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The Rates of Combination of the Isolated Chains of Human Hemoglobin with Oxygen*

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ROBERT W. NOBLE‡ AND QUENTIN H. GIBSON

From the Department of Biochemistry and Molecular Biology, Cornell University, Ithaca, New York 14850

MAURIZIO BRUNORI, ERALDO ANTONINI, AND JEFFRIES WYMAN

From the Institute of Biochemistry and Centre of Molecular Biology of the C.N.R., University of Rome, and the Regina Elena Institute, Rome, Italy

SUMMARY

The rates of combination of the isolated chains of human hemoglobin with oxygen have been measured by flash photolysis and stopped flow methods. The two experimental methods give similar results, and furthermore the data compare well with those obtained recently by the temperature jump relaxation method. The measured association rates are compared with the values predicted from the previously measured equilibrium and dissociation rate constants. A significant discrepancy is noted in the case of the β chains. In addition, the rates of replacement of oxygen by carbon monoxide as functions of the relative ligand concentrations have been measured, and the observed dependences are compared to those predicted from the equilibrium and kinetic constants for the individual liganding reactions.

It is expected that the reactions of the isolated chains of hemoglobin with oxygen and carbon monoxide can be described by the following equations

$$\mathrm{Fe} + \mathrm{O_2} \underset{k}{\overset{k'}{\rightleftharpoons}} \mathrm{FeO_2}; \quad K = k'/k \tag{1}$$

$$\mathrm{Fe} + \mathrm{CO} \underset{l}{\overset{l'}{\rightleftharpoons}} \mathrm{FeCO}; \quad L = l'/l \tag{2}$$

where K and L are the equilibrium constants for the reactions; k' and l' are the association rate constants; and k and l the dissociation rate constants. Previous studies on the equilibrium and kinetics of these reactions (1, 2) have included the direct determination of all of the equilibrium and kinetic constants with the exception of the rate of combination of oxygen, k'. We now describe the measurement of these "on" constants for oxygen by both flash photolysis and stopped flow methods. In addition, measurements of the rates of replacement of O_2 by CO as a function of the relative concentrations of the two ligands will be reported.

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‡ Present address, Department of Medicine and Biochemistry, State University of New York at Buffalo, Buffalo General Hospital, Buffalo, New York 14203.

MATERIALS AND METHODS

Human Hemoglobin

Human hemoglobin was prepared from fresh blood by the toluene method (3) or alternatively by merely lysing the washed red cells with 1 to 1.5 volumes of water followed by high speed (27,000 × *g*) centrifugation for 20 min to remove the stroma.

Isolated Chains of Human Hemoglobin

These were prepared as previously described (2, 4) by following essentially the original procedure of Bucci and Fronticelli (5).

Measurement of k'

k', the on constant for oxygen, was measured with three separate pieces of apparatus. In all cases the measurements were made at pH 7 in 0.05 or 0.1 M phosphate buffer.

"Rome" Flash Apparatus—As described previously (6), this was built around a commercial flash unit (Multiblitz IIIb) equipped to furnish either 100 or 300 joules per flash with flash durations of 100 and 300 μsec, respectively (to 10% peak). The 300-joule flash was sufficiently powerful to photodissociate essentially all of the oxygen from the hemoglobin chains, and this flash energy was generally used throughout this work. The concentrations of the hemoglobin chains used in these experiments were 2 to 5 × 10^{-7} M. The oxygen concentration varied from 0.2 to 2.0 × 10^{-5} M.

Quartz or Pyrex cylindrical cells (50 and 100 mm, with 20 mm diameters) were used as sample compartments. Temperature control was achieved by immersing the cells in a water bath adjusted to the desired temperature for 10 min and then placing the cell in the apparatus and performing the experiment. Time elapsed during the experiment was very short, and measurements of sample temperature at the end of the rate determination indicated that the temperature had remained constant within ±1°.

"Ithaca" Flash Apparatus—This apparatus has been described previously (7, 8). It can furnish flashes with energies from 150 to 2000 joules, with a flash duration of 30 μsec (10% of peak). Measurements were carried out at two protein concentrations. At 2×10^{-5} M heme the reaction was followed at 372 mμ and the flash was filtered with a cylindrical anthracene filter as previously described (9) to prevent the flash radiation from reaching the photomultiplier. At 10^{-6} M heme the reaction was followed in the Soret region of the spectrum and the flash was filtered with a second cylindrical filter containing both anthracene and auramine dye. Oxygen concentrations varied from 0.6 to 2.7×10^{-4} M.

The cells were a 70-mm water-jacketed cylindrical cell for measurements at low protein concentrations and a 10-mm rectangular cell for measurements at high concentration. Temperature was controlled by circulating water from a constant temperature bath, and was monitored with a thermistor.

Because the flash produced by this apparatus was not sufficiently powerful to dissociate the oxygen from oxyhemoglobin or oxygenated chains,[1] the technique of flashing carbon monoxide from the heme groups in the presence of oxygen (10) was used. The total CO concentration in the system was normally twice the heme concentration, but always less than $\frac{1}{5}$th that of oxygen.

Stopped Flow Apparatus—This apparatus was essentially that of Gibson and Milnes (11), with the modification that the hydraulic syringe drive was replaced by a pneumatic system which decreased the mixing time from 3 to 1.5 msec. The apparatus was also equipped with an "on line" computer to facilitate collection and processing of data (12). The concentrations of the hemoglobin chains were 0.5 to 1×10^{-6} M, while that of oxygen ranged from 0.3 to 0.6×10^{-5} M.

The reaction was followed alternately at 412 and 432 mμ. Because of the high rate of dissociation of oxygen from the β^{PMB} [2] chains, the rate of the association reaction for this chain could not be measured by stopped flow methods.

Measurement of Rate of Replacement of O_2 by CO—A solution of oxyhemoglobin was mixed with one of carbon monoxide in a Gibson-Milnes stopped flow apparatus. The oxyhemoglobin was progressively diluted with a deoxyhemoglobin solution, so that the oxygen tension was decreased while the CO tension was held constant. The reactions involved are the following.

$$FeO_2 + CO \underset{k'}{\overset{k}{\rightleftharpoons}} Fe + O_2 + CO \underset{l}{\overset{l'}{\rightleftharpoons}} FeCO + O_2 \quad (3)$$

If conditions are chosen such that the concentrations of both ligands are large compared to that of the heme protein and the concentration of unliganded heme is always small compared to the sum of the concentrations of the liganded hemes, one can assume that

$$\frac{d(Fe)}{dt} = 0$$

and

$$\sum Fe = (FeO_2) + (FeCO)$$

The equation for the change of (FeO_2) with time is

$$\frac{d(FeO_2)}{dt} = \frac{(FeO_2)[kl'(CO) + k'l(O_2) - (\sum Fe)[k'l(O_2)]}{k'(O_2) - l'(CO)}$$

Solving this differential equation, one obtains a first order rate constant for the approach to equilibrium equal to

$$R = \frac{(O_2)k'l + (CO)l'k}{(O_2)k' + (CO)l'}$$

where R is the rate of replacement of O_2 by CO. If further conditions are chosen such that

$$(O_2)k'l \ll (CO)l'k$$

then

$$R = \frac{(CO)l'k}{(O_2)k' + (CO)l'}$$

so that (13, 14, 2)

$$\frac{1}{R} = \frac{(O_2)k'}{(CO)kl'} + \frac{1}{k}$$

Therefore, if the rate of the replacement reaction is plotted against the ratio of the oxygen concentration to the carbon monoxide concentration, a straight line should be obtained with a slope equal to k'/kl' or K/l' and an intercept on the rate axis equal to $1/k$.

Experimental Errors

The values given in the tables are the 95% confidence limits obtained from the standard error of the mean by Student's t distribution (15).

RESULTS

In Table I are presented the values of k' at 20° and pH 7 obtained by the various methods for the different chains. In addition, the values of the activation energies, ΔH^*, obtained with the two flash apparatuses are given. The time course of the reaction and the dependence of the rate on reactant concentration correspond to that of a second order reaction. Within experimental error, the values of k' are independent of O_2 concentration over the entire ranges examined by each of the three methods. The means of the three values of k' and their confidence limits based on their standard errors and Student's t distribution for 2 degrees of freedom are given in the table ("Average k'"). For purposes of comparison, the values of k' as calculated from k, the "off" constant, and K, the equilibrium constant ($k' = Kk$), are given in the last line.

Table II summarizes the results of the measurements of the rates of O_2 replacement by CO. In these experiments the concentration of CO was held at 1×10^{-4} M while what of O_2 was

[1] The inability of 2000 joules to do in Ithaca what 300 joules can do in Rome appears contradictory, and indeed the exact cause of this discrepancy is not known. Possible partial explanations include the following. In the Ithaca apparatus the photomultiplier is carefully shielded from the output of the flash tubes, requiring a shielding of the sample from a substantial portion of the flash energy. No such shielding was used in the Rome apparatus, as the slower reaction times allowed complete recovery of the photomultiplier. The geometries of the two apparatuses are also somewhat different. The quoted flash energies are actually the energies dissipated from the power capacitors, and are not corrected for the possible dissipation of this energy in forms other than light. Furthermore, the emission spectra of the two flash systems may not be identical. Whatever the correct explanation is, it has no effect on the data presented or the conclusions drawn in this paper.

[2] α^{PMB} and β^{PMB} are chains with the sulfhydryl groups blocked by *p*-mercuribenzoate; α^{SH} and β^{SH} have freely titratable sulfhydryl groups.

TABLE I

Rates of reaction of isolated chains of human hemoglobin with oxygen at 20° in pH 7 phosphate buffer and activation energies of reactions as measured by flash photolysis and stopped flow methods

Method	α^{PMB}	α^{-SH}	β^{PMB}	β^{-SH}
$k' \times 10^{-7}$		M^{-1} sec^{-1}		
Flow	4.1 ± 0.4	3.7 ± 0.33		7.0 ± 0.4
Flash (Ithaca)	4.3 ± 0.51	5.7 ± 0.71	4.9 ± 0.38	6.8 ± 0.6
Flash (Rome)	5.5 ± 0.8	5.5 ± 0.4	7.4 ± 2.0	7.5 ± 1.5
Average k'	4.6 ± 1.3	5.0 ± 1.9	6.0 ± 3.7	7.1 ± 0.6
Calculated (Kk)	4.3 ± 2.2	3.4 ± 1.4	3.1 ± 1.0	1.8 $\pm^{5}_{\Delta}$ 0.9
ΔH^*		kcal deg^{-1} M^{-1}		
Flash (Ithaca)	9.1 ± 1.5	8.15 ± 2.2	6.2 ± 1.2	7.6 ± 1.1
Flash (Rome)	10.2 ± 1.3	7.3 ± 3.4	7.5 ± 1.9	8.4 ± 4.3

TABLE II

Comparison of dependence of rate of replacement of O_2 by CO on relative concentrations of two ligands with that predicted from equilibrium and kinetic constants of reactions of isolated chains with O_2 and CO

When the reciprocal of the rate of replacement is plotted against $(O_2)/(CO)$ the slope of the plot, $\Delta[1/R]/\Delta[(O_2)/(CO)]$ should equal k'/kl' or K/l', and the intercept on the $1/R$ axis should occur at $1/R = 1/k$.

	α^{PMB}	α^{-SH}	β^{PMB}	β^{-SH}
		sec		
Actual slope, $\Delta[1/R]/\Delta[(O_2)/(CO)]$	0.27 ± 0.008	0.28 ± 0.55	0.096 ± 0.004	0.32 ± 0.27
Slope predicted from k'/kl'	0.38 ± 0.18	0.39 ± 0.17	0.16 ± 0.11	0.97 ± 0.53
Slope predicted from K/l'	0.36 ± 0.15	0.26 ± 0.09	0.083 ± 0.026	0.24 ± 0.09
Experimental intercept	0.048 ± 0.006	0.083 ± 0.048	0.015 ± 0.003	0.057 ± 0.022
Intercept predicted from $1/k$	0.032 ± 0.010	0.036 ± 0.008	0.0064 ± 0.0016	0.062 ± 0.019

varied from 1.35×10^{-4} M to 0.8×10^{-5} M. The concentration of the isolated chains was approximately 10^{-6} M. The time course of these reactions was that of a pseudofirst order process. Furthermore, no obvious deviation from linearity was evident in the plot of $1/R$ *versus* O_2/CO. Therefore, the slopes and the intercepts of the plots were determined by linear regression analysis. The slope of the plot of $1/R$ *versus* O_2/CO should theoretically be equal to k'/kl' or K/l'; the intercept should equal $1/k$. The values of these three quantities are therefore included in the table for purposes of comparison.

DISCUSSION

The rate of combination of O_2 with the isolated chains of hemoglobin is much faster than its rate of combination with hemoglobin. This is similar to what is found in the case of CO, and confirms the observation that the combination of the chains to form the hemoglobin molecule causes a great reduction in the on velocity constants of the liganding reactions. The value of k' for the hemoglobin chains is also greater than for horse myoglobin (1×10^7 M^{-1} sec^{-1}) (13). Since the isolated hemoglobin chains and myoglobin exhibit no heme-heme interaction, this finding is presumably due to differences in the heme environment resulting from the known differences in primary sequence (16). The activation energies of these oxygenation reactions seem to fall in the range normally associated with reactions of similar speed.

In a number of cases, the values of k' as determined by two different methods differ by more than their confidence limits. This implies that these values do not belong to the same population and is indicative of the existence of nonrandom errors in some or all of the methods used. In view of the large differences in the three methods, the high value of the rate constant, and the difficulties of working with gaseous ligands, the agreement between the various values seems quite satisfactory, but the confidence limits calculated for the values determined by any single method seem definitely to be overoptimistic. However, confidence in the average values of k' given in Table I is increased when these are compared to the values of the same constants obtained recently by Brunori and Schuster by the temperature jump relaxation method (17). This comparison is presented in Table III.

TABLE III

Comparison of average values of k' as determined by flow and flash photolysis methods (Table I) with those obtained by Brunori and Schuster (17) by temperature jump relaxation method

Method	$k' \times 10^{-7}$			
	α^{PMB}	α^{-SH}	α^{PMB}	α^{-SH}
	M^{-1} sec^{-1}			
Flash-Flow	4.5	5.0	6.0	7.1
Temperature jump	5.5	4.8	8.3	6.5

To the extent that the reaction of the isolated chains with oxygen can be correctly represented by the simple scheme

$$Fe + O_2 \underset{k}{\overset{k'}{\rightleftharpoons}} FeO_2; \quad K = \frac{(FeO_2)}{(Fe)\,(O_2)}$$

then it must be that

$$k'/k = K$$

This appears to be very nearly the case for the α chains, although it must be realized that the precision of such a statement is limited by the errors associated with the measurements. However, from Tables I and II it is clear that for the β^{SH} chains and probably for the β^{PMB} chains

$$k'/k > K$$

The discrepancy appears to be well outside the range of random experimental error in the case of β^{SH} chains.

The data from the replacement reactions (Table II) offer another check of the internal consistency of the measured constants. Here again the agreement is reasonably good in the case of the α chains. In the case of the β chains the slopes agree well with the values of K/l' but not with the values of k'/kl'. The situation is similar to that for myoglobin, where again $k'/k > K$ and the displacement reaction is complex.[3]

Brunori *et al.* (2) have already pointed out the existence of certain apparent discrepancies in some of the constants for the reactions of the hemoglobin chains with O_2 and CO. A comparison of L with l'/l showed a large difference in the case of the β^{PMB} chains. In addition, when the replacement of O_2 by CO was studied under conditions $((O_2) \gg (CO))$ such that

$$R = l + \frac{l'k(CO)}{k'(O_2)}$$

the slopes of the plots of R *versus* $(CO)/(O_2)$ in the case of the α^{SH} and β^{PMB} chains were significantly different from the predicted values.

Since the discrepancies are seen primarily with β chains, which are not monomeric, it is tempting to speculate that because of their polymeric state these may react with ligands in a more complex way than is shown in Equation 3. However, similar although somewhat smaller discrepancies have been found for myoglobin, which is definitely a monomer like the α chains. Furthermore, because of the possibility of nonrandom errors in some of the measured reaction constants, it is not possible at present to be certain that the observed discrepancies reflect properties of isolated hemoglobin chains rather than limitations in the experimental methods.

[3] E. Antonini, Q. H. Gibson, and J. Wyman, unpublished observations, quoted by Antonini (18).

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