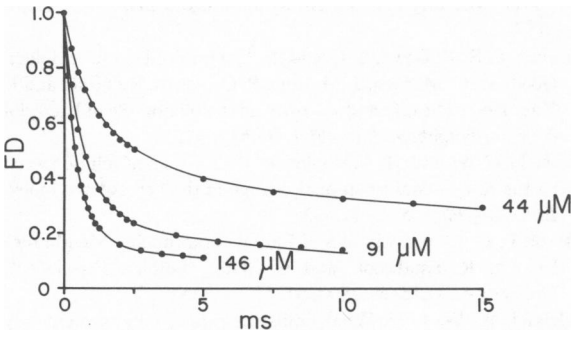


FIGURE 7 Effect of CO concentration on the recombination kinetics observed following photolysis of carboxyhemoglobin in solutions containing 0.332 weight fraction of glycerol. The fraction of deoxyhemoglobin is given by the data points. Total CO concentrations are given to the right of each curve. All other experimental conditions were as given in Fig. 1. The solid curves represent fits of a two-state model described in the text to the data. The best fit values obtained for $K_o$ were $K_o$ = 1,100 $s^{-1}$ for 44 μM CO; $K_o$ = 1,100 $s^{-1}$ for 91 μM CO; and $K_o$ = 1,200 $s^{-1}$ for 146 μM CO.

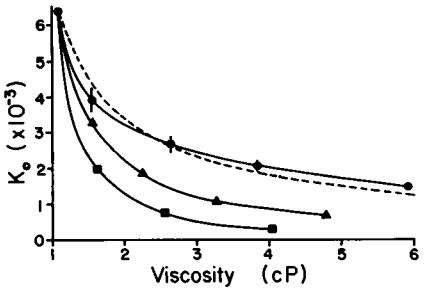


FIGURE 9 Plots of the viscosity dependence of $K_o$, the rate of the R → T conformational change of deoxyhemoglobin. Experimental conditions were as in Fig. 3. Data points represent the best fit values of $K_o$ taken from Table I. The determination of these values is discussed in the text. •, sucrose/borate. ▲, glycerol/borate. ■, ethylene glycol/borate. The solid curves (—) were drawn throught the points. The dashed curve (---) gives the inverse viscosity dependence predicted by Gavish's model, which is described in the text. This curve was scaled so that it passes through the experimental point for borate buffer. The error bars correspond to errors given in Table I.

depolarization of light emitted by fluorophores included in the gel (37). In these experiments, low viscosities similar to those of pure solvent are observed when the fluorophore does not adsorb to the polymer. In general, the microscopic viscosity seen by a molecule appears to depend both on the nature of its interactions with solvent molecules and on the internal order of the solvent. It has been suggested, for example, that formation of clusters of solvent molecules (38) may play a role in producing differences between the macroscopic and microscopic viscosities (39). The experiments of Hare and Lussan (39) show that the microscopic viscosity of aliphatic oils determined using fluorescence depolarization varies by as much as a factor of 30 for oils with the same macroscopic viscosity.

In conclusion, our experiments indicated that increased solution viscosity decreases the rate of the R → T conformational change of deoxyhemoglobin. This result offers further support for the idea that structural fluctuations play an important part in the function of proteins. In sucrose/borate solutions the observed viscosity dependence of this conformational change rate agreed moderately well with the $1/\eta$ dependence predicted by Gavish's adaptation of Kramers's theory. In glycerol and ethylene glycol solutions, conformational change rate fell off more quickly with increasing viscosity than predicted by this model. Changes in the R- and T-state binding rate constants in sucrose solutions, as the fraction of sucrose is varied, seem to be fairly well described by changes in the chemical potential of free CO molecules, due to solubility changes. Data collected for glycerol and ethylene glycol solutions cannot be as well described using the same principle. The increases we saw in tetramer-dimer dissociation that occur when the fraction of solute was increased are qualitatively consistent with Blank's model, which takes into account repulsion between surface charges on the hemoglobin molecule.

## APPENDIX

Our assumption of a constant value of $L$ was based upon consideration of Eq. A1, which was first derived by Thomas and Edelstein (40):

$$\frac{K^{R}_{4,2}}{K^{T_o}_{4,2}} = L. \tag{A1}$$

Discussion of the effect of our viscous solutions on $L$ reduces to a discussion of their effect on $K^{R}_{4,2}$ and $K^{T_o}_{4,2}$, the tetramer-dimer dissociation constants for the R and deoxy T states of hemoglobin. We argue that changes in solution composition should change each of these dissociation constants by the same factor, leaving $L$ unchanged. The free-energy differences between tetramers and dimers determine the values of $K^{R}_{4,2}$ and $K^{T_o}_{4,2}$. Eqs. A2 and A3 taken from Shulman et al. (41) give these differences for conditions of unit activity:

$$\Delta G^{R_4 \to 2D_2} = -RT \ln K^{R}_{4,2} \tag{A2}$$

$$\Delta G^{T_o \to 2D_o} = -RT \ln K^{T_o}_{4,2}. \tag{A3}$$

Next, we need to consider the possible physical origins of changes in these $\Delta G$ values as solution composition is changed. The decrease in dielectric constant, which occurs in our solutions as the fraction of solute is increased, increases the electrostatic forces between charges on the hemoglobin molecules and thus favors dissociation. Because available evidence suggests that the charges on R- and T-state tetramers are quite similar at pH 8.3 (42), it is reasonable to assume that the changes in the $\Delta G$ values in Eqs. A2 and A3 due to this effect will be of the same size. Considering only this effect, $L$ should remain constant as solution composition is changed, since $K^{R}_{4,2}$ and $K^{T_o}_{4,2}$ would then change by the same factor. The effect of interactions between hemoglobin and solution molecules also needs to be considered. These effects could be mechanical, such as the energy of cavity formation that is affected by changes in solution surface tension (43, 44). Here proteins are pictured as cavities in the solution, and tetramer-dimer dissociation could be influenced because the surface area of two dimers is larger by ~26% than one tetramer (34). Here an increase in surface tension would decrease tetramer-dimer dissociation. The details of preferential interactions of dimeric and tetrameric hemoglobin with different solution components should also be considered (44). We make the seemingly reasonable assumption that the differences between R- and T-state tetramers in both surface area and chemical groups exposed on the surface are small on the scale of the differences between tetramers and dimers. In this case, the free energy differences of Eqs. A2 and A3 will change by similar amounts as solution composition is changed, and $L$ will again be left unchanged as we have assumed.

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