Proc. Nat. Acad. Sci. USA
Vol. 70, No. 1, pp. 1–4, January 1973

The Contribution of the α and β Chains to the Kinetics of Oxygen Binding to and Dissociation from Hemoglobin

(heme proteins/ligand binding/protein chemistry)

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Communicated by Leon A. Heppel, October 20, 1972

ABSTRACT **A new type of experiment in which hemoglobin is exposed briefly to oxygen has shown that the half-time of dissociation of oxygen from some partly oxygenated intermediates is about 1 msec at 20° and 10 msec at 2°. The rapid dissociation occurs selectively from one type of chain, provisionally identified as the β-chain. Chains that show the rapid rate of dissociation of oxygen also bind rapidly. It follows that the kinetic equivalent of the Adair equation and the Monod–Wyman–Changeux model are quite unsuited to represent the kinetics of the oxygen–hemoglobin reaction. The reaction of oxygen with hemoglobin closely resembles that of the alkyl isocyanides and differs radically from that of carbon monoxide.**

When the velocity of dissociation of the first oxygen molecule from sheep oxyhemoglobin was measured by Gibson and Roughton (1), and compared with the overall rate of oxygen dissociation in the presence of dithionite, it became clear that the rate of dissociation of oxygen from partly saturated intermediates must be greater than the rate of dissociation from fully liganded hemoglobin. Attempts to fit the time course of the oxygen-binding reaction to an elementary kinetic scheme equivalent to the Adair equation (3), as set out in Eq. **1**, have suggested that the ratio of the rates might be of the order of 100:1 (4), but such estimates are subject to the major uncertainties associated with curve-fitting methods.

$$Hb_4(O_2)_{n-1} + O_2 \underset{k_n}{\overset{k_n'}{\rightleftharpoons}} Hb_4(O_2)_n,\ n = 1,4. \qquad [1]$$

This paper gives a preliminary account of results with a new type of experiment, which will be called the oxygen-pulse technique, that allows the dissociation of oxygen from intermediates to be observed directly. It has been found for human hemoglobin that the ratio of the rates of dissociation of the first and fourth molecules of oxygen is about 1:60. This ratio, however, applies only to one type of chain, tentatively identified as the β-chain. Corresponding figures for the α-chains are not yet available, but the ratio for them must be much smaller. The discovery of a substantial difference between the chains came as a surprise, since although large differences between chains have been demonstrated for the bulky ligand *n*-butyl isocyanide (5), it has been rather generally believed that there is little functional difference between them in their reactions with oxygen. In the only individual reaction so far investigated, the ratio k_4^α/k_4^β was only about 1:2 (6). Investigation of the oxygen–deoxyhemoglobin reaction under chosen conditions has now shown very significant differences between the chains. These differences are great enough to make the use of Eq. **1** in representing oxygen–hemoglobin kinetics physically meaningless. Similar considerations apply, of course, to special forms of Eq. **1** such as the Monod–Wyman–Changeux model, recently applied by Hopfield *et al.* (7) to interpret a wide range of kinetic experiments with hemoglobin.

MATERIALS AND METHODS

Hemoglobin was prepared as described (4) and stripped before use (8). The stopped-flow apparatus and data collection system were as described by DeSa and Gibson (9). Gases and other materials were obtained from the sources specified in (4).

RESULTS

Oxygen dissociation from intermediates

Oxygen pulse experiments were performed by mixing deoxyhemoglobin solutions containing dithionite with solutions of oxygen in the stopped-flow apparatus. On mixing, oxygen begins to disappear from the solution by reaction with dithionite in a zero-order reaction (2) and at the same time reacts with deoxyhemoglobin. The oxygen binding is quickly terminated by the disappearance of free oxygen from the solution, the time required depending on the concentrations of oxygen and of dithionite (typically 1–10 msec.). In these circumstances the population of partially oxygenated intermediates formed, from which oxygen subsequently dissociates, is determined primarily by the kinetic constants for binding the first one or two molecules of oxygen. Such populations differ widely from those established at equilibrium, where, as a result of cooperativity, the species Hb_4 and $Hb_4(O_2)_4$ are heavily represented. The kinetically determined populations, thus, permit the behavior of the partially oxygenated intermediates to be examined. The results of an experiment performed at 2° in 0.05 M phosphate buffer, pH 7.0, are shown plotted in semi-logarithmic form in Fig. 1. The rapid binding reaction, much of which occurs in the dead time of the stopped-flow apparatus, is followed by a deoxygenation reaction, which consists of two clearly separated phases differing in rate by about 20-fold. The relative amplitude of these phases is a function of the peak saturation reached, the proportion of rapid reaction increasing as the peak saturation declines, and approaching 100% below a peak saturation of 0.1. The reaction can be satisfactorily represented by two rates only, of about 100/sec and 6/sec.

The two distinct phases in Fig. 1 might represent the effects of heme–heme interaction upon functionally similar hemes, as

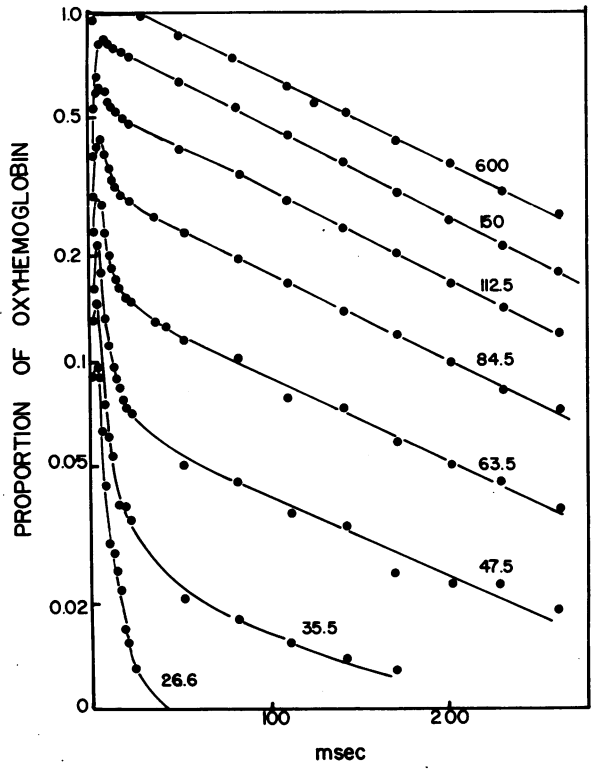

FIG. 1. The time course of the absorbance change at 438 nm in an oxygen pulse experiment performed at 2°. The concentration of hemoglobin was 17 μM (heme basis) after mixing. Hemoglobin was dissolved in 0.05 M phosphate buffer, pH 7.0, containing 0.1% sodium dithionite (before mixing). This solution was mixed with buffers containing oxygen to give the concentrations of oxygen (μM) shown opposite each curve (after mixing).

assumed in the Adair equation and in the Monod–Wyman–Changeux model, or they might be due to interactive effects superimposed upon intrinsically different α and β chains. This point has been investigated by examination of the wavelength dependence of the time course of the deoxygenation reaction. In the neighborhood of suitable isosbestic points there is a well-marked dependence of the apparent rate of deoxygenation upon wavelength; an example is given in Fig. 2. It follows that the biphasic behavior shown in Fig. 1 arises in a large measure from the presence of two chemically distinct species having widely separated rates of oxygen dissociation. By analogy with *n*-butyl isocyanide (5), the more rapid rates may be assigned to the β chains. An additional check on this assignment is possible by use of the effects of inositol hexaphosphate. As shown by Gray and Gibson (10), this compound increases the rate of oxygen dissociation from the β chains, which can be identified spectrophotometrically as shown by Olson *et al.* (6). When inositol hexaphosphate is added to oxyhemoglobin and the overall deoxygenation reaction in the presence of dithionite is examined, a biphasic reaction is observed with a wavelength dependence similar to that exemplified in Fig. 2.

Effect of pH, temperature, and anions on oxygen dissociation from intermediates

Oxygen pulse experiments have been performed under conditions other than those of Fig. 1. At 20° and in 0.05 M phosphate, pH 7.0, the general pattern of Fig. 1 is maintained, but the proportion of the rapid phase is less at any given peak saturation. The rate of the rapid phase is about 1100/sec. The results at pH 6 are closely similar to those obtained at pH 7. In 0.05 M borate buffer, pH 9.0, the proportion of the rapid phase is smaller than at pH 7, and the rate drops to about 500/sec. In 0.05 M Bis-Tris buffer, pH 6 and pH 7, the amplitude of the rapid phase is similar to that seen in borate pH 9, but the rate is about 1000/sec. The addition of 50 μM inositol hexaphosphate greatly increases the amplitude of the rapid phase at pH 6 and pH 7 when added either to Bis-Tris or phosphate buffers. There may be a small increase in the rapid rate also.

Oxygen-binding experiments

In view of the oxygen dissociation behavior just described, wavelength dependence was looked for in the oxygen-binding reaction. The results obtained in the region of the isosbestic point at 585 nm are illustrated in Fig. 3. It is clear that the time course of the absorbance change is a function of the observed wavelength, with fast and slow components disposed as in the case of the oxygen pulse experiments. An approximate analysis of a series of experiments performed with different concentrations of oxygen is presented in Fig. 4, which gives the rates obtained if it is assumed that the time course of the absorbance change may be represented by the sum of two exponentials. The results suggest a rate of about 2.5×10^6 $M^{-1}sec^{-1}$ for binding to the fast component (β-chains), and a dissociation rate of about 100/sec, similar to that observed directly in the oxygen-pulse experiments. The slow component has a nonlinear dependence on oxygen concentration, and a much lower dissociation velocity. The results shown in Fig. 4 exactly parallel those reported by Olson and Gibson (5) for *n*-butyl isocyanide.

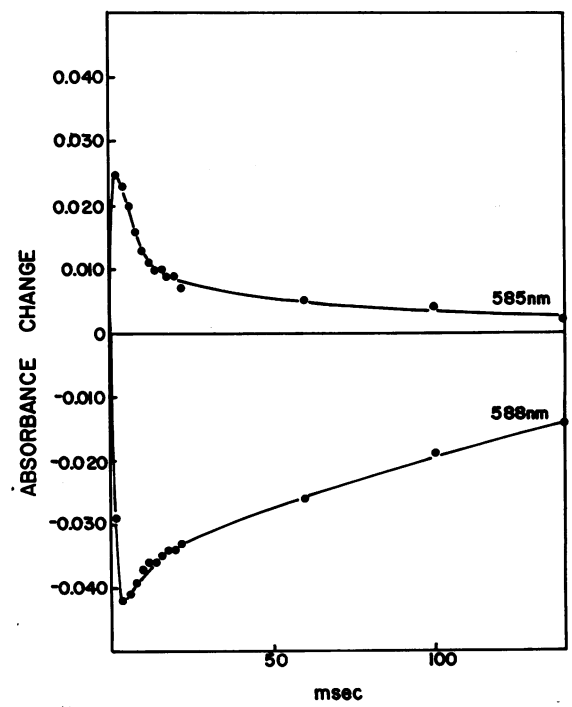

FIG. 2. The time course of the absorbance changes at 585 and 588 nm in an oxygen pulse experiment. Conditions are as for Fig. 1, but hemoglobin concentration was 35 μM, optical path 2 cm, oxygen concentration 124 μM after mixing, dithionite 0.4%.

DISCUSSION

The results just presented show that there are large and hitherto unsuspected differences between the α and β chains in their reaction with oxygen. It follows that Eq. **1**, which assumes chain equivalence, cannot be used to represent the kinetics of the oxygen-binding reaction. The Monod–Wyman–Changeux model, as recently applied by Hopfield *et al.* (7) for this purpose, is equally inapplicable, and constants derived from either scheme are without physical meaning. The simplest realistic scheme, which incorporates chain differences, appears to be that used by Olson and Gibson (5) for *n*-butyl isocyanide, but even this scheme requires that nine equilibrium constants be assigned. Preliminary examination of the oxygen data suggests that there may be appreciable α–β interactions, as well as the major change in the tetramer that occurs when an organic anion is released. As compared with *n*-butyl isocyanide and carbon monoxide, extra data can be obtained for oxygen by use of the dithionite reactions, and it is hoped that these extra data may permit at least a plausible determination of the rate constants in the scheme used by Olson and Gibson (5).

Brunori *et al.* (11) have recently deduced from comparisons of the carbon monoxide equilibrium curve of hemoglobin in the light and in the dark that the dissociation velocity constants for carbon monoxide from partly liganded intermediates cannot be much greater than those from fully liganded carbon monoxide–hemoglobin. Their result is widely different from those with oxygen and *n*-butyl isocyanide. It is interesting to note that the absolute rate of dissociation of the fourth molecule of carbon monoxide (i.e. the rate of dissociation from Hb_4CO) would be about 30,000 times less than the rate of dissociation of oxygen from $Hb_4(O_2)\beta$, and it may be that the rate-limiting step in the dissociation process is different for the two types of ligand. Taken together, the present results and those of Brunori *et al.* (11) strongly support the conclusion of Roughton (12) that the equilibrium curves for oxygen and carbon monoxide cannot be superimposed by application of a scale factor.

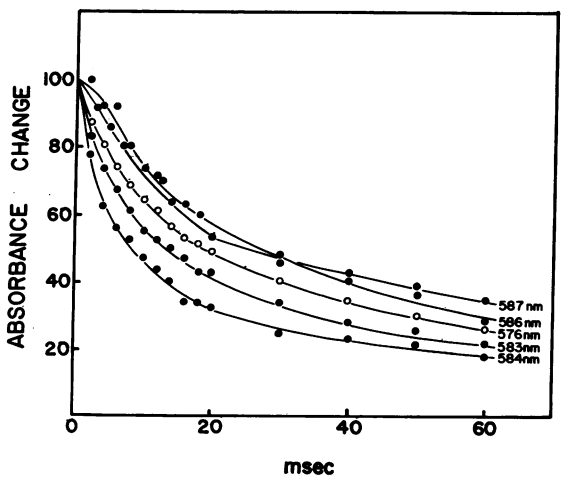

FIG. 3. The time course of the absorbance change observed on oxygen binding at wavelengths near the 585-nm isosbestic point. Conditions: 0.05 M phosphate buffer, pH 7.0, 2°, 2-cm optical path, hemoglobin 30 μM, oxygen 31 μM. The excursions have been normalized to permit ready comparisons. Data obtained at 576 nm, remote from the isosbestic point, have been included.

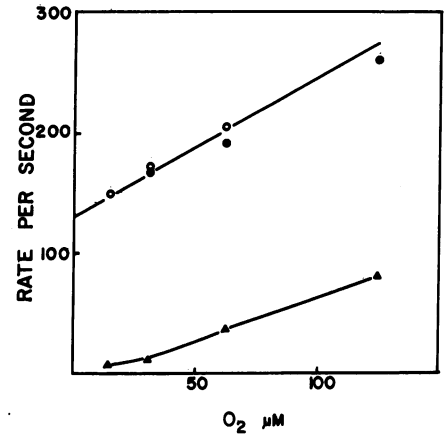

FIG. 4. Rates of absorbance change observed during oxygen binding. The time course was treated graphically to yield two rates for each oxygen concentration used. The *open circles* are for data at 576 nm, the *filled circles* are for data at 585 nm. The *crosses* were derived from data at 576 nm. The experimental conditions were those of Fig. 3, with oxygen concentrations as shown on the abscissa.

The oxygen pulse experiments show beyond reasonable doubt that the large values of the oxygen-dissociation velocity constants suggested as a result of curve-fitting work have a real physical existence even though they were derived from an unsuitable model. This not only complicates kinetic work, but if the kinetic differences carry over to the equilibria, modifies the interpretation of equilibrium constants derived from the extremes of the equilibrium curve. At low saturation the higher affinity chain will be examined, and at high saturation the lower affinity chain will be examined. The apparent interaction energy derived from Hill plots may, therefore, be modified appreciably. Since $K_1 = 2(A_1 + B_1)$, where $K_1 = k_1'/k_1$ of Eq. 1 and A_1 and B_1 are the intrinsic equilibrium constants for binding of the first ligand molecules to α and β chains, respectively, it is possible to estimate, very roughly, the relative affinities of the two types of chain. Roughton and Lyster (15) have given K_1 at 20° in 0.6 M phosphate, pH 7, as 0.049/mm of Hg for human hemoglobin, but in a personal communication made several years later Prof. F. J. W. Roughton informed the author that experiments with more dilute hemoglobin in 0.1 M phosphate had given a value of 0.1/mm of Hg for K_1. Adoption of the latter value, and application of the only temperature coefficient for K_1 available, that of Paul and Roughton (13) for sheep hemoglobin, gives K_1 at 2° as 0.18/μM (converting into concentrations). If B_1 is about 0.025/μM, A_1 would be about 0.065/μM. Clearly a direct determination of K_1 under the conditions of the kinetic experiment would be desirable, but it seems likely, nevertheless, that the α chains may have a higher affinity for oxygen than the β chains at low fractional saturations.

The new experiments explain clearly why K_1 calculated from kinetic data by Gibson (4) was substantially smaller than that observed by Roughton. The kinetic constants necessarily took into account the high rate of dissociation of the β chain, whereas Roughton was primarily studying the α chain, which has a higher affinity. The new experiments, however, do

nothing to account for the discrepancy between the kinetic results of Berger *et al.* (14) and those of Gibson (4) for the oxygen-binding reaction. Berger *et al.* observed a lag at the beginning of the oxygen-binding reaction that was reproduced in some measure by the Monod–Wyman–Changeux model as applied by Hopfield *et al.* (7). The new results indicate clearly that there can be no such lag, and suggest that the observations of Berger *et al.* (14) may have included an instrumental artifact in the earliest stages of the reaction.

This work was supported by United States Public Health Services Grant GM 14276-07. I thank Drs. S. J. Edelstein and J. K. Moffat for helpful discussions, and for reading the draft of this paper.

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