Cooperative Nitric Oxide Binding by Manganese Hemoglobin

IMPLICATIONS FOR THE ROLE OF STERIC CONTROL IN HEMOGLOBIN LIGATION*

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The reaction of NO with managanoglobin, manganese porphyrin-substituted hemoglobin (MnHb), shows close correspondence to the reaction of CO with hemoglobin (Hb) in all features examined. All measurements are consistent with cooperative binding of NO by MnHb, associated with a T → R conformational change in the tetramer. An important conclusion based on a comparison of rates of NO ligation by Hb and MnHb is that steric control of access to the metal center by a ligand cannot be responsible for the change in ligation rate caused by the hemoglobin T → R transformation.

The apparent second order rate constant for the (NO + MnHb) combination reaction is about 35 mm⁻¹ s⁻¹ (pH 7 and 20°C), compared with about 120 mm⁻¹ s⁻¹ for the (CO + Hb) reaction. Both reactions are autocatalytic and liganding is associated with similar changes in reactivity with bromothymol blue (faster with MnHb than with MnHbNO) and β-mercuribenzoate (slower with MnHb than with MnHbNO). Binding of 8-hydroxy-1,3,6-pyrenetrisulfonate to MnHb is stronger with MnHb than with MnHbNO). Binding of 8-hydroxy-1,3,6-pyrenetrisulfonate to MnHb is stronger than to MnHbNO, although the difference is less than that between Hb and HbCO. The dissociation of NO from MnHb in the presence of dithionite is slow (0.005 s⁻¹), suggesting a static dissociation constant of the order of 10⁻⁷ M⁻¹.

As with HbCO, MnHbNO is photosensitive with a similarly high quantum yield. In both cases, the products of partial photodissociation react 20 to 30 times faster with their respective ligands than the products of complete photodissociation. Spectrophotometric evidence of an R → T conformational transformation upon NO photodissociation is also presented.

The tetramer-dimer dissociation constants of met-MnHb, MnHbNO, and MnHb are 1 x 10⁻⁸, 1 x 10⁻⁹ M⁻¹, and too small to measure accurately with the ultracentrifuge. Dimeric MnHb, like dimeric Hb, reacts 20 to 30 times faster with ligand than the tetramer does.

The reduction of Mn⁺⁺ porphyrin of MnHb by dithione and the oxidation of MnHb by ferricyanide are both markedly biphasic reactions. Analogy with the corresponding reaction of Hb suggests that chain differences are responsible.

In recent years it has been found that the old procedure of replacing the heme iron in hemoglobin by other metals (1) can give important insights into the properties of this protein (2). Some characteristics of managanoglobin, manganese-substituted hemoglobin, have previously been reported (3–5). We have presented preliminary observations of reversible NO binding by MnHb (6, 7), have studied the crystal structure of (8) and N₂ binding by MnHb (7, 9) and have demonstrated cooperativity in the oxidation-reduction reaction (6, 10, 11).

These several studies suggested that the binding of NO to MnHb might show a close parallel to cooperative hemoglobin ligation. The system (MnHb + NO) is isoelectronic with (FeHb + CO). Correspondingly, model compounds confirm that in both cases the diatomic molecule binds in a linear fashion (12, 13) and we have found the (Fe-Hb) and (Mn-NO) linkages to be comparable photodissociable (14). Similarly, model studies show that the high spin, five-coordinate Mn⁺ and Fe⁺ porphyrins of the unliganded protein should be stereochemically equivalent (15, 16). These structural correspondences lead to the conclusion that NO binding by MnHb should be analogous, not to NO binding by Hb (17, 18), but to CO binding since the Fe-NO linkage is chemically and geometrically different (14).

The binding of CO to Hb and of NO to MnHb is too strong to be readily measured by equilibrium methods, but in both cases, and unlike the binding of NO to Hb, the rates of reaction are readily measured by stopped flow techniques or after flash photodissociation. In this paper, we describe the kinetics of the reaction of NO with MnHb as well as the use of reactions regarded as probes of hemoglobin conformation. We find that the similarities with CO binding to Hb extend to the finest details of the process. These include cooperativity, photosensitivity, and a conformation change corresponding to the T → R transition which is directly observable upon NO photodissociation. As an important consequence of these similarities, a comparison of the rates of NO binding by Hb and MnHb supports the conclusion that steric control of access to the metal center cannot be responsible for the changes in ligation rates caused by the T → R transformation and provides a basis for discussing the possible role of steric hindrance in these changes.

EXPERIMENTAL PROCEDURES

Materials—MnHb was prepared as described elsewhere (19). Samples were shipped to Ithaca and stored at 0°C. MnHb was prepared by taking a portion of stock MnHb and diluting with deoxygenated buffer. Sodium dithionite was added (0.6 mg/ml) and the solution passed through an anaerobic column of Sephadex G-25 (10 x 1 cm) equilibrated with the same buffer. The colored band was collected in a syringe without exposure to air and used as a stock solution. With a glass syringe, oxygen penetrates very slowly and the solution could be used for several days.

Solutions of NO—These were prepared by filling a glass tonometer

* The abbreviations used are: MnHb, manganese hemoglobin (managanoglobin); Hb, terremoglobin; met-Hb, terremoglobin; HbCO, carboxyhemoglobin; HbNO, nitrosylhemoglobin; MnHb, met-MnHb, manganese hemoglobin; bis-Tris, 2,2'-bis(hydroxymethyl)-2,2',2'-nitroloethanol; TPP, tetraphenylporphyrin.
with NO (Matheson). Stock solutions were obtained by injecting appropriate deoxygenated buffers through a three-way stopcock and shaking vigorously. With a 1-liter tonometer pressure, uncertainties are about 2 to 3% in this method using 20-ml portions of buffer.

Methods—Stopped flow experiments were performed using a Duram-Gibson apparatus, interfaced to a computer as previously described (20). Flash photolysis experiments were performed using a 100-J photographic flashlamp, Multiblitz III, Mannesman, Koeln, BRD. The flash has a decay rate of about 2300 s⁻¹. The flash was screened by a suitable yellow filter (usually Corning No. 3-71 for experiments in the Soret). Observations were made with light from a tungsten lamp and a Bausch and Lomb small grating monochromator (f4) using a nominal band width of 3 to 5 nm. This was backed with a blue glass filter, usually Corning No. 7-59, to exclude stray light from the monochromator, permitting observations to be made before the flash had decayed.

RESULTS

Binding of NO to MnHb—The Soret maximum of MnHb (434 nm, ε = 152 mmol/cm⁻¹) is blue-shifted (424 nm, ε = 154 mmol/cm) upon addition of NO. Stopped flow binding kinetics were typically followed at 434 nm, and an example of a progress curve is shown in Fig. 1, corresponding to (intrinsic) J = 35 mm⁻¹ s⁻¹ in 0.05 bis-Tris, pH 7 (20°C). Under similar conditions, the hemoglobin + NO reaction is about 700 times faster, the hemoglobin + O₂ reaction is about 100 times faster, and the hemoglobin + CO reaction is about 4 times faster. The rate of the MnHb-NO reaction is accurately proportional to the NO concentration, and the reaction itself is homogeneous and closely pseudo-first order, except that there is often a small and variable amount of rapidly reacting MnHb (about 3% in the example of Fig. 1). This rapid phase is present even when the MnHb is reduced under anaerobic conditions and excess dithionite is removed by passage through an anaerobic Sephadex column; if reduction is performed by adding small amounts (10 µg/mL) of dithionite and the solution is used directly, a larger rapidly reacting fraction is observed.

Six preparations were closely examined in a search for an accelerating time course and in two cases a clear effect was observed (leaving aside the initial rapid burst). Fig. 2 is a plot of the apparent rate constant against the proportion of the reaction completed. It shows an increase of 30% over the course of the reaction, which is qualitatively and quantitatively similar to the effect observed in CO binding to Hb by Gibson and Bughton (21). Repetition with exactly one-half the concentration of NO and collecting data over twice as long a period gave an accurate reproduction of the absorbance data used in constructing Fig. 2 within 1% (0.003 in absorbance) over the 90% of the reaction followed. In three cases, no acceleration was seen, and in one the rate constant decreased monotonically.

Although the result of Fig. 2 could not be reproduced at will, evidence presented in subsequent paragraphs indicates that a change from the T to R state occurs upon NO binding by MnHb and that the binding rate in the R state is about 20 times more rapid than in T. The result presented in Fig. 2 is, correspondingly, more probable than the other results mentioned, which we interpret as resulting from minor chemical degradation of some of the protein during reconstitution.

A limited exploration of the effect of pH and of ionic composition on the rate of NO binding to MnHb in stopped flow experiments gave rates of 35 mm⁻¹ s⁻¹ in 0.05 M phosphate, pH 6, and 50 mm⁻¹ in 0.05 M borate, pH 9. At pH 7, the addition of 100 µM inositol hexaphosphate slowed the rate of 26 mm⁻¹ s⁻¹ (Fig. 1). All of these results are analogous to those seen in the CO + Hb reaction.

Flash Photolysis of MnHbNO—MnHbNO is readily photodissociated, in sharp contrast to HbNO which under most conditions shows a very low apparent quantum yield. Fig. 3a gives the result upon mixing MnHb (1 µM monomer after mixing) with NO, and Fig. 3b gives the result after flash photolysis of the preformed MnHbNO. The absorbance excursions (ΔA at t = 0) are identical in both cases, indicating effectively complete photodissociation. Experiments were performed in which the light intensity was varied using a shutter, with MnHbNO and HbCO photolysed in turn. These indicated that the quantum yields for dissociation are of the same order for the two systems; since the spectra of MnHbNO and HbCO are rather different, no attempt at exact numerical estimates of the relative quantum yield was made. Thus, as we have discussed elsewhere (14), the isoelectric nature of the two systems, (MnHbNO) and (FeHbCO), is reflected not only in the structures of the liganded metalloporphyrins but also in the properties of their photoexcited states.

Unlike the results of the flow experiments, the recombination reaction after photolysis is markedly biphasic, consistently showing an appreciable rapid phase and, thereafter, following closely on the results of the stopped flow experiment (Fig. 3). In the flash experiment the extent of the rapid phase is reproducibly large (~45%, in Fig. 3b) and varies with concentration, features which differentiate this observation from the small (~3%) and variable burst in stopped flow.

Analogous results are observed after photolysis of HbCO. It is known that unliganded Hb is in effect fully tetrameric, "Hb₄", but that Hb₄(CO)₂ is appreciably dissociated into dimers, Hb₄(CO)₂; the rapid phase in CO recombination after photolysis of carboxyhemoglobin is associated with a rapid binding of CO by the photolytically prepared (Hb₄)₂ dimers.

\[ \text{MnHbNO} + \text{NO} \rightarrow \text{MnHb} + \text{NO} \]

\[ \text{HbCO} + \text{NO} \rightarrow \text{HbNO} + \text{CO} \]
whether there are spectrophotometric differences between the
differentiation within the slow component. It is not known
between the rapid and slow components rather than from
wavelength was small and seemed to derive from differences
point between MnHb and MnHbNO at 430 nm. The effect of
were sought by exploring in the region of the Soret isosbestic
in the rate of NO binding, analogous to those reported for Hb,
using an observing beam at 434 nm. Possible chain differences
these experiments was about 20 times greater than the rate
MnHb rose to 65%. The rate of the rapid component in all
photolysis flash was attenuated to give only 29% breakdown
15% breakdown (Fig. 4), the proportion of rapidly reacting
MnHbNO. The percentage of rapidly recombining material
was only -15% that upon full flash, but excursions in the bottom
curve are scaled to give the same initial ΔA as after full flash.
Conditions are as in Fig. 2, but the path length is 2 mm.

(22). This suggested that the rapid phase during NO recombina-
tion in Fig. 3b might similarly be associated with NO
binding by (MnHb)2 dimers, and that the fraction of rapid
reaction after photolysis of nitrosylmanganoglobin should fol-
low the equilibrium fraction of liganded dimers and, thus, be
concentration-dependent. Using the same stock of reagents,
but a shorter path length and 16-fold greater concentration of
MnHb, the proportion of rapidly reacting material was re-
duced to 13% (Fig. 4), in agreement with this prediction.

Gibson (23) showed that, on partial flash photolysis of
HbCO, intermediate compounds were formed which reacted
more rapidly (30 times faster) with CO than did deoxy-Hb.
Using the same relatively concentrated solution of MnHb, the
photolysis flash was attenuated to give only 29% breakdown
of MnHbNO. The percentage of rapidly recombining material
rose from 13 to 30% and, on further reducing the light to give
15% breakdown (Fig. 4), the proportion of rapidly reacting
MnHb rose to 65%. The rate of the rapid component in all
these experiments was about 20 times greater than the rate
measured in stopped flow runs.

All of the experiments described in detail were performed
using an observing beam at 434 nm. Possible chain differences
in the rate of NO binding, analogous to those reported for Hb,
were sought by exploring in the region of the Soret isosbestic
point between MnHb and MnHbNO at 430 nm. The effect of
wavelength was small and seemed to derive from differences
between the rapid and slow components rather than from
differentiation within the slow component. It is not known
whether there are spectrophotometric differences between the
α- and β-manganese chains; there certainly are functional
differences in the azide Mn133Hb reaction (9), and structural
chain differences in Mn135Hb, as shown by x-ray diffraction
(8). We return to the question of chain differences below.

Dissociation of NO from NO MnHb—This very slow reac-
tion was studied by mixing MnHbNO with dithionite. The
dithionite reacts rapidly with NO in solution, thus preventing
the rebinding by MnHb of dissociated NO. At 20°C, the
reaction was closely first order with a rate constant of 0.004/
s. This rate was independent of dithionite concentration in the
range 0.1 to 1% and could be accelerated by cross-illumina-
tion of the solution. The acceleration showed reasonable
proportionality to the intensity of illumination as measured
with a photomultiplier and over a 2.3-fold range of light the
rate increased 2.5 times. As the absolute rate in the light was
15 times that with the monochromatic observing beam alone,
It seems assured that the rate of the reaction of dithionite
with NO is sufficiently great to prevent significant NO rebind-
ing by MnHb. Combining the combination and dissociation
rates is not strictly justifiable, but suggests a dissociation
constant of the order of 10⁻¹¹ M for NO.

Reactions Related to Conformation Changes in MnHb—
Taken together, the kinetic experiments on NO binding to
MnHb suggest that the reaction is cooperative. It is then
natural to ask if it is accompanied by a conformation change
as has long been believed true of hemoglobin ligand reactions
(24, 25). A number of reactions are generally regarded as
providing an index of hemoglobin conformation, and several
of these have been run in parallel experiments with NO,
MnHb, MnHb, COHb, and Hb.

(i) The reaction with bromothymol blue was introduced by
Antonini et al. (26) as an index of hemoglobin conformation,
and experiments were performed under the conditions they
describe. The results shown in Fig. 5 offer a close parallel to
the results reported for hemoglobin, the unliganded MnHb
reacting faster than the NO complex and showing a greater
total amplitude of absorbance change. The reaction of hemo-
globin with bromothymol blue is heterogeneous, with many
binding sites/molecule, and the same is true of the manganous
protein.

(ii) Reaction with p-mercuribenzoate occurs 80 to 100 times
faster with liganded than with unliganded hemoglobin. Gibson
(27) has shown that this difference probably depends upon
protein conformation. Experiments with MnHb, one of which
is summarized in Fig. 6, show that the rate of reaction of the
—SH group at β93 is numerically closely similar to that found
in a control experiment with the same reagent solution using
Hb, and a similar acceleration is observed after binding of NO
to MnHb as is observed on adding CO to Hb. Although not
FIG. 6. Reaction of p-mercuribenzoate (50 μM) with Hb (C), MnHb (E), and MnHbNO (W). Conditions: protein, 20 μM in monomer, 0.05 M P, pH 7.0, 20°C. Ordinate is log (AA, (AA, - AA(t))) at 255 nm.

shown in the figure, the speeding up of the reaction was actually rather greater with MnHbNO than with Hb.

(iii) Tetramer-dimer dissociation of hemoglobin has been studied in detail in recent years and it is now agreed that hemoglobin in the deoxy conformation is less dissociated than in the liganded state (28-30). A series of ultracentrifuge determinations using the method of Crepeau et al. (28) were kindly made for us by Dr. Richard Crepeau and Dr. Stuart Edelstein on MnHb, MnHbNO, and Mn+Hb with the results given in Table I. In interpreting these results it should be noted that the sensitivity of the optical system is such that dissociation constants of about or below 1 x 10^-5 M should be regarded as equal to, or less than, that number. Thus, although deoxyhemoglobin may give an experimental number of this order, linkage indicates that the true constant is probably of the order of 3 x 10^-13 M (31). The table shows averages of at least six determinations on three samples. The dissociation constants are, in each case, in the order Mn+ < Mn2+NO < Mn3+, and addition of inositol hexaphosphate reduces the values for Mn2+NO and Mn3+ just as for HbCO and methemoglobin (Tienfley et al. (32)).

The differences in tetramer-dimer dissociation for the derivatives found by Dr. Crepeau (Table I) point to a quaternary structure change upon ligation or oxidation of MnHb, although the evidence is qualitative rather than quantitative. If the Mn3+ globin, for example, is contaminated with 3 to 5% of MnHb which has been sufficiently denatured so that it remains dimeric, a spurious value for K4,2 will be obtained. As shown in Table I, inositol hexaphosphate has less effect on K4,2 for the Mn3+ protein than on the other derivatives. If the value of K4,2 in Table I really applied to native derivatives, contamination by a small amount of permanently dimeric MnHb.

(iv) MacQuarrie and Gibson (33) have shown that the fluorescent compound 8-hydroxy-1,3,6-pyrenetrisulfonic acid will bind differentially to deoxy- and liganded hemoglobin, probably at the site which accepts 2,3-diphosphoglycerate in deoxyhemoglobin. Attempts to demonstrate analogous effects with manganoglobin gave inconsistent success, for reasons which have not been identified. Some preparations showed 6- to 10-fold stronger binding of pyrenetrisulfonate to free manganoglobin than to the NO complex, but the difference was quite small in others. In kinetic experiments, a change in fluorescence on binding NO was observed which was abolished by adding inositol hexaphosphate to the system. There was little delay in the fluorescence increase as compared with the absorbance change. It was much less than that seen in the (CO + Hb) reaction, in experiments with 0.05 M bis-Tris buffer (pH 7), 12 μM manganese, and 5 μM pyrene sulfonate.

Rate of the T — R Transition in Unliganded MnHb—The experiments described in the previous section leave little doubt that the liganded and unliganded forms differ in structure. By analogy with the natural hemoprotein, this transition might occur slowly enough to be observed following rapid removal of NO from the Mn3+ complex by flash photolysis (34-36). Evidence for a spectrophotometric change accompanying the T-R transition was looked for by following absorbance changes near to the isosbestic point (429 nm). No isosbestic point was found, but instead, after a short lag period, there was a decrease in absorbance with a half-time of about 250 μs. This change is not associated with ligand binding because its rate is quite unaffected by changing the concentration of NO from 50 to 100 μM (Fig. 7, A and B). It does not represent the rate of removal of ligand by the flash, which, as followed at 424 nm, is represented by Curve C. This has a half-time of about 80 μs and shows no sign of the lag period at the beginning of Curves A and B. Under the conditions of Curve A, the half-time for rebinding of NO in the rapid phase of the reaction (due to dimers) is 3 ms and in the slow phase it was 70 ms; these times are outside the range of Fig. 7 altogether. The reaction followed in Curves A and B is, however, accelerated somewhat, about 2-fold, by the addition of 250 μM inositol hexaphosphate.

All of these results are compatible with the identification of the change at 429 nm as due to the R-T transition of the unliganded Mn3+ compound. The initial lag in Curves A and B arises because the R-T transition does not begin until more...
than 1 molecule of NO has been removed from a tetramer; the rate of the later part should represent the rate of the transition, about 3500/s. This is somewhat less than the rate for the corresponding transition in the iron protein (30).

Finally, when the energy input to the solution was decreased, the amplitude of the absorbance excursion following photolysis as measured at 429 nm decreased in proportion to the slow component of the recombination reaction as measured at 424 nm, not in proportion to the total amount of NO released by the flash, supporting the identification of the 429-nm change with the R-T transition.

Other Reactions of Manganoglobin—The reaction of MnHb with oxygen was examined with several preparations. The reaction was always slow, typically requiring a few seconds for half reaction of preparations made by reduction using minimal dithionite. The rate was not proportional to oxygen concentration and did not increase much on substituting oxygen-equilibrated for air-equilibrated buffers. Wavelength dependence was looked for as a means of detecting a Mn-O2 complex analogous to the Mn NO one, but none was found, consistent with results from a model Mn- porphyrin-O2 complex (37). No details of these experiments are presented because the rate of oxidation was found to be about 100 times slower ($t_{1/2} \sim$ several minutes) when the preparation of manganoglobin was passed through an anaerobic Sephadex G 50 column after reaction with dithionite. These observations suggest that oxygen interacts directly with MnHb only slowly, if at all. The more rapid oxidation when solutions of MnHb are reduced with excess dithionite and then aerated may be associated with the H2O2 produced by the dithionite reduction of O2 or with a catalytic effect of metal ions introduced with the dithionite.

The kinetics of reduction of MnHb was observed by mixing a deaerated solution of the protein with dithionite. The reaction was markedly biphasic, with about 45% of rapid phase at both 468 and 434 nm. In bis-Tris, pH 6, and with 4.8 pM Mn3+, 0.2% dithionite, 20°C, the rates of the phases were 0.095 and 0.0068/s and were little influenced by a 3-fold increase in dithionite concentration. This may be explained if it is assumed that the likeness to hemoglobin extends to the finest features of the reaction which have so far been studied. Although equilibrium studies are not yet possible technically because of the high affinity of manganoglobin for NO, there is satisfactory kinetic evidence for cooperativity in the reaction. There are four lines of evidence: (i) Partial photolysis of NO manganoglobin yields a product which recombines about 20 times faster than manganoglobin reacts with NO in stopped flow experiments. (ii) The detailed time course of NO binding shows an accelerating trend in some preparations. Both results (i.e. i and ii) may be explained by supposing that partially saturated intermediates react faster with NO than unliganded, T state manganoglobin does. (iii) Full photolysis of dilute MnHbNO solutions yields a large proportion of rapidly combining products, and the proportion is a function of MnHb concentration. This may be explained if it is assumed that the MnHbNO dissociates into dimers and that, as in hemoglobin (22), the MnHb dimer produced by photolysis is noncooperative and reacts rapidly, with a rate characteristic of the R quaternary state; quantitative comparisons with ultracentrifuge data give a value of $K_d$ for MnHbNO from kinetic experiments about half that shown in Table I. (iv) Spectrophotometric evidence for the R-T transition has been obtained from the absorbance changes observed upon rapid removal of

### DISCUSSION

The heme iron of hemoglobin can be replaced by manganese with minimal perturbations of protein structure (8), and the kinetic experiments described in this paper show that ligand binding by MnHb offers basic parallels to hemoglobin-ligand reactions: MnHb binds NO in a second order reaction without any evidence of spectrophotometric intermediates. The reversibility of the NO reaction is demonstrated by the experiments with dithionite. The dithionite reaction is closely first order, is independent of dithionite concentration over the range examined, and again proceeds without demonstrated intermediates to yield a product with the spectrum of MnHb.

In describing the NO binding reaction of manganoglobin, we have noted that the closest analogy is not with NO binding to hemoglobin, but with CO binding. This analogy may be related to studies of model compounds. Upon binding a nitrogenous base, an Mn3+ porphyrin adopts the five-coordinate geometry imposed by the heme-pocket of hemoglobin and provides a close structural analogue to a pentacoordinate Fe3+ porphyrin. For example, in Mn3+ (1-methylimidazole) (TPP), the Mn3+ lies 0.515 Å out of the mean plane, and the distance between w of the histidine and the mean plane is 2.71 Å (15); corresponding distances in Fe3+ (2-methylimidazole) (TPP) are 0.42 and 2.58 Å (16). The pentacoordinate Mn3+ porphyrin will add NO as a sixth ligand and, as mentioned above, the low spin (Mn3+ + NO) and (Fe3+ + CO) are isoelectronic and are also structurally similar (12, 13).

Detailed examination of the NO binding reaction shows that the likeness to hemoglobin extends to the finest features of the reaction which have so far been studied. Although equilibrium studies are not yet possible technically because of the high affinity of manganoglobin for NO, there is satisfactory kinetic evidence for cooperativity in the reaction. There are four lines of evidence: (i) Partial photolysis of NO manganoglobin yields a product which recombines about 20 times faster than manganoglobin reacts with NO in stopped flow experiments. (ii) The detailed time course of NO binding shows an accelerating trend in some preparations. Both results (i.e. i and ii) may be explained by supposing that partially saturated intermediates react faster with NO than unliganded, T state manganoglobin does. (iii) Full photolysis of dilute MnHbNO solutions yields a large proportion of rapidly combining products, and the proportion is a function of MnHb concentration. This may be explained if it is assumed that the MnHbNO dissociates into dimers and that, as in hemoglobin (22), the MnHb dimer produced by photolysis is noncooperative and reacts rapidly, with a rate characteristic of the R quaternary state; quantitative comparisons with ultracentrifuge data give a value of $K_d$ for MnHbNO from kinetic experiments about half that shown in Table I. (iv) Spectrophotometric evidence for the R-T transition has been obtained from the absorbance changes observed upon rapid removal of

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**FIG. 8. Reaction of MnHb (4.8 μM) with K3Fe(CN)6 (0.1 mM) 0.05 M bis-Tris·HCl, pH 6.0, 20°C. 0), observed progress curve; $\bigcirc$), rapid component obtained by subtraction of contribution from slow component.**
NO from MnHbNO by flash photolysis.

It is not appropriate to stress too strongly the numerical values reported here. The operations of preparing native globin and synthesizing manganoglobin involve somewhat severe treatment of the protein, and the final preparations are somewhat variable and may be slightly inhomogeneous. However, none of the conclusions rests upon detailed analysis of reaction records, and it is usually possible to identify artifactual contributions.

The rapid binding of NO by partially saturated MnHb intermediates is analogous to results for binding of CO by Hb, and differs from observations of NO binding to Hb. Casoely and Gibson (17) have recently shown that the successive rate constants for the binding of the 4 NO molecules to hemoglobin are statistically related, although the intermediate Hb4(NO)4 combines rapidly with CO. That is, the intrinsic rates of the reactions Hb4 + NO and Hb4(NO)3 + NO are the same, whereas the reaction Hb4(NO)3 + CO is 30 times faster than the reaction Hb4 + CO and a similar rate enhancement obtains for (MnHb)4(NO)3 + NO.

Perutz (39) has suggested that both the rates of ligand binding to the individual chains and the change in rate associated with the T → R transition are determined by steric factors which control the access of a ligand to the heme iron. The observations of Casoely and Gibson (17), however, by themselves, suggest that such factors and, indeed, the details of the structures seen by x-ray do not determine the rate of binding, and the present results are consistent with this suggestion. The R structure of manganoglobin has been shown by x-ray diffraction to closely correspond to that of hemoglobin, and all the considerations given above indicate that the same correspondence should obtain for the T state. Thus, the two different types of kinetic behavior, slow and cooperative or rapid and noncooperative, are exhibited by a single ligand, NO, interacting with equivalent structures in MnHb and Hb. This would seem to show beyond a reasonable doubt that access to the metal atom predicted from the x-ray crystallographically studied T and R forms does not provide the rate-determining step during ligation in solution. Rather, the influence of the T state structure must be exerted after initial encounter between ligand and metal, in agreement with the idea that steric features inferred from the x-ray studies of the crystals do not give a sufficient guide to accessibility of the hemes to ligands or that such features are not important contributors to the ensemble of structures occurring in solution, or both.

These considerations are open to a somewhat more detailed analysis. Austin et al. have emphasized the complex nature of the heme-ligation process (40). They report evidence for multiple successive barriers on the pathway of a ligand (L) from the solvent to the metal-ion binding site, the first probably associated in some part with desolvation of L, and the last certainly associated with formation of the Fe-L bond. Comparisons of results for CO and O2 suggest that the barrier heights are different for different ligands.

According to this model, in fluid solutions near room temperature the observed second order rate constant for ligation does not correspond to an elementary process. Rather, it is a complex function of the rates of population and depopulation of all the potential wells which define the individual barriers (Ref. 40, Equation 47). In this picture CO binding is slow relative to O2 binding because of differences at several stages in the binding process. Similarly, in T state Hb the decreased binding rate of CO and to a lesser extent of O2, could arise from a change in the properties of any or all barriers and wells. However, since our studies indicate the structure of MnHb to be indistinguishable from that of Hb, we suggest that the difference in the binding of NO by MnHb and Hb could only occur in the last step (k6), in the notation of Ref. 40, involving ligand-metal bond formation. In other words, comparison of NO binding by Hb and MnHb shows that the NO can reach the vicinity of the manganese at least ~700 times faster than it is bound. Thus the ~30 times decrease of the NO binding rate by T state MnHb as compared to the R state protein cannot be thought to arise from restricted access of NO to the metal site. The functional identity of MnHb and Hb similarly means that restricted access cannot account for the quaternary structure influences on the rates of ligation by Hb, a conclusion which is anticipated in earlier studies of fluorescence quenching by O2 (41).

The above argument requires one qualification. The Mn-NO linkage is approximately linear (13), as with Fe-CO (12), and in contrast to the bent geometry of Fe-O2 and Fe-NO (13). A linear linkage must experience close contacts with His E11 which would be lessened for the bent geometry. Thus, it is possible that enhanced steric hindrance in the T state could reduce the observed ligand on-rate by reducing the actual rate of bond formation (k6), even though the access steps leading to the metal-ligand encounter are unaffected. This suggestion is further consistent with the large influence of the T→R switch upon on-rates for ligands forming linear linkages (Fe-CO, Mn-NO) and the lesser influence O2 on-rate (Fe-O2 is bent). However, it is certainly important to keep in mind that the x-ray studies which indicate that there is insufficient room for a sterically unhindered linear linkage (43, 44) were performed on the R quaternary structure; we are unaware of a comparison of steric effects in liganded R and T forms would be able to reliably indicate any greater hindrance in the T state.

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