Vol. 171, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1990 Pages 306-312

QUATERNARY STRUCTURE AND THE GEMINATE RECOMBINATION OF CARP HEMOGLOBIN WITH METHYLISOCYANIDE

Debkumar Bandyopadhyay, Kevin N. Walda,[+]
Douglas Magde,[+] Teddy G. Traylor,[+] and Vijay S. Sharma

Department of Medicine and [+]Department of Chemistry
University of California at San Diego, La Jolla, California 92093

Received June 18, 1990

The kinetics of geminate recombination were studied for the methylisocyanide derivative of carp hemoglobin. Carp hemoglobin is of interest because it has been established that the fully liganded form switches between a high affinity R state at pH 9 and a low affinity T state at pH 6 in the presence of IHP. Geminate recombination was observed on both the picosecond and the nanosecond time scales under all conditions; however, only a small variation is observed in the rates and the yields of geminate recombination as the protein switches from the R to the T state. Taken together with overall "on" and "off" rates, the data indicate that the change from the R to the T configuration affects bond breaking most, but also influences subsequent escape from the protein as well as both entry into the protein and bond formation. There is some reason to postulate tertiary conformational change in the T state on the microsecond time scale following ligand escape from the protein.

A multistep mechanism for the combination of ligands to heme proteins was first proposed by Austin et al. (1) to explain their low-temperature flash photolysis study of carboxymyoglobin. Since then further details of the reaction mechanism have been explored and the original mechanism has been extended in order to explain the fast and ultra-fast reactions of the oxygen, nitric oxide, and various alkylisocyanide derivatives of myoglobin and hemoglobin (2-14). Those efforts concentrated on either single chain heme-proteins (myoglobin or the α or β chains of hemoglobin) or on fully liganded human hemoglobin (HbA), with the latter results applicable primarily to the high affinity R state of cooperative tetrameric heme-proteins. Very few studies have been made on the T state of tetrameric hemoglobin.

It is difficult to produce liganded hemoglobin in the T state for flash photolysis studies. To overcome this difficulty, Murray et al. (12,13) studied the geminate rebinding of carbon monoxide to hemoglobin in which cobalt had been substituted for iron in the β chains. Those metal hybrids switch to the T state in the presence of allosteric effectors such as inositol hexaphosphate (IHP) and bezafibrate (BZF). Since concern is sometimes expressed about the effect of changing the metal, it is desirable to compare the results with other methods of generating liganded T states. One alternative approach is to study hemoglobin from a nonhuman species in which the fully liganded form can be switched to the T state by allosteric effectors. The best known example is carp hemoglobin: in the presence of IHP at pH 6.0 the quaternary structure of COHb(carp) switches to the T state (15). Unfortunately, our preliminary measurements on COHb(carp) observed no geminate recombination at any pH either in the presence or in the absence of IHP and, therefore, could not distinguish any difference there might be between R and T state geminate reactivities for CO. Fortunately, Lin et al. (16) have reported that isocyanide derivatives of

0006-291X/90 $1.50

Hb(carp) also switch to the T state at pH 6.0 in the presence of IHP, even when fully liganded. In this communication we report picosecond and nanosecond geminate recombination studies on methylisocyanide derivatives of Hb(carp) at various pH values with and without IHP. The aim is to investigate the effect of quaternary structure on the kinetics of geminate recombination of ligands with hemoglobin.

MATERIALS AND METHODS

The pH 5.9 buffer was 0.1 M bistris, and the pH 9.0 buffer was 0.05 M borate. Methylisocyanide (MeNC) was prepared by the method reported in the literature (17). Carp hemoglobin was prepared by lysing carp blood by the water-toluene method (15). Hemolysate free from 2,3-diphosphoglycerate (2,3-DPG) was prepared by Dintzi's method as described by Garlick et al. (18). The isocyanide derivative of Hb(carp) was prepared by adding a calculated volume of deoxygenated concentrated protein directly to a gas-tight optical cell (2 mm light path) containing a known volume of deoxygenated buffer. One μL of concentrated dithionite solution was added to ensure complete deoxygenation. A calculated volume of MeNC (~250-fold excess) was cannulated into the cell to generate the MeNCHb(carp). The final Hb concentration was approximately 35-45 μM as calculated from the absorption at 540 nm. Static absorption spectra were recorded before and after each experimental run to check for degradation of protein. In the nanosecond experiments protein degradation was very rare. In the picosecond experiments, turbidity in the protein solution was occasionally observed. Such experiments were discarded.

The instrumental procedure and data treatment used for flash photolysis were described earlier (11). In both the picosecond and the nanosecond experiments the excitation laser energy was attenuated to produce about 5% photolysis, so that transient signals were dominated by contributions due to dissociation of only one ligand from each tetrameric protein. The picosecond data were fit by single exponentials. The biphasic reaction time course in the nanosecond time domain was analyzed as the sum of two exponential decays. The error terms listed in Tables 1 and 2 represent one standard deviation as calculated by least-squares analysis of a typical set of kinetic data. The overall accuracy of the constants is estimated to be 10 to 20%.

RESULTS

Typical picosecond transient absorption spectra are displayed in Figure 1 and a typical nanosecond kinetic absorbance trace is shown in Figure 2. Tables 1 and 2 summarize the results measured for the observed rates of change of absorbance and the extent of recombination, estimated as $(\Delta A(0)-\Delta A(\infty))/\Delta A(0)$, with $\Delta A(0$ *or* $\infty)$ determined from the measurements on the particular time scale. The changes between pH 6.0 (with IHP) and 9.1 in the rates and the yields of geminate recombination on both time scales are small, but in the direction that might be expected if the tetramer exists in the T conformation at low pH, as appears to be well established. In the first few picoseconds at low pH in the presence of IHP, there is somewhat less recombination (75% as much) than is observed at high pH, as well as a small change in the rate of disappearance of transient absorption, but no qualitative change in the appearance of either spectra or kinetics.

In contrast to the monophasic reaction time course observed (within present resolution) in the picosecond time range, the nanosecond reaction time course is clearly biphasic under all conditions. When the decay curves are fit to the sum of two exponentials, the rates assigned to the two phases differ by a factor between 6 and 10 under all conditions, that is at both pH 5.9 and pH 9.1 and with or without IHP. The zero-time amplitudes of the two phases are approximately equal under all conditions, according to a least squares analysis. Changing from the R to the T state does not affect these qualitative features of the geminate recombination. However, the total amount of geminate recombination is smaller at lower

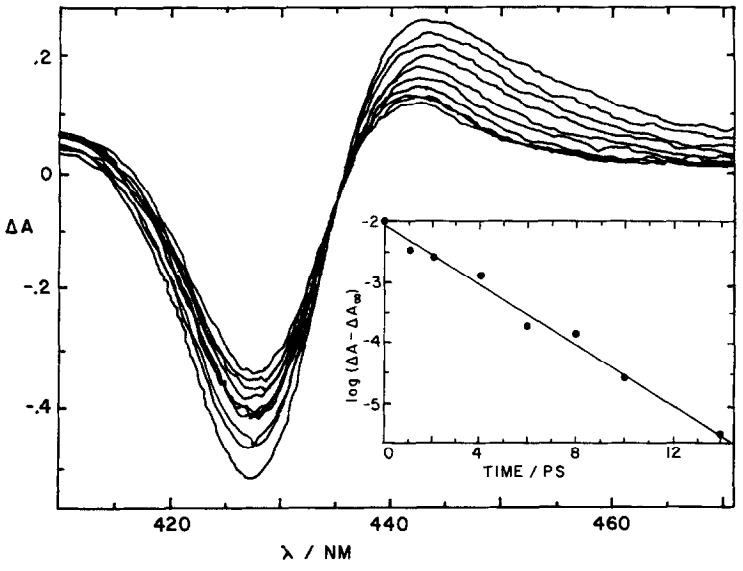

Figure 1. Picosecond transient difference spectra of MeNCHb(carp). The change in absorbance is plotted vs. wavelength for time delays between the pump and probe of 0 (top-most at 442 nm), 1, 2, 3, 6, 8, 25, 40, 75, and 302 ps; [Hb] = 36 μM; [MeNC] = 11.8 mM; pH 9.1; photolysis energy was ~70 μJ at 314 nm. The inset shows the linear dependence of ΔA-ΔA(∞) vs. time for the region near 440-445 nm.

pH and is reduced further by the addition of IHP at low pH. At pH 9.1, IHP has no effect on geminate recombination.

There are very few data in the literature that can be compared directly with these results. In the only other work on isocyanide derivatives of tetrameric Hb, Olson et al. (9) fit nanosecond photolysis measurements on HbA to multiple exponentials, using deconvolution to find rates for processes shorter than their laser pulse, but found no rates faster than 10^9 $s.^{-1}$ Their deconvolutions did extract a decay component with a rate between 10^8 and 10^9 $s,^{-1}$ as well as a slower geminate process observed over hundreds of nanoseconds. The latter was a single phase for small isocyanides in HbA, in contrast to our observations on Hb(carp), which is biphasic over the same time range. Other measurements on a different protein,

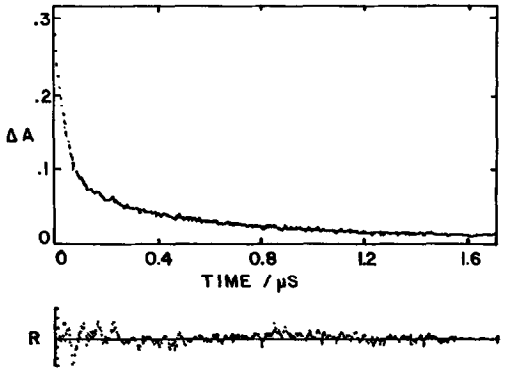

Figure 2. Nanosecond transient absorption kinetics of MeNCHb(carp) at 440 nm: A, absorbance change vs. time along with best fit to sum of two exponentials; R, residuals for fit in A, each division representing 1.33% deviation. Conditions as in Figure 1.

Table 1. Rate constants for the picosecond geminate recombination of MeNC to R- and T-state carp hemoglobin at 22°

State	pH	IHP(mM)	$k_{g,ps}$ (s^{-1})	%Return
T	5.9[a]	2.00	$(1.60 \pm 0.01) \times 10^{11}$	31
R	9.1[b]	0.00	$(2.30 \pm 0.01) \times 10^{11}$	42

[Hb] = 35 μM; [MeNC] = 12 mM.
[a] 0.10 M bistris buffer.
[b] 0.05 M borate buffer.

MeNCMb(sperm whale), gave picosecond recombination rates that were reported to be about 3 to 5 times slower than the values in Table 1 (11).

DISCUSSION

Even without making any assumption regarding the exact details of the reaction mechanism involved in photolyses of liganded hemoglobin on the picosecond or the nanosecond time scales, it is obvious from the data presented above that a change in the quaternary structure of the MeNCHb(carp) complex produces relatively small changes in the amount of geminate recombination and the rate at which it occurs.

It is known (16) that the ligand binding affinity for MeNCHb(carp) is reduced 34-fold between the R and the T states, measured with the same allosteric effector, buffer choice, and pH as used in the present study. At low pH in the presence of IHP, ligand binding was noncooperative and the tetramer existed in the low affinity form (T state) regardless of the number of ligands bound. At high pH the ligand binding was only slightly cooperative, and affinity was high (R state) regardless of the degree of ligation. The reduced affinity in the T state might be attributed to an *increased* probability of dissociation (increased "off" rate constant, k) at low pH; or it might be due to *decreased* probability of overall bimolecular combination (decreased "on" rate constant, k') at low pH. In fact, both parameters were characterized and found to vary in the direction required to account for the affinity change (16). Interpretation of the kinetic

Table 2. Rate Constants for the Nanosecond Geminate Recombinations of MeNC to R- and T-State Carp Hemoglobin at 22°C

State	pH	IHP(mM)	$k_{1,g,ns} \times 10^{-7}$ (s^{-1})	$k_{2,g,ns} \times 10^{-6}$ (s^{-1})	ΔA(t=0)	%Return
T	5.9[a]	2.00	1.56 ± 0.05	2.52 ± 0.05	0.041	25
	5.9[a]	0.00	1.62 ± 0.05	2.03 ± 0.05	0.041	33
R	9.1[b]	2.00	2.53 ± 0.07	2.05 ± 0.05	0.029	39
	9.1[b]	0.00	2.55 ± 0.07	2.09 ± 0.05	0.034	40

[Hb] = 45 μM; [MeNC] = 12 mM.
[a] 0.10 M bistris buffer.
[b] 0.05 M borate buffer.

measurements is complicated by the fact that two phases were observed for the bimolecular combination at all pH conditions. The two phases contributed approximately equal amplitudes (as we observed also in this study for the nanosecond geminate process). For the ligand dissociation, the faster k increased by 12 and the slower k increased by 9, when the protein was converted from the R to the T state. Thus, the change in "off" rates was about an order of magnitude and accounted for the majority of the affinity change. For the ligand association, the faster k' was unchanged by the conversion from R to T, but the slower k' decreased by 13. Thus, the change in "on" rates, in some vague "average" sense, accounted roughly for the remainder of the affinity change. It is tempting to speculate that the phases observed in the geminate recombination correlate with the phases observed in the bimolecular kinetics and interpret each phase in terms of some specific assignment (such as to α and β chains) having different kinetics; however, there really is insufficient evidence to be certain on this point, particularly in view of the fact that even single chain proteins, such as human β_4 tetramers, yield biphasic nanosecond geminate recombination with methylisocyanide (9).

The measurements of the geminate recombination processes reported here permit further interpretation of k and k'. A simplified mechanism for dissociation and combination can be written as

$$\mathrm{HbL_4} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [\text{geminate pair}] \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \mathrm{HbL_3} + \mathrm{L} \quad ,$$

$$k = k_{off} = k_1 [k_2 (k_{-1} + k_2)] = k_1 \,\mathrm{Prob}(escape) \quad ,$$

$$k' = k_{on} = k_{-2} [k_{-1}(k_{-1} + k_2)] = k_{-2} \,\mathrm{Prob}(bond\text{-}making) \quad .$$

Although there remain numerous questions about details of geminate recombination still to be resolved, the general idea that a thermalized pair exists for many nanoseconds in the protein seems certain. The "off" rate constant can be written as the product of a primary bond-breaking step multiplied by the probability, Prob, of escape from the protein. The "on" rate constant can be written as the product of the rate of successful diffusive encounter and entry into the protein multiplied by the probability of bond formation once the ligand has already entered the protein. The nanosecond process we observe almost certainly must be part of the kinetic pathway in thermal reactions; the picosecond process might be partly (or even entirely) relevant only to recombination following flash photolysis.

It is apparent that the efficiency with which the geminate pair either forms a bond or terminates with ligand escape from the protein does change, but by less than a factor of two, when the protein is switched from the R to the T state. Consequently, it seems that the dominant effect on k due to switching from R to T is a destabilization of the ligand bond with a consequent increase in the probability of thermal bond disruption. This is in accord with Raman spectroscopic studies (19) that correlate R to T changes quite directly with proximal strain at the iron. An additional small contribution, less than a factor of two, is due to the fact that once the bond breaks, the ligand is more likely to escape from the T state protein. At the same time, one may infer that k' is reduced in the T state at least partly because of the reduced likelihood of bond formation following entry of a ligand into the protein. In our measurements, this contributes less than a factor of two and leaves us with the need to account for another factor of perhaps 2 or 3 reduction in k' in the T state. It is possible that the ligand has more facile entry into the protein in the R state. Some effect of that sort is consistent with the increased probability for ligand escape in the T state, but the

effect is not quite large enough to account for the required enhancement unless there is considerable asymmetry between exit and entry. The most plausible way to account for such an assymetry is to postulate that, even in the absence of cooperativity among chains, loss of ligand is accompanied by conformational relaxation in the protein on the time scale of microseconds, that is, between the time scale on which geminate recombination is characterized in photolysis experiments and the time scale for bimolecular combination. If this occurs, then the geminate pair formed by the photolysis of a liganded T state would have a slightly different tertiary conformation than that which confronts a ligand entering from the solvent. Consequently, the geminate pair formed by photolysis could have a greater probability for bond formation and a lower probability for ligand escape than the pair that is formed by ligand entry from the outside.

Hofrichter et al. (5), Murray et al. (12,13), and Marden et al. (10) studied the effect of quaternary structure on the rates and yields of geminate recombination in metal hybrids of HbA [α_2(Co)β_2(Fe) and α_2(Fe)β_2(Co)] as well as in native HbA. In the absence of allosteric effectors IHP and BZF, α_2(Fe–CO)β_2(Co) behaved like R-state hemoglobin (geminate recombination ~40 percent). The addition of either allosteric effector produced a marked reduction in the amount of geminate recombination. For conditions where there are no dimers or R-state tetramer, the geminate recombination was estimated to be ~1 %. A similar conclusion regarding the geminate yields in the T state of carboxy HbA was reached by Marden et al. (10). These low *estimates* for geminate yields of the T-state COHb correlate with the difference of ~60 in the rate constants of the rate limiting step (bond formation) for combination reactions in the two quaternary structures. The difference between those results and the geminate process we observe in the MeNCHb(carp) system may well be related to the fact that CO ligation is controlled much more by bond formation than is ligation of any other ligand, all of which are limited largely by diffusion and entry into the protein.

CONCLUSIONS

Laser photolysis of methylisocyanide with the R and T states of carp hemoglobin reveals small, but definite, differences in geminate recombination yields and kinetics. The geminate process is primarily controlled by the tertiary structure of the ligand binding site, which apparently does not differ much in the two liganded quaternary structures. The differences between the R and the T states are reflected in all rate constants; but the largest single contribution is associated with actual bond breaking, at least in this system. It is likely that there is some small tertiary relaxation after the ligand escapes from the protein and before bimolecular combination commences.

ACKNOWLEDGMENTS

This work was supported by Grants #HL 31159, HL 13581 and RR 02352 from the National Institutes of Health, and CHE-8715561 from the National Science Foundation.

REFERENCES

1. Austin, R. H., Beeson, K. W., Eisenstein, L., Frauenfelder, H., and Gunsalus, I.C. (1975) *Biochemistry* **14**, 5355-5373.

2. Alberding, N., Chan, S. S., Eisenstein, L., Frauenfelder, H., Good, D., Gunsalus, I. C., Nordlund, T. M., Perutz, M. F., Reynolds, A. H., and Sorensen, L. B. (1978) *Biochemistry* **17**, 43-51.

3. Dlott, D. D., Frauenfelder, H., Langer, P., Roder, H., and DiIorio, E. E. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 6239-6243.

4. Morris, R. J., Gibson, Q. H., Ikeda-Saito, M., and Yonetani, T. (1984) *J. Biol. Chem.* **259**, 6701-6703.

5. Hofrichter, J., Henry, E. R., Sommer, J. H., Deutsch, R., Ikeda-Saito, M., Yonetani, T., and Eaton, W. A. (1985) *Biochemistry* **24**, 2667-2679.

6. Campbell, B. F., Magde, D., and Sharma, V. S. (1985) *J. Biol. Chem.* **260**, 2752-2756.

7. Jongeward, K. A., Marsters, J. C., Mitchell, M. J., Magde, D., and Sharma, V. S. (1986) *Biochem. Biophys. Res. Commun.* **140**, 962-966.

8. Gibson, Q. H., Olson, J. S., McKinnie, R. E., and Rohlfs, R. J. (1986) *J. Biol. Chem.* **261**, 10228-10239.

9. Olson, J. S., Rohlfs, R. J., and Gibson, Q. H. (1987) *J. Biol. Chem.* **262**, 12930-12938.

10. Marden, M. C., Hazard, E. S., Kimble, C., and Gibson, Q. H. (1987) *Eur. J. Biochem.* **169**, 611-615.

11. Jongeward, K. A., Magde, D., Taube, D. J., Marsters, J. C., Traylor, T. G., and Sharma, V. S. (1988) *J. Am. Chem. Soc.* **110**, 380-387.

12. Murray, L. P., Hofrichter, J., Henry, E. R., Ikeda-Saito, M., Kitagishi, K., Yonetani, T., and Eaton, W. A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2151-2155.

13. Murray, L. P., Hofrichter, J., Henry, E. R., and Eaton, W. A. (1987) *Biophys. Chem.* **29**, 63-76.

14. Petrich, J. W., Poyart, C., and Martin, J. L. (1988) *Biochemistry* 27, 4049-4060.

15. Tan, A. L., DeYoung, A., and Noble, R. W. (1972) *J. Biol. Chem.* **247**, 2493-2498.

16. Lin, M. J., Noble, R. W., Winterhalter, K. H., and DiIorio, E. E. (1988) *Biochim. Biophys. Acta* **954**, 73-81.

17. Casanova, J. J., Werner, N. D., and Schuster, R. E. (1966) *J. Org. Chem.* **31**, 3473-3481.

18. Garlick, R. L., Davis, B. J., Farmer, M., Fynn, H. J., Fynn, U. E. H., Noble, R. W., Powers, D. A., Riggs, A., and Weber, R. E. (1979) *Comp. Biochem. Physiol.* **62A**, 239-244.

19. Friedman, J. M. (1985) *Science*, **228**, 1273-1280.