The Reaction of Oxygen with Hemoglobin and the Kinetic Basis of the Effect of Salt on Binding of Oxygen*

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SUMMARY

The reaction of hemoglobin with oxygen has been studied by stopped flow methods, and, under suitably restricted conditions, it can be adequately represented by a system of four consecutive reversible reactions. The numerical values given to the eight rate constants permit a satisfactory fit to combination and dissociation velocity data, and yield an equilibrium curve of the appropriate form. The distribution of rates among the various steps in the reaction requires that cooperativity in oxygen binding be attributed primarily to deviations of the successive dissociation velocity constants from their statistical values, and is consistent with the idea that the major change in reactivity occurs after 1 ligand molecule has dissociated from saturated hemoglobin. The difference in affinity between hemoglobin in phosphate buffers and hemoglobin freed from salts is due to reduction in the rate of dissociation of the 2nd, 3rd, and 4th molecules leaving oxyhemoglobin. The rate of dissociation of the 1st molecule from saturated hemoglobin is not changed.

About 12 years ago Gibson and Roughton (1) showed that the reaction between carbon monoxide and sheep deoxyhemoglobin could be satisfactorily described, within the limits of experimental error, by a simple four-step consecutive reaction scheme, and suggested possible sets of values for the rate constants involved. Attempts were then made to collect and analyze similar data for the reaction of deoxyhemoglobin with oxygen, and to correlate kinetic and equilibrium findings on the same samples of hemoglobin, but these experiments were much less successful, and only the equilibrium results have been published in detail by Roughton (2).

One major difficulty, briefly mentioned in a review article (3), was that equilibrium was approached in two phases, the slower of which depended on protein concentration rather than on ligand concentration, and so could not be accommodated within a scheme which did not contain protein concentration terms. Further, the properties of the subunits which might arise from dissociation of hemoglobin were unknown, as was the relative degree of dissociation of free and ligand-bound hemoglobins. A second difficulty was in analysis of the results which called for computations much too extensive to be possible by the methods then available, and so made it impossible to know if failure to reconcile experimental results with simple models was due to real inadequacy of the models or to insufficient arithmetical trials. In the meantime, great advances in all these points have taken place, and it has now been possible to show that the binding of oxygen by hemoglobin can be described in terms of four simple reversible reactions, provided that the measurements are made and analyzed under carefully controlled conditions.

EXPERIMENTAL PROCEDURE

**Human Hemoglobin**—This was prepared from freshly drawn blood taken during the course of routine examination of students. The plasma was removed by washing with 1% NaCl, and the erythrocytes were hemolyzed by adding 6 volumes of water. The stroma was removed by centrifugation. Hemoglobin freed from 2,3-diphosphoglycerate was prepared by the method of Benesch, Benesch, and Yu (4). Deoxyhemoglobin solutions were prepared by repeated evacuation and flushing with pure N₂, using the strong stock hemoglobin solution (about 3 mM). Suitable dilutions were then made with buffers deoxygenated by bubbling with pure N₂.

**Sheep Hemoglobin**—This was prepared from blood supplied by the Cornell slaughterhouse. The electrophoretic type (fast was used in these experiments) was determined from the rate of dissociation of oxygen from saturated hemoglobin.

**Kinetic Measurements**—These were made using the apparatus of Gibson and Milnes (5) with some modifications. The observing beam was obtained using a 150-Watt quartz iodine tungsten lamp (General Electric type 1958) supplied with direct current from a highly regulated constant-current power supply constructed in the laboratory. The photomultiplier output was applied to an operational amplifier whose output was digitized with an analogue-to-digital converter (Digital Equipment Corporation, Maynard, Massachusetts, model 130E) and transferred to a PDP 8/I computer (Digital Equipment Corporation) equipped with a 1-MHz quartz crystal clock (model KW8/I) under control of a program similar to that described by DeSa and Gibson (6).

In addition to performing the kinetic determinations with various concentrations of oxygen, control runs were made with nitrogen-bubbled buffer before and after the kinetic runs proper, as well as runs in which an air-equilibrated buffer was mixed with air-equilibrated hemoglobin solution. These runs served to fix the absorbance change corresponding to the range 0 to 100% saturation with oxygen and also allowed the saturation
it can be carried out with monochromator and other settings. The action is quite closely second order, and has the advantage that separation by 6.5 msec, on Curve C, D. At the end of a kinetic run to be estimated approximately (within ±1.5%). In the kinetic runs, the apparatus dead time was defined as the interval between closing of a switch on the stopped flow apparatus and the collection of the first data sample by the computer. This time was measured by a control experiment, carried out immediately after the hemoglobin experiments, in which myoglobin was allowed to combine with CO. This reaction is quite closely second order, and has the advantage that it can be carried out with monochromator and other settings identical with those used in the oxygen-deoxyhemoglobin experiments. The actual data points collected in each experimental run were taken from the computer and displayed using a storage oscilloscope to provide a visual check on the functioning of the equipment.

**Data Analysis**—This was performed using the PDP 8/I computer to drive a TR48 analogue computer (Electronic Associates Inc., Long Branch, New Jersey) through an interface constructed in the laboratory. The program used the method of steepest descents incorporating Marquardt’s (7) modification to find the best-fitting set of kinetic constants. The program ran continuously, selecting a new set of starting points for the parameters varied with a pseudo-random number generator, after each minimum had been located. It was found that, with the families of kinetic curves available, many local minima existed, but one solution (or group of closely related solutions) was usually clearly better than the others. The program was arranged to simulate the experimental procedure as closely as possible, the comparisons between the observed points and the computed values being instituted after the computer had run for a period corresponding to the dead time. In this way an extrapolation of experimental data to zero time was required, and the only operation performed on the data before beginning computation was to convert the changes in absorbance to changes in oxyhemoglobin concentration, as measured from the first observed point. Trials with model data generated by digital computation showed that the program was able to fit the data satisfactorily with the correct values of the parameters. As with real data, multiple minima were found, although the known parameter values gave a better fit. Because of the occurrence of multiple minima at least 50 complete cycles of the curve-fitting program were run with each set of experimental data.

**RESULTS**

A family of curves showing the time course of oxygen binding by deoxyhemoglobin is given in Fig. 1. These results were compared with the simplest set of four reversible reactions

\[
Hb_4 (O_2)_{k-1} + O_2 \xrightarrow{k_n} Hb_4 (O_2)_n, \quad n = 1 \text{ to } 4
\]  

using the fixed value for \( k_1 \) of 50 per sec required by measurements of the rate of the replacement reaction

\[
Hb_4 (O_2)_{k-1}(CO)_{k-1} \xrightarrow{k_1} Hb_4 (O_2)+(CO)_{k-1}
\]  

as followed by mixing oxyhemoglobin with a solution of 0.2% sodium dithionite saturated with CO, and the value of \( 3.3 \times 10^9 \text{ M}^{-1} \text{sec}^{-1} \) for \( k_1 \) obtained as described by Gibson (3). The remaining six velocity constants were varied freely, usually within the range 0 to 2000 per sec for the dissociation velocity constants and 0 to \( 1.6 \times 10^8 \text{ M}^{-1} \text{sec}^{-1} \) for the combination velocity constants, the limits in any particular experiment depending on the scaling of the analogue computer. Kinetic results alone proved insufficient to define a unique set of six kinetic constants in the system, and they were therefore supplemented by equilibrium data. Accurate equilibrium curves determined by gasometric methods are not available for human hemoglobin under the conditions of the kinetic experiments. The pressure of oxygen required for half-saturation was therefore determined in the laboratory for the solutions used, and the assumption was made that the precise shape of the equilibrium curve was the same as that found by Roughton and Lyster (8) for human hemoglobin at the same pH but with higher concentrations of both hemoglobin and phosphate buffer. The affinity of the dilute solutions was such that the oxygen tension required for half-saturation was within 1 mm of mercury of that reported by Roughton and Lyster. With the added requirement that the equilibrium curve should also be fitted, the computer output of sets of six kinetic constants was drastically reduced, most sets of starting values failing to lead to an acceptable solution, and only a single satisfactory set of rate constants was obtained. Although about 20 kinetic experiments have been performed so far, only three are directly comparable with the experiment shown in Fig. 1, some having been performed with sheep hemoglobin, and others with human hemoglobin at different tempera-
FIG. 2. A, comparison of the oxygen equilibrium curve of human hemoglobin as observed by Roughton and Lyster (8) (points) and as calculated from the kinetic constants given in the legend to Fig. 1 (line). B, comparison of the oxygen equilibrium curve of stripped hemoglobin examined in 0.05 M 2-bis(hydroxy-methyl)-2,2',2''-nitrilotriethanol-Tris buffer, pH 7.0, as observed in spectrophotometric titrations (points) and as calculated from the kinetic constants given in the legend to Fig. 1 (line).

FIG. 3. The reaction of stripped human deoxyhemoglobin (53 μM) with residual phosphate equivalent to 4% of binding sites for 2,3-diphosphoglycerate with: A, 62 μM O₂; B, 31 μM O₂; C, 15.5 μM O₂; D, 7.8 μM O₂. The reaction was followed in a 2-mm cuvette at 435 nm at 20° in 0.05 M bis-Tris buffer, pH 7.0. The ordinate is percentage of saturation with oxygen, the abscissa, time in milliseconds. The points are observed, the lines were calculated using the scheme of Equation 1 with the values: $k_1 = 14.7$, $k'_1 = 35.2$, $k_2 = 15.8$, $k'_2 = 35$ per μM sec and, $k_1 = 150$, $k_2 = 15.7$, $k_3 = 138$, $k_4 = 50$ per sec.

Effect of Ionic Strength—It has been known for some time that polyphosphates bind to hemoglobin, and the effect of 2,3-diphosphoglycerate has been recently and clearly defined by Benesch, Benesch, and Yu (9). Although the effects of removing phosphates on oxygen affinity are large under suitable conditions, no satisfactory kinetic explanation for them has so far been given, Gibson and Parkhurst (10) noting only a moderate increase in the rate of CO binding. Analysis of a family of curves for oxygen binding by hemoglobin freed from phosphate, together with spectrophotometric data for oxygen equilibrium on the same sample, gave the results of Fig. 3 and Line D in Fig. 2. An excellent fit was obtained with the values given in the legend to Fig. 3, the root mean square residual being only ±0.56% in saturation for the combined results. The value of $k_4$ derived from the replacement reaction was 50 per sec just as for untreated hemoglobin, but the rate of deoxygenation in the presence of dithionite was 19 per sec as compared with the 40 per sec observed when the stripped hemoglobin was made up in 0.1 M phosphate buffer, pH 7, instead of in 0.05 M 2-bis(hydroxy-methyl)-2,2',2''-nitrilotriethanol-Tris buffer at the same pH. The rate of the deoxygenation reaction calculated from the rate constants in the legend to Fig. 3 was 18 per sec, in good agreement with experiment. Comparison of the rate constants for stripped and unstripped hemoglobin shows that the greatest differences are in the values of the dissociation velocity constants other than $k_4$ which are substantially smaller for stripped hemoglobin. The net effect of all the changes is to give a substantially higher affinity for oxygen while retaining much of the cooperativity in oxygen binding.

DISCUSSION

The rate constants given in the legend to Fig. 1 are able to account with good precision (better than 1% saturation) for the kinetic and equilibrium behavior of human hemoglobin in solutions strong enough so that dimerization is not significant, and the general kinetic behavior during oxygen binding is similar to that reported in the literature (e.g. Hartridge and Roughton (11) and Berger et al. (12)). There are two points,
however, which cannot be fitted into the simple framework of Equation 1. The first concerns the equilibrium curve in the range 0 to 2% saturation. Observations by Roughton and Lyster (8) give a substantially higher value for the first equilibrium constant $k_1 = k'/k$, than that derived from the kinetic experiments. They reported a value of 0.049 per mm of pO$_2$, as compared with the value of 0.017 per mm per pO$_2$ derived from the kinetic experiments. A possible, although purely speculative explanation is that there is normally a small fraction of hemoglobin present with much higher affinity than the bulk of the pigment. This would greatly influence the bottom of the dissociation curve, but would be virtually without effect on the kinetic observations. A second discrepancy is with a detail of the results of Berger et al. (12) who reported that the apparent second order velocity constant for the binding of oxygen by deoxyhemoglobin increased during the course of the reaction. The stopped flow apparatus used in the present experiments does not permit observations to be made so soon after flow stopping (0.3 msec), as does that of Berger et al., so that the point could not be examined directly. The rate constants reported here would give a time course of oxygen binding during the 1st msec after mixing which would be appreciably faster than reported by Berger et al.

The finding that the experimental results can be fitted by the simple scheme of Equation 1 does not require that that scheme be accepted as a model of the reaction. The values of the rate constants may be used in rough comparisons with the values predicted by some models, although such comparisons are far from rigorous. Thus, the numbers obtained do not appear at all like those expected for the model of Monod, Wyman, and Changeux (13), which predicts a monotonic change in rate constants as the combination and dissociation reactions proceed. They seem in better agreement with Antonini (14) who has developed the idea that much of the cooperativity of ligand binding by hemoglobin derives from interactions between pairs of hemes. It is, of course, clear that the tetramer must be the basic functional unit in hemoglobin, if only because 12 in Hill’s hemes. It is, of course, clear that the tetramer must be the basic functional unit in hemoglobin, if only because 12 in Hill’s equation is near 3, and in keeping with this it proved impossible to obtain a good fit to the data reported here with a dimer model even if the rates of the replacement reaction (Equation 2) and of deoxygenation by dithionite were totally disregarded and all four kinetic constants allowed to vary freely.

Examination of the rate constants for oxygen dissociation from hemoglobin in phosphate buffer shows that the observed over-all rate is severely limited by the rate of dissociation of the 1st oxygen molecule from saturated hemoglobin ($k_4$) which is much smaller than the other three constants, especially when statistical weights are applied. In stripped hemoglobin examined in 2-bis(hydroxymethyl)-2,2',2''-nitrotrierethanol-Tris buffer, the position is much different. The first off constant ($k_4$) is the same, but the other three are much smaller than in phosphate. It is this difference which provides the kinetic explanation of the observed difference in affinity between stripped and unstripped hemoglobins.

It is also interesting to compare the combination velocity constants for oxygen with those for carbon monoxide. All the rates for oxygen are substantially greater, but the rate constants for the binding of the first 3 oxygen molecules are in the same proportions as those suggested for the binding of the first 3 molecules of carbon monoxide. The rates for the binding of the 4th molecule are in quite different proportions, however, the 4th molecule of carbon monoxide binding much more rapidly, relatively, than the 4th molecule of oxygen. It is reasonable to suppose that the changes in the protein are much the same for both ligands but that there is more opportunity for the expression of a large effect after 3 molecules of carbon monoxide have bound because the rates are slower than for oxygen, where the rates for binding of the first 3 ligand molecules may already be approaching an upper limit set by diffusion. Whatever the reason for the difference, the results suggest a need for caution in using results with carbon monoxide to predict the behavior of hemoglobin with oxygen and vice versa.

Finally, it should be stressed that although considerable, and it might perhaps be said unusual, effort has been expended in trying to locate sets of rate constants able to fit the results, there is not, and cannot be, complete assurance that the values given here are truly unique. They are also derived from a scheme which must represent an oversimplification, and at best must arise by the superposition of kinetic constants corresponding to many more intermediates than the five considered here.

REFERENCES

The Reaction of Oxygen with Hemoglobin and the Kinetic Basis of the Effect of Salt on Binding of Oxygen
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