Allosteric Transition and Ligand Binding in Hemoglobin Chesapeake*

QUENTIN H. GIBSON AND RONALD L. NAGEL†

From the Department of Biochemistry and Molecular Biology, Cornell University, Ithaca, New York 14850, and the Department of Medicine, Division of Hematology, Albert Einstein College of Medicine, Bronx, New York 10461

SUMMARY

The human hemoglobin (Hb) mutant Chesapeake (α2ε2→ε2α2ε2) is characterized by increased oxygen affinity, a Bohr effect, and a lowered value of n in Hill’s equation in the midrange of saturation. The data presented here demonstrate that, in addition, the dissociation of oxygen from oxy Hb Chesapeake proceeds with a rate of 15 per s and that the carbon monoxide binding proceeds with a slightly accelerating time course at a rate of $3.5 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$. The reactions of bromthymol blue and p-hydroxymercuribenzoate with oxy and deoxy Hb Chesapeake demonstrate that the R = T interconversion does occur in this mutant. Finally, oxygen binding and the oxygen equilibrium curve can be satisfactorily accounted for by a Monod, Wyman, Changeaux model (in its simplest form) with a small value of L (5 to 10) and a normal value of c. These results are in agreement with the analysis of Edelstein (230.224-227) and the predictions of Shulman et al. (SHULMAN, R. G., OGAWA, S., AND HOPFIELD, J. J. (1972) Arch. Biochem. Biophys. 151, 68-74) that the functional properties of Hb Chesapeake can be explained by an early conversion from the T to the R state as ligand binding progresses.

The mutant hemoglobin Chesapeake (α2ε2→ε2α2ε2) was studied some years ago by Nagel et al. (1) who found that, as compared with hemoglobin A, it had an increased oxygen affinity, a lowered value of n in Hill’s equation, and an increased rate of combination with carbon monoxide. Since then, several other high affinity mutants and chemically modified hemoglobins have been examined (8), and several new tools for the study of hemoglobin-ligand interactions have become available. Additional experiments covering a wider range of properties of hemoglobin Chesapeake have been carried out at intervals during the last 4 years and are now reported here. The results are in good quantitative agreement with predictions made by Shulman et al. (9) using the simplest form of the allosteric model of Monod et al. (10).

EXPERIMENTAL PROCEDURE

Hb Chesapeake was obtained from blood samples provided for us as a generous gift by Dr. S. Charache of Johns Hopkins University. The mutant hemoglobin was separated from Hb A either by starch block electrophoresis, in Veronal buffer, 0.05 M, pH 8.0, or more frequently by chromatography on DEAE-cellulose. A Chromaflex glass column with two separable portions was used. The resin was DE-52 (Whatman) equilibrated and developed with Tris-HCl buffer, 0.05 M, pH 7.5. After the Hb A and A had traveled to the lower portion of the column, this was removed, and the remaining band of Hb Chesapeake, attached to the top, was eluted with the same buffer to which 0.5 M NaCl was added.

Sample preparations were by the procedure of Benesek et al. (11). Oxygen equilibrium determinations were performed as described elsewhere (12). Stopped flow determinations were made using a Durrum-Gibson apparatus equipped for data collection as described by DeSa and Gibson (13). The methodology of CO replacement reaction with attention to chain contribution, oxygen pulse experiments, and the reactivity of Hb toward p-hydroxymercuribenzoate and bromthymol blue has been described elsewhere (14-10).

Concentrations in terms of heme were determined spectrophotometrically.

Inositol hexaphosphate and bis-tris were obtained from Sigma and Aldrich, respectively. The Manox brand of sodium dithionite was a gift from Holdman and Harden, Miles Plating, Manchester, England.

RESULTS

Oxygen Binding—The reaction between Hb Chesapeake and O2 was followed by stopped flow at 460 nm (Fig. 1). Oxygen concentrations were varied between 8 µM and 64 µM (Fig. 1).

Curves that fitted the experimental points satisfactorily were computed with $k_1 = 18.5 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, $k_1 = 50/s$, $k'_a = 33 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$, and $k_a = 60/s$. Other rate constants were related statistically to $k'_a$ and $k_a$ for combination and dissociation velocities, respectively.

The reaction between hemoglobin and ligands is described in terms of the equation: $Hb X_{n-1} + X \xrightarrow{k_a} Hb X_n$ ($n = 1, 4$), where $X$ is the ligand; $k$ is used for reactions with oxygen and $l$ for reactions with carbon monoxide. The equilibrium constants are given by $K_a = k'_a/k_a$, i.e. they are association constants.
FIG. 1. Reaction of oxygen with deoxyhemoglobin Chesapeake. Concentration 38.5 pM in heme after mixing, in 0.1 M phosphate buffer, pH 7.0, 19°, 460 nm. Oxygen concentration adjacent to curves.

FIG. 2. Replacement of oxygen by carbon monoxide. The ordinate is the reciprocal of the observed initial rate of the replacement reaction (5); the abscissa is oxygen concentration (mM). The concentration of hemoglobin was 30 pM, and the reaction was followed at 560 nm, in 0.05 M phosphate buffer, pH 7.0, at 20° using 0.46 mM CO as the replacing reagent. The points are experimental. The lines marked α and β show the behavior expected of individual chains as calculated from the results of analyzing the over-all time course of the replacement reaction as described by Olson and Gibson (14) using $k_\alpha = 8.3 \pm 0.1$ per s, $k_\beta = 21 \pm 0.4$ per s, $t_1/k_\alpha = 0.2 \pm 0.03$ per s, $t_2/k_\alpha = 0.18 \pm 0.05$ per s; the root mean square residual was 0.00056 in absorbance. The symbol △ shows the reciprocal of the rate of the deoxygenation of Chesapeake by dithionite. The symbol △ shows the corresponding value for Hb A.

Oxygen Dissociation from Liganded Hemoglobin Measured by Displacement with Carbon Monoxide—The reaction was measured and the results were analyzed as described by Olson and Gibson (14) in experiments which also included measurements at 570, 572, and 578 nm (Fig. 2). The results showed a close similarity to those obtained with hemoglobin A both in absolute rates and in the relative rates observed at the three wavelengths. There appears to be a significant effect of the α chain substitution on the dissociation of oxygen from liganded hemoglobin Chesapeake, where the rate is about two-thirds of that observed for hemoglobin A.

Carbon Monoxide Binding—Fig. 3 shows the result for the binding of CO by Hb Chesapeake followed at 432 nm. Gross biphasicity is observed at low ionic strength. The fast component has a rate constant of $3.4 \times 10^6$ M$^{-1}$ s$^{-1}$ and the slow component 3.6 $\times 10^5$ M$^{-1}$ s$^{-1}$. The reaction between Hb Chesapeake and CO in the presence of inorganic phosphates proceeds with a slightly accelerating time course and a rate constant of about $3.5 \times 10^5$ M$^{-1}$ s$^{-1}$. This value is in excellent agreement with the calculated value of $4 \times 10^5$ M$^{-1}$ s$^{-1}$ derived by Shulman et al. (9), assuming a simple allosteric model for Hb Chesapeake.

Dissociation of Oxygen from Oxyhemoglobin in Presence of Dithionite—This reaction has not been studied over a range of conditions. At pH 7 in 0.05 M phosphate buffer the reaction was closely exponential, after removal of oxygen from physical solution as determined from experiments with solutions equilibrated with widely different partial pressures of oxygen. The rate was 15 per s, in close agreement with the calculated value of 13 per s derived from the proposal of Shulman et al. (9). Although the replacement reaction for Hb Chesapeake is quite similar to that of Hb A, the reaction with dithionite differs widely. The rate for Hb A is about 35 s$^{-1}$ under the conditions of the experiment shown in Fig. 2. The rate for Hb Chesapeake is much lower and does not differ greatly from the initial rate for the replacement reaction. The difference between the rates of
FIG. 4. Reaction of Hb Chesapeake with p-hydroxymercuribenzoate. The reaction was followed at 255 nm, with 15 μM Hb Chesapeake in 0.05 M phosphate buffer, pH 7.0, and 25 μM p-hydroxymercuribenzoate (before mixing) at 20°C. ●, COHb Chesapeake; ○, deoxy Hb Chesapeake.

The dithionite and replacement reactions reflects the differences in cooperativity between Hb A and Hb Chesapeake.

**Oxygen Pulse Experiments**—These were carried out in 0.05 M phosphate buffer, pH 7, with and without 100 μM inositol hexaphosphate. In phosphate alone, the rate of deoxygenation remained constant at 15 per s until the peak saturation had been reduced (by lowering the oxygen concentration) to 20%. At a peak saturation of 10% a rapid phase of deoxygenation appeared, accounting for about one-quarter of the reaction. The rate of the rapid phase was about 50 per s. For hemoglobin A under similar conditions, the amplitude and rate of the rapid phase were 60% and 1100 per s, respectively.

In the presence of inositol hexaphosphate there was an increase in the proportion of rapidly reacting hemoglobin to 20% at a peak saturation of 25%, and to 35% at a peak saturation of 13%. The rate was about 300 per s or one-quarter of the rate seen with hemoglobin A. The rate of the slow component was increased from 15 to 22 per s. This effect, too, is much less than that observed with hemoglobin A.

Wavelength dependence in the region of the isosbestic point at 585 nm was sought. Definite effects were observed only in the presence of inositol hexaphosphate: the relative proportion of the slower reaction was greater at wavelengths to the longer wavelength side of the isosbestic point, as with hemoglobin A.

**Reaction with p-Hydroxymercuribenzoate and Bromthymol Blue**—Hemoglobin Chesapeake in the oxy and deoxy state was reacted with two reagents believed to be sensitive to the structural differences between T and R conformers.

The reaction between p-hydroxymercuribenzoate (25 μM) and CO hemoglobin Chesapeake (15 μM) is very fast with an initial rate of about 100 per s (Fig. 4). The reaction of deoxyhemoglobin Chesapeake with the same ratio of reactants was considerably slower with an initial rate of about 10 per s. The reaction of carboxyhemoglobin Chesapeake with p-hydroxymercuribenzoate is comparable to the results obtained by Antonini and Brunori with Hb A (15), but the rate of reaction of deoxyhemoglobin is appreciably more rapid. Bromthymol blue also distinguishes between the liganded and unliganded state of Hb Chesapeake (Fig. 5). The reaction between a 20 μM solution of bromthymol blue and 60 μM deoxy Hb Chesapeake at pH 7.0, followed at 620 nm, is much faster than the same reaction with CO Hb Chesapeake. This again in general agreement with the results for Hb A of Antonini et al. (16).

**Oxygen Equilibrium**—The oxygen equilibrium of Hb Chesapeake is shown in Fig. 6. The experimental points do not lie upon a straight line in a Hill plot. A similar result was actually reported in Fig. 1 of Nagel et al. (1) (but was not commented upon at the time). As shown there and in Fig. 6 of the present paper, the points below 50% saturation have a steeper slope than the rest of the curve.

The experimental points shown in Fig. 6 can be fitted satisfactorily by a curve computed on the assumption that there is a statistical relation between the equilibrium constants for the
second, third, and fourth binding sites. The best fitting values for $k_1$ and $k_4$ are given in the corresponding legend.

**DISCUSSION**

In contrast with hemoglobin A, it seems that the behavior of hemoglobin Chesapeake in its reactions with oxygen and carbon monoxide can be accounted for by a relatively simple scheme, applying the allosteric model of Monod et al. (10) in its original form without taking into account chain differences. This conclusion has been reached not by assuming the model, but as a result of analysis in terms of arbitrary kinetic and equilibrium parameters of the Adair scheme which were found to lead naturally toward it.

In the case of oxygen the starting point was the observation that the family of combination progress curves of Fig. 1 could be quite well represented by neglecting altogether the reverse (dissociation) reactions, and treating the results as simple second order one step reactions. The implication is that none of the oxygen dissociation velocity rates can be large. This conclusion was supported directly by the results of oxygen pulse experiments, which showed much smaller rates of dissociation of oxygen from partly saturated intermediates than is true of hemoglobin A (20-fold slower), and by the time course of oxygen dissociation on mixing oxyhemoglobin Chesapeake with dithionite and dithionite plus CO, which gave similar rates, again in contrast to hemoglobin A.

A more realistic representation requires inclusion of the dissociation velocity for oxygen, and that the presence of 4 hemes be taken into account. The value of $k_4$ was obtained from a photolysis experiment with carbon monoxide and the ratio $k_1/k_4$ and $k_4$ from the rate of replacement of oxygen by carbon monoxide. It was then calculated that $k_4$ is $3.3 \times 10^4 \text{m}^{-1} \text{s}^{-1}$. The crude assumption were made that chain differences may be neglected, and that kinetic behavior changes abruptly from deoxy-like to liganded after a definite number of ligand molecules have been bound. An excellent fit was obtained (Fig. 1) when the change in behavior was assumed to follow binding of the first oxygen molecule. The corresponding oxygen equilibrium curve at first appeared to offer difficulty, since the $n$ value reported for Chesapeake by Nagel et al. (1) was only 1.3, and the calculated $n$ value approaches 2 below 50% saturation. It was noticed, however, that the few data points of Nagel et al. (1) at low saturations tended to deviate from a straight line. Reinvestigation showed that this deviation was real (Fig. 6) and in good agreement with expectation.

For carbon monoxide binding in phosphate buffer, pH 7, agreement was also obtained with the same assumption of a change from deoxy to liganded behavior after binding of 1 molecule of CO, fitting the observed progress curve with only one disposable constant ($'p'$), just as for the oxygen case where only $k'_1$ was varied.

These rate and equilibrium constants are equivalent to those developed from the model of Monod et al. with a small value of $L$ (perhaps 5 to 10) and a normal value of $e$. The kinetic behavior can be described in general terms of the two-state model just as suggested by Shulman et al. (7). (There is full agreement between the values observed here for CO binding and the dithionite reaction and the values calculated by these authors.) The result is in excellent accord also with the x-ray crystallographic work of Greer (17), who found an essentially normal deoxy structure and many minor changes scattered throughout both chains in the liganded form. These changes were attributed to quaternary rather than tertiary structure, and would not be expected to show up in the function of the unconstrained R state. The results do not, however, accord with the model of Ogata and McConnell (18) which calls for large chain differences in affinity in the T state; it should be noted, however, that our experiments and theirs were performed under different conditions.

The foregoing account applies to experiments using neutral phosphate buffers. In buffers of low ionic strength, carbon monoxide binding becomes biphasic with large fractions of the reaction occurring 10 or more times faster than in phosphate buffer. The same result, which implies the coexistence of two deoxy forms not in rapid equilibrium, has been observed with several high affinity mutant and chemically modified hemoglobins (e.g. Olson and Gibson (14)). There is no wavelength dependence of the rapid phase in the region of the isosbestic point, and it cannot be associated with a specific type of chain.

The behavior of Chesapeake can be further modified by addition of inositol hexaphosphate, which apparently delays the allosteric transition until more than 1 molecule of ligand has bound. Chain differences then appear in oxygen pulse experiments, and rates of dissociation of oxygen from $\beta$ chains of several hundred per second can be observed. Its behavior thus becomes more like that of hemoglobin A (19).

The generally simple interpretation applied above agrees qualitatively with the results of experiments on binding of $p$-mercuribenzoate, bromthymol blue, and haptoglobin, all of which show the occurrence of an R-T transition; the experiments with haptoglobin and deoxy Hb Chesapeake show that L is not large in phosphate buffers.

The allosteric model also provides a satisfactory qualitative explanation for the observation of Nagel and Gibson (20) that both liganded and deoxyhemoglobins Chesapeake bind to haptoglobin.

In conclusion, the functional properties of Hb Chesapeake can be well accounted for by an early conversion from the T to the R state as ligand binding progresses. The transition is advanced by high pH and low ionic strength and retarded by the presence of organic phosphates.

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