Geminate Recombination of CO in Rabbit, Opossum, and Adult Hemoglobins*

(Received for publication, September 13, 1984)

Blair F. Campbell‡, Douglas Magde§, and Vijay S. Sharmas

From the ‡Department of Chemistry and the §Department of Medicine, University of California, San Diego, La Jolla, California 92093

The geminate recombination of CO with Hb following dissociation by a 10-ns laser pulse has been studied as a function of pH (9.2 and 7.0 without inositol hexaphosphate and 6.0 with inositol hexaphosphate) and temperature (5–35 °C). The hemoglobins studied included adult, Rothschild, rabbit, opossum, and carp. Despite significant differences in their structural and functional properties, the first four of these hemoglobins show similar trends in the yields, rates, and activation energies of the geminate recombination. The nature of the "cage recombination" in hemoglobin is discussed in the light of such findings. Neither a slow diffusion model nor a model based upon a specific non-heme binding site accounts for the observations.

When light is absorbed by hemoglobin that is bonded to carbon monoxide, the iron-carbonmonoxide bond frequently breaks resulting in a large change in the visible absorption spectrum of the hemoglobin. Studies in which absorption changes were measured some milliseconds after irradiation by light have shown that about 50% of the photons absorbed by HbA at ambient conditions result in photolysis. Recent nanosecond measurements, however, show that the yield of bond cleavage at nanosecond times is higher, perhaps 80%, and that fast "geminate recombination" produces the lower apparent yields observed in milliseconds experiments (1–6).

The recombination observed in milliseconds experiments is always analyzed in terms of a second-order, bimolecular kinetic process in which CO diffuses through solution and then combines with hemoglobin. The recent nanosecond experiments have discovered a kinetic process, independent of CO concentration, in which some of the CO recombines with the heme group before it leaves the protein. This is termed geminate recombination. Although it is now generally agreed that geminate recombination is the major factor in determining absorption changes in the nanosecond range, a number of puzzles remain regarding both the mechanism of geminate recombination itself and the significance of geminate recombination in relation to overall equilibrium binding affinities.

The geminate recombination of CO continues for a few hundred nanoseconds after photolysis even though any freely diffusing CO should escape from a volume the size of the protein in less than 10 ns, as has been pointed out (6). Such relatively prolonged "cage recombination" requires some mechanism for trapping the CO molecules in the protein.

Several groups have studied the details of the recombination kinetics for a specific hemoglobin (generally HbA1) under particular conditions in order to determine the trapping mechanism. Hasinoff (7) and Lindqvist (8) have argued that small ligands diffuse through the entire protein volume returning perhaps several times to the heme site where they may recombine on any encounter. The long duration of the random walk prior to recombination or escape into solution was attributed to an unexpectedly high viscosity within the protein matrix. In support of their model they made two arguments: 1) certain previous experiments implied very slow diffusion of oxygen through (quite different) proteins, and 2) their diffusion models predicted $\exp(-kt^{1/2})$ kinetics, which seemed to agree with the oscilloscope traces they measured in recombination studies. Implicit in their diffusion model is the prediction that all diatomic ligands will behave similarly and all varieties of hemoglobin be generally similar.

Catterall et al. (3) have examined the recombination kinetics using as trial fitting functions both $\exp(-kt^{1/2})$ and double exponentials and found two exponentials necessary and an $\exp(-kt^{1/2})$ fit unsatisfactory. Frauenfelder et al. (9) have found that the kinetics of single chain heme proteins in glassy solution at low temperatures require a distribution of exponentials to yield a satisfactory fit. Hofrichter (6) has examined the whole Soret band as a function of time and has fit the observed kinetics to exponentials, only one of which lies in the nanosecond time range. He and others (10) too have suggested that the CO binds to non-heme binding sites within the protein, and this accounts for the long duration of geminate recombination.

In order to explore the reasons for such diverse interpretations of experiments which address the same issue, we have undertaken a comparison study of the nanosecond kinetics of different hemoglobins under a range of conditions. We have also used signal averaging to improve the precision of our measurements over that of some earlier studies. Given the potential heterogeneity of complex protein systems, we are doubtful that any study, however precise, on a single system will determine a kinetic model unambiguously. We believe that at the present time comparison studies offer more insight into the nature of the cage recombination than further parameterization of a single decay. Specific binding sites on the protein should result in significant differences in fast recombination kinetics of different proteins, while diffusion kinetics would presumably be independent of the detailed protein sequence.

*This work was supported in part by Grants HL31159, AM18781, AM17548, and RR7510 from the National Institutes of Health and the National Science Foundation. Computer support was provided by a National Institutes of Health Research Resource. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: HbA, normal adult hemoglobin; bis, 2-[(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)-propane-1,3-diol; IHP, inositol hexaphosphate.

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METHODS AND MATERIALS

Hemoglobins of carp, opossum, rabbit, human adult, and Rothschild were studied. Freshly drawn blood was lysed by the water-toluene method and the hemoglobin stripped on a Dintzis' column. Hemoglobin solutions thus obtained were deoxygenated in gas tight cells (Precision T6-61 ES 2 mm path length), then diluted with buffer equilibrated with 1 atm of CO. A small amount of dithionite added to the buffer removed the last traces of oxygen. Absorbance measurements at 540 nm were used to calculate the concentration of HbCO. During the runs the cell holder was maintained at constant temperature by a water circulator. Runs were made at temperatures of 5, 12, 20, 27, and 35 °C. The buffers used were: for pH 9.2, 0.01 M boric; pH 7.0, 0.1 M bis-tris; pH 6.0, 0.1 M bis-tris with 0.5 mM IHP.

An XeCl excimer laser (Lumonics 861T) provided photolysis pulses of a few millijoules at 308 nm. Extent of the prompt photolysis was less than 10% in all cases. Sample absorbance was probed with a pulsed Xe lamp. The probe light was passed through a copper sulfate liquid filter and a Corning 5-57 glass filter. The lamp was imaged on a 3 x 10-mm portion of the sample irradiated by the laser. The probe light then focused onto the entrance slit of a small monochromator (J-Y H20) and detected by a photomultiplier (RCA 931A). A commercial 100 x amplifier (Pacific Precision AD-6) shielded by a copper box amplified the photocurrent. A Biomation 6500, interfaced to a CAMAC-based Z-80 microprocessor, digitized the resulting signal (2 ns/point, 1024 points), and summed 100 photolysis events/experimental run. The pretrigger mode of the Biomation measures photocurrent prior to photolysis, allowing photolysis levels to be calculated from absorbance changes at 438 nm using the reported extinction coefficients. Data were transferred from the Z-80 to a DEC VAX 11/750 computer for subsequent analysis.

RESULTS

Because of the small fraction of CO dissociated, all of the kinetic results reported below refer to recombination of the fourth CO with a triliganded, tetrameric hemoglobin.

The observed time course of CO rebinding between 40 and 500 ns after photolysis was fit, using a least-squares algorithm (11) to the equation:

\[ A_t = A_\infty + C_1 e^{-\lambda t} \]

where \( A_t \) and \( A_\infty \) are absorbances at time \( t \) and at the end of the geminate reaction and \( \lambda \) is the first order geminate rate constant. \( C_1 \) is the extrapolated zero time absorbance change associated with the geminate recombination. In addition to the recombination rate \( \lambda \) itself, one is also interested in the fractional recombination yield \( \phi \). This may be calculated from the parameters in Equation 1.

\[ \phi = \frac{C_1}{A_\infty + C_1} \]

The reactions were monitored at 438 nm, the isosbestic point for slower tertiary structure changes (2, 6). The use of Equation 1 yields randomly distributed residuals and is consistent with the studies of Hofrichter et al (6) whereas fits to either a power law or an exponential in the square root of time were less successful. Catterall's (3) double exponential we attribute to his use of phosphate buffer, which enhances differences between \( \alpha \) - and \( \beta \)-chains. We cannot rule out completely the possibility that two exponentials having rather similar decay times might remain hidden in our data, but that refinement is not pertinent to the central issues discussed below. The rate constants we measure for adult hemoglobin are in good agreement with those measured by Hofrichter (6).

Typical recombination curves are shown in Figs. 1 and 2. Fig. 1 shows the results of an experiment on opossum hemoglobin for different values of the pH. The portion of photodissociated carbon monoxide that quickly rebinds to the iron is less at pH 6 than at pH 9.2. There is an evident competition between the process of rebinding and the process of escape from the protein. Prompt rebinding by geminate processes is reduced at pH 6 and in the presence of IHP and the escape of CO is favored.

Fig. 2 shows the effect of temperature on rabbit hemoglobin at pH 7. Low temperature favors the rebinding process and again this can be explained by postulating competition between rebinding and escape. In fact, Duddell (1) based his initial identification of geminate recombination primarily upon the argument that the escape process would be more efficient at higher temperatures and the extent of recombination \( \phi \) lower, in accord with our and his observations.

At least five curves like each one of those in Figs. 1 and 2 (each itself the average of 100 photolyses) were collected for each of the 45 different conditions of species, pH, and temperature. The rate constants \( \lambda \) obtained as the average over all runs for each condition are plotted against 1/T as a semilog plot in Fig. 3 for the three hemoglobins studied in most detail.

Fig. 4 shows the corresponding summary of results for \( \phi \), expressed as the percent CO undergoing prompt recombination. The geminate reaction rates were found to be independent of CO concentration (950-300 μM).

Parameters at 20 °C for four of the hemoglobins are collected in Table I. The recombination fraction in carp was too small to permit analyzing the decay to obtain accurate rate constants. This was true at all temperatures (5-35 °C) and all values of pH. In the case of Rothschild hemoglobin, it proved difficult to obtain reproducible results, presumably because Rothschild is an unstable hemoglobin. Nevertheless, we established that it showed similar rate constants and yields and exhibited the same general trends with variation in pH and temperature as the other hemoglobins. Limited availability of this mutant made it impossible to refine the measurements at this time. The parameter values for Hb Rothschild in Table...
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FIG. 2. Effect of temperature on the kinetics of rabbit hemoglobin at pH 7.0: (a) 308 K, (b) 293 K, (c) 278 K.

FIG. 3. Logarithmic plots of rate constants for geminate recombination versus 1/T for opossum, normal adult, and rabbit at pH 9.2 (O), pH 7.0 (Δ), and pH 6.0 with IHP (□).

I have relatively large associated uncertainties and should be taken as tentative.

DISCUSSION

The hemoglobins used in the present study differ widely in their primary structure and functional behavior. In rabbit hemoglobin one finds larger residues in the heme pocket of the α-chains as a result of the substitutions (expressed as HbA → rabbit): Leu → Val-29 (B10), Leu → Phe-48 (CD6), and Ser → Thr-49 (CD7) (12). This hemoglobin shows an unusually large α,β-chain difference in its ordinary, millisecond CO combination reaction (13). In the α-chains of opossum hemoglobin, the usual His at position E7 and the Val at E11 are replaced by Gln and Ile, respectively (14). At low pH in the presence of IHP, carboxy Hb opossum undergoes a structural change to the T state as shown by its combination reaction with the last CO (15). This implies either that the fully liganded form is already in the T state or that the triliganded species undergoes facile conversion to T following photolysis. A similar structural transition is shown by carp Hb at low pH (16). Hb Rothschild involves substitution of Arg for Trp at position B37 (C3) and it exists as dimers in the fully liganded state (17). These varied hemoglobins allow one to test whether geminate recombination is influenced by such variations in protein structure.

As described in the Introduction, the confinement of CO to the protein for hundreds of nanoseconds calls for an explanation and has been attributed either to binding somewhere on the interior of the protein or to a slow diffusion in a viscous medium. Our results are inconsistent with both those interpretations, at least in their simplest forms.

From infrared data, Maxwell and Caughey (10) have inferred a specific donor-acceptor interaction between the distal histidine and ligand. One possibility might be that this is the interaction which keeps the ligand trapped in the heme pocket after photolysis. We reject this particular suggestion because of the similar activation energy and high percentage of re-
binding in opossum hemoglobin, which has a distal glutamine in its α-chain, in place of the usual distal histidine. More broadly, the similarity in activation energies among the three hemoglobinls argues against a specific site being responsible for ligand trapping. In any case, future suggestions for specific binding sites must be consistent with nanosecond recombination data and should be tested using appropriate mutants or hemoglobin variants which differ in amino acid sequence.

The similarity of activation energies for different proteins is a point in support of the high viscosity diffusion model. However, it seems to be the only support that this model has. The problem with the high viscosity diffusion model is that it would predict not only similar activation energies but also similar diffusion rates independent of protein structure. Yet the most striking feature of our data in Fig. 3 is that the rates vary but activation energies do not. Furthermore, we concur with the other studies which did not find the \( \exp(-kT') \) variation but activation energies do not. Consequently, our data argue against a pure diffusion model.

The answer may involve more emphasis on movement of the protein rather than movement of the CO, perhaps along with some rather nonspecific weak interactions of ligands with a number of different groups in the protein. The protein forms an adequate cage around the ligand and the protein must overcome an energy barrier as it moves to allow the dissociated ligand out. This model involves neither a specific binding site nor free diffusion. It allows variations in detailed protein structure to affect the rate at which the ligand leaves the protein, but predicts generally similar activation energies characteristic of protein conformational changes.

The above discussion would become much sharper if a specific model for the geminate recombination process were available. All such models will envision some sort of competition between rebinding and escape into solution.

\[
\text{Hb} + \text{CO} \rightarrow \text{Hb.CO} \quad \text{and} \quad \text{Hb.CO} \rightarrow \text{Hb} + \text{CO} \tag{3}
\]

An equation of the form of Equation 3 is the assumption, often implicit and unstated, of all discussions of geminate recombination. The question is whether Equation 3 merely describes the stoichiometry of the reaction or is taken to imply a reaction mechanism. Given additional assumptions, Equation 3 may be considered as an explicit model, the simplest possible mechanism. If only the concentration of HbCO is monitored, a single exponential recovery is predicted with

\[
k_e = k_{3a} + k_{51} \tag{4}
\]

and

\[
\phi = k_{31}/(k_{3a} + k_{31}) \tag{5}
\]

It is straightforward to calculate \( k_{3a} \), the escape rate, and \( k_{31} \), the rebinding rate, from the data of Figs. 3 and 4. The results of that decomposition are collected in Figs. 5 and 6.

A number of inferences follow immediately from Figs. 5 and 6. 1) The rate of escape \( k_{3a} \) generally exceeds the rate of rebinding \( k_{31} \), reflecting the fact that geminate recombination captures less than half of the CO initially dissociated, except in rare cases such as opossum at low temperatures and high pH. 2) The temperature dependence of the geminate recombination is predominately in \( k_{23} \), which supports Duddell's initial reasoning (1) that the temperature dependence observed was related to more facile escape at higher temperature. The recombination proper \( k_{31} \) has a low activation energy, suggesting that there is no large barrier along the reaction coordinate between whatever it is that is represented by Hb-CO and the bound ligand state. 3) For any given species, \( k_{3a} \) is affected very little by changes in pH, even with the addition of IHP. However, in every case \( k_{3a} \) is clearly lowered at pH 6.0 in the presence of IHP, conditions favorable to tense state conformations. Whatever control of affinity is exercised by conformational changes is controlled predominately by the ligand binding proper as reflected in \( k_{32} \). Nanosecond geminate recombination measures these rates prior to tertiary relaxation. Consequently, it does not reflect the additional reduction of \( k_{31} \) which may accompany tertiary changes resulting from photolysis. 4) Although the above features are common to all three species, the actual rates themselves differ but show no obvious pattern. Carp Hb4(CO)4 has no measurable geminate recombination either at pH 6.0 + IHP where it exists in the \( T \) state or at pH 9.2 where it is in the \( R \) state (16). Opossum Hb4(CO)4, on the other hand, follows the pattern of human Hb4(CO)4, although like carp it also undergoes an \( R \rightarrow T \) transition at pH 6 + IHP and exhibits an important substitution at the ligand binding sites as well. For opossum, \( k_{3a} \) is consistently about half what it is for HbA, which may reflect the effect of this substitution. Comparing the two human hemoglobins, the main effect of dimerization in Hb Rothschild is to lower \( k_{31} \) indicating significantly increased constraints at hemes in dimers. It is interesting to note in this connection that Mill and Ackers (18) have ob-

![Fig. 5. Logarithmic plots of \( k_{3a} \) rate constants versus \( 1/T \) for opossum, normal adult, and rabbit at pH 9.2 (○), pH 7.0 (△), and pH 6.0 with IHP (□).](image)

![Fig. 6. Logarithmic plots of \( k_{3a} \) rate constants versus \( 1/T \) for opossum, normal adult, and rabbit at pH 9.2 (○), pH 7.0 (△), and pH 6.0 IHP (□).](image)
erved that the unliganded subunit in triliganded tetramers Hb$_4$(L)$_2$ has ligand affinity which is significantly higher than that of dimers. A similar trend, that is, low $k_2$, low $k_{23}$, and low CO affinity is displayed in Hb rabbit. 5) In the mammalian hemoglobins, the rates $k_2$, and $k_{23}$ are roughly comparable, so that small changes in either have a significant effect on ligand affinity. Perhaps in human, rabbit, and opossum hemoglobin nature has selected for a condition in which the protein is susceptible to refined control by way of both processes. A small change in either $k_2$, or $k_{23}$ will have a significant effect on the escape of ligand. In the case of carp, where very little geminate recombination is observed at any pH, either $k_2$ is much slower or $k_{23}$ is much faster (or both) than in the mammalian hemoglobins and small changes in the rate constant have little effect.

Although observations 1 through 5 may be discussed in terms of the simple model implicit in mechanism (Equation 3), they offer little reason to suppose that the simple mechanism is more than a crude approximation. In fact, the variation of geminate recombination with species and conditions suggests that the elementary model, a literal interpretation of Equation 3, is oversimplified. For the reasons proposed earlier under “Discussion,” it is unlikely that the intermediate Hb-CO is a single entity, either a particular complex at a nonheme binding site or representative of a randomly diffusing CO in a homogeneous “interior of the protein.” Heterogeneity in Hb-CO may be related to the complex recombination behavior observed at low temperatures (9). It is tempting to resolve the hypothetical Hb-CO into multiple intermediates.

Dlott et al. (19) have recently reported the identification of what they term Process I, said to be related to one of the intermediate recombination occurring on a faster time scale than we have considered, before the ligand moves away from the iron very far at all. It will be most interesting to learn the results when those measurements are extended from isolated subunits to tetrameric species possessing full cooperative behavior.

Hemoglobin is of interest, in part, as an example of control in an allosteric protein. Kinetic studies carried out at different conditions can probe the effects of conformation on binding affinity. Depending upon the extent of photolysis, millisecond studies are able to measure recombination either in the $R$ state or after quaternary relaxation to the $T$ state. The nanosecond studies of geminate recombination probe recombination prior to tertiary relaxations. It will be valuable to compare the geminate recombination with the observed millisecond rate constants in order to explore the dependence of tertiary relaxation in the control of ligand binding. In setting out to make that comparison, we found that it is necessary to re-measure and re-evaluate certain of the millisecond recombination results. Our data for the millisecond processes and the comparison with nanosecond processes will be presented in another place.

REFERENCES