MANGANESE HEMOGLOBIN: ALLOSTERIC EFFECTS IN STOPPED FLOW FLASH PHOTOLYSIS AND SEDIMENTATION MEASUREMENTS

Q. H. Gibson,[*] B. M. Hoffman[†,1] R. H. Crepeau,[*] S. J. Edelstein,[*,2] and C. Bull[†]

[*] Section of Biochemistry and Molecular Biology
Cornell University
Ithaca, New York 14850

and

[†] Department of Chemistry
Northwestern University
Evanston, Illinois 60201

Received May 10,1974

Summary. Conformational differences between manganese hemoglobin and its liganded derivatives are observed in the tetramer-dimer dissociation equilibrium, in the binding of 8-hydroxy-1,3,6-pyrenetrisulfonate (an analogue of 2,3-diphosphoglycerate) and bromthymol blue, and in the reaction with p-hydroxymercuribenzoate. The reaction of manganese hemoglobin with NO is readily followed by stopped flow, the resulting NO derivative is photodissociable, and rapid kinetic measurements exhibit the hemoglobin allosteric interaction.

Introduction. The redox and ligand-binding equilibria of manganese hemoglobin have been shown to exhibit the hemoglobin allosteric interactions (1). We here report a series of observations which demonstrate that the conformational properties of MnHb[3] differ from those of its derivatives and that these differences are equivalent to those observed for the naturally occuring iron protein. Furthermore, the time course of the reaction MnHb with NO can be readily followed and the resulting MnHbNO is observed to be photodissociable. Through the use of stopped flow and flash photolysis techniques, we have studied the kinetic allosteric effects in MnHb reactions.

Materials and Methods. The preparation and properties of MnHb and its derivatives were given previously (1). Stopped flow kinetic determinations were made with the apparatus of Gibson and Milnes (2) in the version manufactured

(1) Recipient of an NIH Career Development Award.
(2) Alfred P. Sloan Foundation Fellow.
(3) Abbreviations: Hb, ferrohemoglobin; MnHb, manganohemoglobin; IHP, inositol hexaphosphate; PTS, 8-hydroxy-1,3,6-pyrenetrisulfonate; PMB, p-hydroxymercuribenzoate; DPG, 2,3-diphosphoglycerate.

by Durrum Instrument Co., Palo Alto, California. Flash photolysis was performed using apparatus similar to that described by Gibson (3). Sedimentation equilibrium experiments were performed with an analytical ultracentrifuge equipped with a scanner and on-line computer system (4). Data were fitted to a two-term exponential equation to obtain tetramer-dimer dissociation constants (5).

Results

Sedimentation. Tetrameric liganded hemoglobin dissociates into dimers with a dissociation constant ($K_{4,2}$) of 1-2 x 10^{-6}M, and this value is sensitive to the presence of IHP. Although Hb is observed to dissociate with a constant at least an order of magnitude lower than liganded Hb, the values are near the limits of detection by direct methods and are independent of IHP. Recent results based on CO binding equilibria indicate an actual value of $K_{4,2}$ = 3 x 10^{-12} for Hb (6,7).

Table I gives dissociation constants for MnHb and its derivatives. As for Hb, the value of $K_{4,2}$ of MnHb is insensitive to the presence of IHP and is so low as to lie at the limit of detection. In the absence of organic

Table I

Sedimentation Equilibrium Values
for Tetramer-Dimer Dissociation Constants ($K_{4,2}$)*

Form	[IHP] = 100 μM	[IHP] = 0
MnHb	6 x 10^{-7} M	5 x 10^{-7} M
MnHbNO	6 x 10^{-7} M	2 x 10^{-6} M
$Mn^{III}Hb$	8 x 10^{-7} M	3 x 10^{-5} M

* 20°C; 0.05 M Bis-Tris + 0.1 M NaCl; for experimental details, see text.

phosphates either binding of NO or, most noticeably, oxidation to $Mn^{III}Hb$ increases $K_{4,2}$. Addition of IHP reduces the $K_{4,2}$ of both MnHbNO and $Mn^{III}Hb$ by an amount similar to the reduction observed with HbCO (unpublished).

Conformational Probes. Conformational differences between hemoglobin and its liganded forms are also manifested in the affinity and/or rates of binding small molecules at sites other than the metalloporphyrin. PTS, a fluorescent DPG-analogue (8), binds to MnHb and is partially released upon NO binding, demonstrating that the affinity of MnHb for PTS, and therefore DPG, is reduced upon ligation. In addition, as is true for hemoglobin (9), bromthymol blue interacts reversibly with MnHb with the velocity of combination and affinity considerably higher than for any of its derivatives.

In Hb the sulfhydryl groups at position β-93 undergo a change in reactivity upon heme ligation, the rate of reaction with PMB being much higher in the liganded form (10). When MnHb (~ 1μM, in tetramer) is mixed with 25μM PMB in phosphate (0.05M, pH 7, 20°), reaction with these sulfhydryl groups occurs with a half-time $t_{1/2} \sim 200$ msec. When a similar experiment is performed with MnHbNO, $t_{1/2} \sim 5$ msec. This increase in sulfhydryl reactivity upon ligand binding is comparable to that for Hb.

Ligation by Stopped Flow. Both Hb and MnHb bind NO. The association reaction for Hb is very rapid ($k = 1.7 \times 10^7 M^{-1} sec^{-1}$ (11)), and is only observed with difficulty by stopped flow. However, NO binding to MnHb is readily followed (Fig. 1a) and, as expected, the association velocity is proportional to [NO]. The reaction is homogeneous, except for variable small amounts of rapidly reacting material which seem largely associated with the reduction procedure.

In these preliminary measurements, we have not yet reliably observed an acceleration in rate during the time course of reaction. Such acceleration, observed in the combination of Hb with O_2 and CO, is interpreted as one expression of homotropic linkage or "cooperative" ligand binding (12).

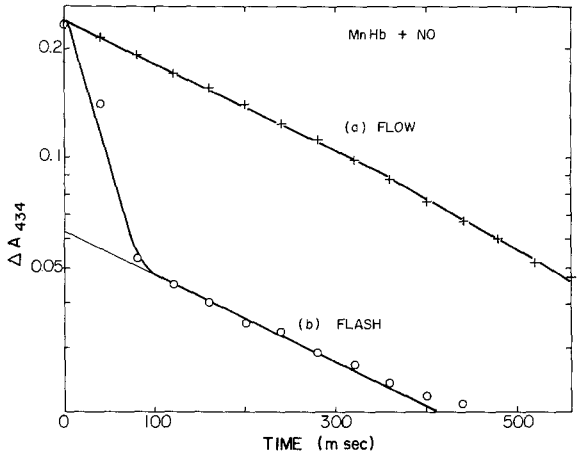

Figure 1. Time course of the reaction of MnHb (1μM in monomer) with NO(~100μM). a) Stopped flow; b) Upon flash photolysis of the final solution after flow. Conditions are 0.05 M P_i, pH 7.0, 20° and 2 cm path length.

However, the rate of combination of MnHb with NO is appreciably reduced (~ 1/5) by the addition of IHP, thus kinetically demonstrating that ligand binding is linked to the binding of organic phosphates.

Flash Photolysis. MnHbNO is readily photodissociated, in sharp contrast to HbNO which under most conditions cannot be observed to photodissociate. Fig. 1a gives the results upon mixing MnHb (1μM monomer after mixing) with NO and Fig. 1b the result after flash photolysis of the preformed MnHbNO. The absorbance excursions (ΔA at t = 0) are identical in both cases, indicating 100% photodissociation. However, unlike the flow experiment, association after flash-dissociation is markedly biphasic with roughly 70% of the absorbance change occuring rapidly and the remainder at the same rate as in the flow experiment. The amount of rapid reaction depends upon [MnHb] and is reduced to ~ 20% at 15μM (monomer), Fig. 2a. This concentration-dependent rapid reaction thus appears to be related to the dissociation of MnHbNO into dimers, implying that unliganded dimers react more rapidly than do unliganded tetramers.

NO binding to MnHb after flash dissociation gives further evidence of

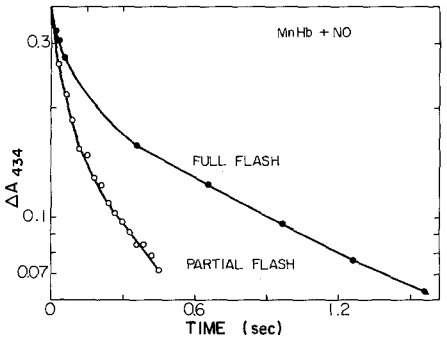

Figure 2. Reaction of MnHb (15μM, monomer) with NO(~70μM) after full (upper curve) and partial (lower curve) flash photolysis of MnHbNO. The actual OD excursion upon partial flash was only ~15% that upon full flash, but excursions in the lower curve are scaled to give the same initial ΔA as after full flash. Conditions are as in Fig. 2, but the path length is 2mm.

homotropic linkage similar to that of Hb. Fig. 2a shows the progress of the recombination of MnHb with NO after total photodissociation. Fig. 2b is the recombination reaction after only partial dissociation; the initial excursion (ΔA) was 15% that of the full flash, but the curve has been scaled to give the same value at zero time as in that upon full flash. The binding of NO to MnHbNO which is partially (~ 15%) dissociated is substantially faster than that after full dissociation. This increased rate of binding for the "last" NO demonstrates cooperative ligand binding to MnHb, analogous to results for Hb (13).

Discussion. Despite the fact that MnHb cannot reversibly bind molecular oxygen, the preliminary results presented here and elsewhere (1) indicate a high degree of similarity between the molecular properties of MnHb and those of Hb. Allosteric effects have now been observed both in ligand binding and oxidation equilibria and in the kinetics of ligand binding. These results, plus the sedimentation and PTS, PMB, and bromthymol blue binding studies demonstrate that MnHb undergoes a conformational change upon ligation equivalent to that of Hb. The ability to observe combination of NO both in flow and after photodissociation gives an added dimension to our ability to

use MnHb as a means of probing the mechanism of hemoglobin function.

Acknowledgments: We are indebted to Anne Griffin for technical assistance. C.B. is a predoctoral trainee of the USPHS, administered by the National Institutes Institutes of Health, training grant GM00626. This work was supported by the National Institutes of Health, Grants HL-13531 (BMH), GM-14276-08 (QHG), and HL-13591 (SJE) and by the National Science Foundation, Grant GB8773 (SJE).

References

1a. Hoffman, B. M., Gibson, Q. H., Bull, C., Crepeau, R. H., Edelstein, S. J., and McDonald, M. J., Ann. N.Y. Acad. Sci., in press.
b. Bull, C., Fisher, R. G., and Hoffman, B. M., BBRC, 000, 0000-0000.
2. Gibson, Q. H., and Milnes, L., Biochem. J., 91, 161-171 (1964).
3. Gibson, Q. H., (1956) J. Physiol., 134, 112-113.
4. Crepeau, R. H., Edelstein, S. J., and Rehmar, M. J., Anal. Biochem., 50, 213-233 (1972).
5. Taisky, M. and Edelstein, S. J. (1973), J. Mol. Biol., 75, 735-739.
6. Edelstein, S. J., Rehmar, M. J., Olson, S. J., and Gibson, Q. H., (1970), J. Biol. Chem., 245, 4372-4381.
7. Thomas, J. O. and Edelstein, S. J., (1972), J. Biol. Chem., 247, 7870-7874.
8. MacQuarrie, R. and Gibson, Q. H., (1972) J. Biol. Chem., 247, 5686-5694.
9. Antonini, E., Wyman, J., Moretti, R., and Rossi-Fanelli, A., (1963) Biochem. Biophys. Acad., 71, 124-138.
10. Antonini, E., and Brunori, M., (1969) J. Biol. Chem., 244, 3909-3912.
11. Gibson, Q. H., and Roughton, F. J. W., (1965) Proc. Roy. Soc. B., 163, 197.
12. Roughton, F. J. W. (1948) Barcroft Memorial Symp., Butterworth, London, 83-95.
13. Gibson, Q. H. (1956) J. Physiol., 134, 123-134.