KINETIC DIFFERENCES AT LOW TEMPERATURES BETWEEN R AND T STATE CARBON MONOXIDE-CARP HEMOGLOBIN

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ABSTRACT We use the low-temperature recombination kinetics of carbon monoxide with carp hemoglobin to determine that the R and T states of hemoglobin exhibit different low-temperature geminate recombination kinetics. The peak of the fitted Gaussian activation energy spectrum is at 1.5 kcal/mol for R state and 1.8 kcal/mol for T state. The distribution in activation energies is fit well by the Agmon-Hopfield linear strain model. The T state is fit with a stronger elastic constant than R, and has a larger displacement of the protein conformation coordinate than does the R state, indicating that the T state does have a significantly greater rigidity and also stores more strain energy in its conformational states than does R hemoglobin. The pre-exponential in the activation energy spectrum is only a factor of two greater in the R than the T state and the low-temperature activation energy spectrum does not correctly predict the difference in the on rates for R and T states at 300°K, indicating that processes removed from the binding site are important in cooperativity.

INTRODUCTION

Here we examine the low-temperature recombination kinetics of carbon monoxide-carp hemoglobin (CO-carp Hb) following laser photolysis to determine if the low-temperature kinetics are of importance in understanding cooperativity in Hb. According to the Monod-Wyman-Changeux (MWC) theory of cooperativity, allosteric proteins can exist in two distinct states (Monod et al., 1965), the R (for relaxed, or tight binding) state and the T (for tense, or weak binding) state. If one hopes to study the two discrete states of the protein, then it is necessary to have a protein that may be chemically locked into both the R and T states.

Perutz (1977) have shown via x-ray and optical studies that Hb exists in two discrete structural states whose equilibrium ratio is affected by the concentration of organic phosphates such as inositol hexaphosphate (IHP). Noble and co-workers (1970) have shown that for CO-carp Hb at pH 6.0 and below, in the presence of 1.0 mM IHP, the protein is locked in the T state, while for pH >6 and in the absence of organic phosphates the CO-carp Hb is in the R state. The shift between the R and T states is indicated by a characteristic difference spectra in the region around the Soret band. Carp Hb was chosen for these experiments because the carboxy form is easily put into both the R and T states (Noble et al., 1970). This protein seems to give strong support to the ideas of MWC since in either the R or the T state cooperativity is abolished.

To further understand the mechanism of cooperativity in Hb we need to know where the energy of cooperativity is stored. It is known from the x-ray crystallography that several salt bridges break upon going from the deoxy (T) state to the carboxy (R) state, but it is not clear how this energy, if it is the energy associated with the cooperative transition, is transmitted to the binding site. Here we use the simple model of Agmon and Hopfield (1983) to interpret the low-temperature kinetics from the viewpoint of the energetics of conformation distributions in an attempt to localize the origin of cooperativity in carp Hb.

METHODS

The carp Hb was dissolved in 75:25% glycerol water (vol/vol) solutions to insure a clear sample at low temperatures. The Hb was buffered in sodium acetate (10 mM) titrated with NaOH. The pH of the buffers was measured at room temperature. Acetate buffer was chosen because of the relative pH stability under temperature change (Douzou, 1977) and its buffering range. The R state buffer was free of phosphates and was titrated to pH 6.8, while the the T state buffer was titrated to pH 5.5 in the presence of 6 mM IHP. The IHP was acquired from Sigma Chemical Co. (St. Louis, MO) and used without further preparation.

Purified carp Hb was a generous gift of Dr. Robert Noble (Department of Biochemistry and Medicine, the State University of New York at Buffalo, Buffalo, NY) and used without further treatment, except for the dilution into the proper buffer. Myoglobin solutions where prepared from freshly dissolved sperm whale myoglobin (Sigma Chemical Co.), centrifuged to remove nondissolved protein. Samples were kept under a carbon monoxide atmosphere and kept reduced by a 100 μM solution of sodium dithionite.

Final protein concentrations were ~90 μM in heme content. Samples were mounted between glass slides with 1.5 mm Teflon spacer. The holder was mounted in an atmospheric access variable temperature cryostat (Janis Cryogenics), the temperature was measured by both a silicon diode

and a copper-constantin thermocouple, and controlled by a analogue temperature controller to ±0.5°K during the run. The excitation pulse was a frequency doubled Nd:YAG laser (Molectron MY-32; Molectron Corp., Sunnyvale, CA) run at 2 Hz for most runs. The laser pulse width (full width at half-maximum) is 20 ns. The laser power was adjusted so that the transmission changes were <10% at the monitoring wavelength of 436.8 nm. Since we remained in the small signal regime it was not necessary to take the log I/I_0 of the signal to convert to absorbance. Unity in terms of the fraction of photolyzed molecules was defined as the signal size 20 ns after the flash at 80°K, a temperature where the fractional recombination is <1% for the first 100 ns. The monitoring light source was a Hg:Xe arc lamp (Photochemical Research Associates Inc., Ontario, Canada). The monitoring wavelength of 436.8 nm (a mercury line) was selected by 5-nm bandwidth interference filters and attenuated so that no appreciable steady state photolysis of the sample occurred.

Transmission changes were detected by a photomultiplier (Hamamatsu 928; Hamamatsu Corp., Middlesex, NJ) with a 100 MHz bandwidth active base and stored by a Biomation 6500 transient recorder. A computer provided the averaging and transferred the files to a Digital Equipment VAX computer for further analysis (Digital Equipment Corp., Marlboro, MA). At low temperatures the kinetics are highly nonexponential and cover at least 5 decades in time, thus it was necessary to take three sets of data at each temperature at acquisition rates of 2 ns/point, 200 ns/point, and 20 μs/point and concatenate the files on the computer. The total time range covered was 8 decades.

Least-square fits of theoretical fits to the data were done by a Manquadt's compromise, an algorithm that uses a gradient search and analytical expansion which allows for arbitrary functions (Groth, E., personal communication). Standard deviations for the data used to evaluate the goodness of fit were derived from the pretrigger noise. Adequate fits had a reduced chi-squared of <2. Because of the fact that five variables are present in the fit function, there are several valleys where the fitting routine claims convergence. These fits then must be viewed as suggestive guides as interpreted by the model of Agmon and Hopfield and not in any manner proof of the correctness of their model.

Fig. 1 shows the difference spectra for CO-carp Hb, R (pH 6.8) vs. T (pH 5.5 and 6.0 mM IHP), 10 mM acetate buffer in 75% (vol/vol) glycerol water agree well with literature values (Tan et al., 1973) for R and T state CO-carp Hb. This indicates that the glycerol does not affect the points of the R and T equilibration. In Fig. 2. we have plotted representative recombination curves for R and T state Hb at 100°K, 140°K, and 180°K. To see more clearly the differences between the two states in Fig. 3 we have coplotted R and T state kinetics at 100°K and 140°K. It can be easily seen that the R state is consistently faster than the T state. As expected, the data show highly nonexponential recombination, with the T state showing a greater curvature vs. time on a log-log plot than the R state.

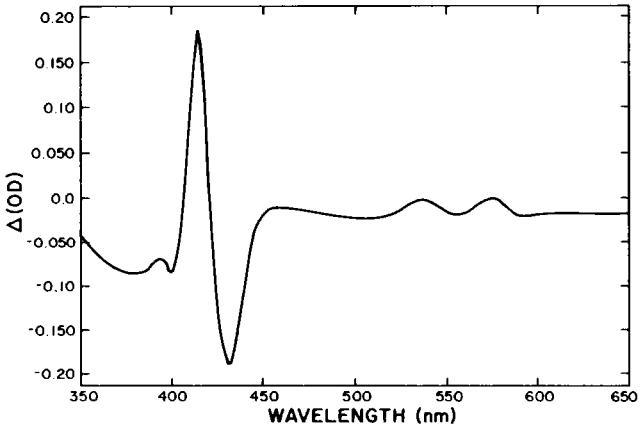

FIGURE 1 Difference spectrum between R and T state carboxy-carp Hb. Buffers used were 10 mM pH 6.8 acetate (R) and 10 mM pH 5.5 acetate + 6 mM IHP (T). The solvent used was 75% glycerol/25% water (vol/vol).

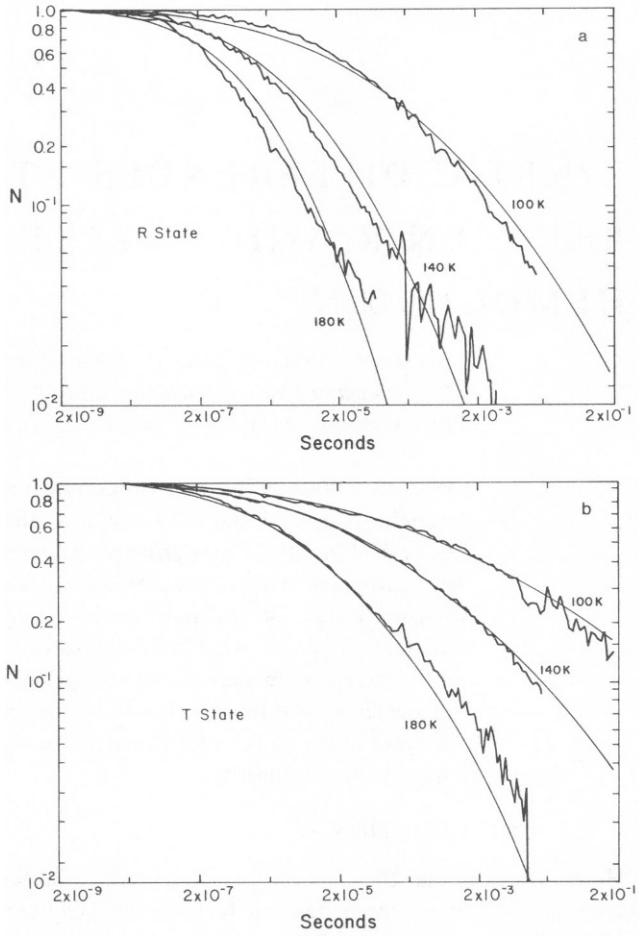

FIGURE 2 The recombination of CO-carp Hb after laser photolysis. In these log time vs. log intensity change plots, we have normalized the data so that 1.0 is equivalent to the signal at 80°K, 20 ns after the laser flash. (*a*) Low-temperature recombination kinetics for R state Hb. The solid lines are fits to the Gaussian approximation of Agmon and Hopfield (1983). (*b*) The low-temperature recombination kinetics for T state Hb. Again, solid lines are Gaussian fits. Values for the simulation are taken from the average values in Table I.

We also measured the low temperature kinetics of carboxy sperm whale myoglobin in our two buffers (data not shown). No difference outside of error limits could be detected, indicating that the kinetic differences seen are due to the two conformational states of the Hb and not some artifact of the buffers, the pH differences or the IHP. The myoglobin data agreed well with previous data (Austin et al., 1975) taken at pH 7.5, but does not show the pH effect seen in (Dorster et al., 1982). We do not understand the difference between these two experiments. It is possible that the different buffers used in the two experiments plays a role.

DISCUSSION

The nonexponential kinetics observed here are typical of heme protein recombination kinetics and are naturally fit by assuming that the recombination is due to a distribution

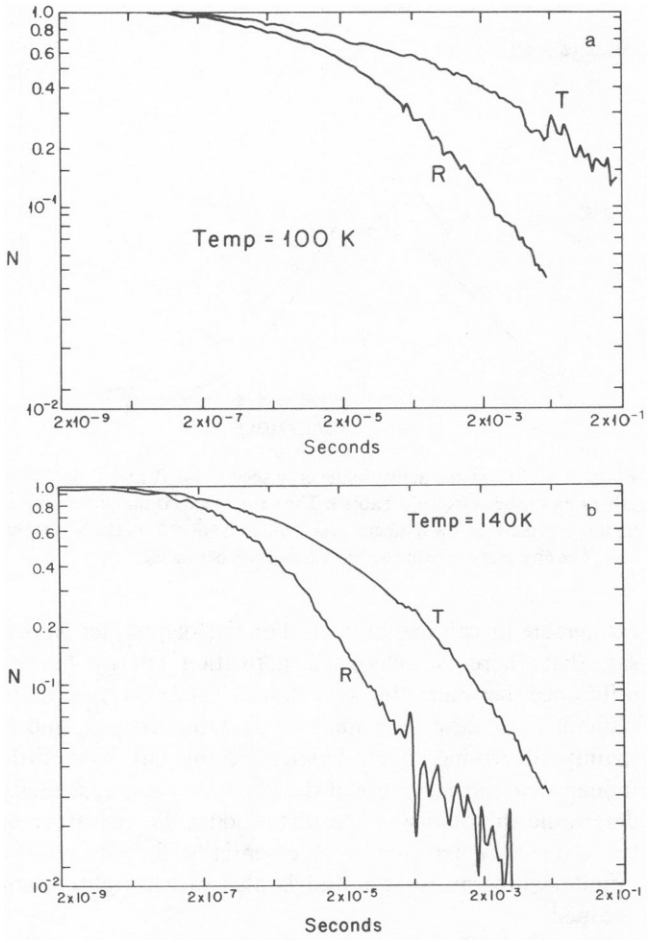

FIGURE 3 Comparison of R and T state Hb kinetics at 100°K (*a*) and 140°K (*b*).

of activation energies. That is, we assume that the Arrenhius relation holds

$$k = A \exp [-E/k_b T], \tag{1}$$

where k is the exponential recombination rate, E is the activation energy barrier for recombination, T is the absolute temperature, k_b is the Boltzmann constant, and A is the preexponential rate. Eq. 1 gives only a single rate and an exponential recombination. To handle nonexponential processes, we can assume

$$N(t) = N(0)A \int_0^\infty g(E) \exp [-kt] \, dE, \tag{2}$$

where k is the Arrenhius rate in Eq. 1, $g(E)$ is the probability that one can find a molecule with internal activation energy E, and $N(0)$ is the number of molecules photolyzed by the laser pulse at $t = 0^+$. It is assumed that at low temperatures $g(E)$ is static and exchange does not occur among different activation energies within the molecule. Further, we assume that the pre-exponential A is a single value and not distributed.

There exist several very strong attempts to understand these kinetics. The basic concept of a distribution of activation energies was provided by Frauenfelder (Austin et al., 1974). This idea has proven very fertile in understanding the low-temperature kinetics and in unifying the data from x-ray crystallography, electron paramagnetic resonance and Mossbauer data on protein conformation (Hartmann et al., 1982).

A recent addition to these ideas has been the analysis of Agmon and Hopfield (Agmon and Hopfield, 1983) who retain the idea of a distribution of activation energies and attempt to derive a functional form from an analysis of the barriers associated with the two states of iron, low spin ($S = 0$) carboxy and high spin ($S = 2$) decarboxy. There are two main assumptions to the theory of Agmon and Hopfield. One assumption is that the reaction of the CO and the iron is nonadiabatic. This assumption may be true for the case of CO recombination, although this assumption surely needs more experimental verification (Gerstman et al., 1981; Frauenfelder, H., and P.G. Wolynes, manuscript submitted for publication). A further assumption in the Agmon-Hopfield theory is that the conformational rigidity f (defined below) is the same in the oxy and deoxy state, as long as the protein remains R or T. This also may not be true, although it seems reasonable to us that a local property like the iron distance with respect to the heme plane is more likely to change then is a global property like the bulk modulus. Finally, Agmon and Hopfield assume a linear restoring force, at variance with the x-ray data. Young and Bowne have developed an alternate approach (Young, R.D., and S.F. Bowne, manuscript submitted for publication) that assumes an adiabatic reaction and changes in the conformation rigidity between the photolyzed species and the activated complex, and they further assume that the conformation rigidity is not quadratically related to the conformation energy. A careful comparison of the two models should prove fruitful in interpretation of our data.

Agmon and Hopfield derive a Gaussian activation energy that has the advantage of naturally accounting for the deviations from a power law kinetics, which a strict exponential activation energy spectrum is unable to do. Further, the stiffness of the nuclear coordinates that is built into the theory can be used to calculate the energies of conformational states and should be subject to experimental tests.

In the model of Agmon and Hopfield model the distribution in activation energies arises from the transition between the $S = 2$ surface of the deoxy state and the $S = 0$ surface of the carboxy (or oxy) state, and response of the protein strain energy to the presumed change in protein conformation in the two states. The carboxy ($S = 0$) electronic potential surface is modeled by a Morse potential, and lies a distance D_e kcal/M below the zero potential value, and the deoxy state is modeled by a simple exponential repulsive state that lies a distance Δ below the zero

potential (vacuum) surface. The net binding potential for the ligand, if there is no protein strain, is then $D_e - \Delta$.

Agmon and Hopfield then assume that there is extra energy stored in the protein structure, removed from the iron-CO or O_2 bond. Perhaps the best evidence for this being true is the startling fact that in carp Hb there is no change in the iron-ligand stretch frequencies between the R and the T states, despite the difference in binding free energy (Nagai et al., 1980). The difference associated with strain energy is

$$V(x) = \tfrac{1}{2} f x^2 - \tfrac{1}{2} f (x - x_o)^2, \qquad (3)$$

where f is the elastic constant of the protein and x_0 is the shift in mean coordinate position of the potential surface that occurs upon binding CO, in our case. Agmon and Hopfield assume that the probability for the protein to have been in the conformational state x is frozen in at a glass transition temperature T_g

$$P(x) = (4\pi k_b T_g f)^{1/2} \exp(-f x^2 / 2 k_b T_g). \qquad (4)$$

The barrier height E for ligand recombination is derived from the relation

$$E(3 - E/D_e) = \tfrac{1}{2} f x_o (x_o - 2x) + \Delta. \qquad (5)$$

The mapping from x to E implied by Eqs. 4 and 5 is the source of Agmon and Hopfield's distribution in activation energies. Since the quartic terms cancel in Eq. 3, the distribution of activation energies predicted by this model is essentially Gaussian in energy. This model remains speculative and must be viewed as an approximation in a complex system, yet it does afford useful insights.

We have done least-squares fits to our data using this model and the results of these fits are shown as the solid lines in Fig. 2. In the fits, we let D_e, Δ, f, x_o, and A be variables, and we constrained the fit so that D_e would be greater than Δ, on physical grounds. We assumed T_g for the protein of 200°K. Table I shows the parameters that best fit our data as determined by our computer and Fig. 4 shows the activation energy spectrum for both the R and the T states derived from these values.

The activation spectrum is significantly different for the R and T states, implying a significant change in the nuclear coordinate equilibrium energy for the two states. Since there are five variables it is not too surprising that a reasonable fit can be obtained. For the skeptic, let us just say that there is indeed an activation energy barrier difference between the two states. The fits are more difficult to achieve than may be thought, since D_e and Δ mainly determine mean barrier height but have little influence on the curvature of the fit, while f and x_o strongly determine the ability of the fit to model the curvature of the data. The parameter A essentially determines the infinite temperature rate and is also rather tightly constrained.

TABLE I
SUMMARY OF FITTING PARAMETERS

R state	T state
$D_e = 16.1.5$	18.0 ± 0.3
$\Delta = 9.7 \pm 0.4$	16.1 ± 0.6
$f = 13.0 \pm 0.6$ kcal/mol · au^2	16.1 ± 0.3 kcal/mol · au^2
$x_o = 0.88 \pm 0.1$ au	1.05 ± 0.3 au
$1/2 f x_o^2 = 5.0$	8.9

Energies are expressed in kilocalories per mole, distances in atomic units (au = 0.053 nm).

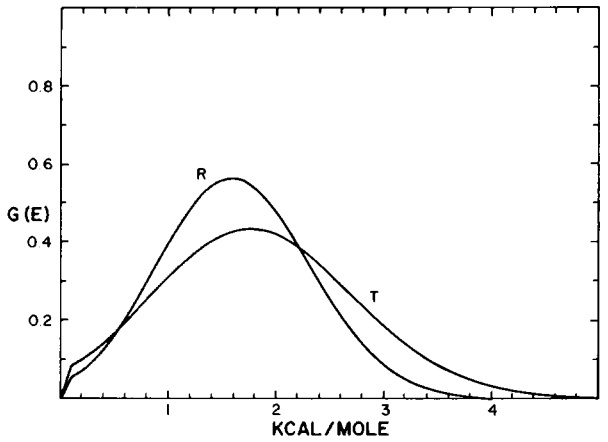

FIGURE 4 Plot of the activation energy spectra for R and T state carp Hb, using the best fits from Table I. The vertical axis is the probability of finding a protein at low temperatures with activation E on the horizontal axis. The energies are expressed in kilocalories per mole.

The physically relevant parameters in terms of conformational states are the derived values for f, the force constant, and x_o, the nuclear rearrangement displacement. As can be seen from Table I the two terms are quite different in the two states. The R state has a softer force constant and a smaller x_o, and a more peaked $g(E)$ compared with the T state.

As a rough indication of the energy stored in the conformational distribution, we show in Table I the energy difference $\tfrac{1}{2} f x_o^2$ between the oxy and deoxy states. The T state then appears to have 3.97 kcal/mol more stored energy than the R state. This energy is presumably put into protein conformation and not into the iron-ligand bond. Thus, it represents modification of binding strength along the lines predicted by the MWC theory of cooperativity and indicates clearly how the conformation can influence important biological functions. Identification of where this energy is stored is not provided by the kinetics. Raman seems to indicate that the energy is not stored in the iron-ligand bond since the stretch frequency is the same in the R and T states (Spiro, T., manuscript submitted for publication; Nagai and Kiagawa, 1980). Recent x-ray crystallography (Brzozowski et al., 1984) on T state hemoglobin where the alpha subunits are oxygenated seems to also indicate that in the T state the protein puts more restraints on the movement of the iron from the oxygenated to the deoxy state.

Finally, it is interesting to ask if these results explain the kinetics at room temperature. The energies shown in Table I seem to indicate that the $g(E)$ stores enough energy to drive cooperativity, but a further check would be to show that the activation energy spectrum also controls the kinetics at physiological temperatures, where the ligand undergoes diffusion into the surrounding medium. Here we will use Frauenfelder's relaxation model (Austin et al., 1975) and assume that if the distribution is the sole barrier to rebinding, then the recombination rate observed at temperatures where the relaxation rate k_r is much faster than the fastest barrier rate is

$$N(t) = N(0) \exp(-k't), \tag{6}$$

where the rate k' in the exponential is an average rate. This can be calculated by assuming that the protein rapidly, on a time scale less than the shortest recombination time, samples all possible conformational states. In this model the mean rate k' is

$$k' = A' \int_0^\infty g(E) \exp(-E/k_bT)\, dE, \tag{7}$$

where A' includes the possibility that the pre-exponential can change once relaxation occurs.

We thus expect that the recombination rates of R (k_R) and T (k_T) Hb at high temperatures should scale as the ratio of these mean rates, all other processes being equal. Numerical integration of the $g(E)$s for the two states leads to the prediction that k_R/k_T at 280°K should be 2.5, while the measured rates, as shown in Fig. 5, are $k_R/k_T = 30$. The experimental recombination rates of the two states is in fair agreement with the data of Noble and his coworkers (Pennelly et al., 1975), who used an aqueous buffer. This failure of the inner barrier to predict the room temperature on rates seems to indicate that the outer barriers, as discussed in Alberding et al., 1978, must also be of importance in determining the rates, and possibly the energetics, of cooperative behavior.

In conclusion, although there is a difference in the kinetics at low temperatures between the R and T states, and one particular model says that the differences can contain the energies of cooperativity, the failure to explain the measured room temperature kinetics indicates that outer processes remote from the iron group may be controlling cooperativity.

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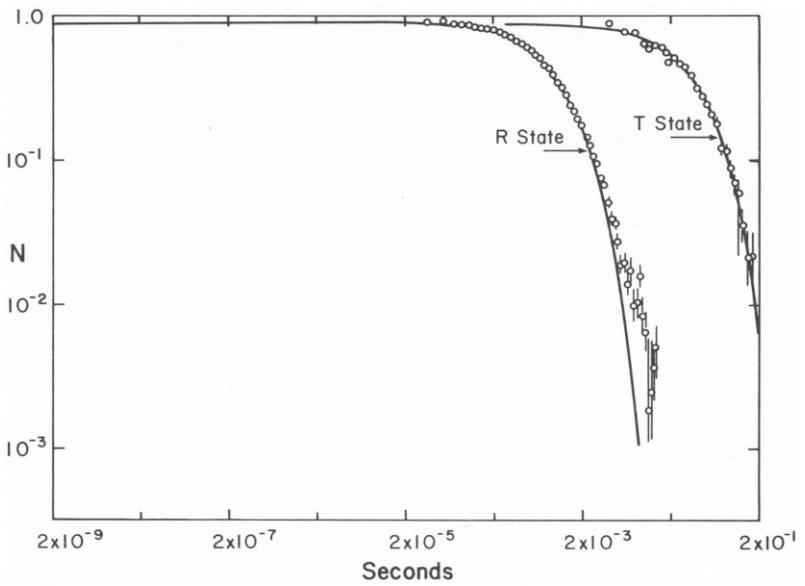

FIGURE 5 Recombination data for R and T state carp Hb at 280°K, where the recombination is now second order in CO concentration and pseudo–first order in time. The solid lines are single exponential fits to the data. The time constant of the T state is 40.6×10^{-3} s, while that of the R state is 1.3×10^{-3} s.

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