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KINETICS OF CARBON MONOXIDE AND OXYGEN BINDING TO HEMOGLOBIN IN HUMAN RED BLOOD CELL SUSPENSIONS STUDIED BY LASER FLASH PHOTOLYSIS

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The reaction kinetics of the binding of CO and O_2 to hemoglobin (Hb) in human red blood cell (RBC) suspensions have been examined using a 300 ns dye laser to photodissociate HbCO or HbO_2. Fast (halftime ~10 μs) and slow (~5 ms) processes were seen after photolysis. The results indicate that neither the rate constants nor the activation energies for the binding of CO to the fast reacting form of Hb in the RBC are significantly different from that measured in solution in spite of the different environments. Rate constants determined for O_2 binding in RBC were intermediate between rates observed for reaction with fast and slow reacting forms of Hb and probably consist of contributions from each. The slow recombination of CO and O_2 probably has contributions both from reaction with slow reacting forms of Hb and from ligand that had diffused away from the RBC after photolysis.

1. Introduction

It is now clear that the uptake of O_2 by the red blood cell (RBC) is not limited by the rate of binding to hemoglobin (Hb) [1–5]. It is instead largely limited by mass transport of O_2 to and through the RBC [1–5]. For example the rate of O_2 uptake of the human RBC measured by stopped-flow is some 40 times slower (with a $t_{1/2}$ of 80 ms in 0.125 mM O_2, 25°) than that of Hb [1] in solution. None the less it is of interest to compare the kinetics of ligand binding to Hb in the RBC to that in solution. In the RBC the [Hb] is about 21 mM (all [Hb] are given on a heme basis) which is some 1000 fold higher than is generally used in solution kinetic studies [6]. In solution the ligand binding kinetics are complicated by dimer formation [6–8] due to low [Hb]. It is also known that the viscosity in the RBC is about 10 times that of water [9]. In addition to Hb at a concentration of 33.5 g per 100 mL of RBC the RBC contains a wide variety of other chemical components [10]. Kinetic studies at high [Hb] increase the rates of the second order processes some 20 fold higher than is usually studied and further out of the range of first order processes. A previous kinetic study [11] of the recombination of CO with Hb in horse RBC suspensions using a flash lamp for photolysis was limited to times greater than 15 μs and consequently missed the initial part of the reaction which proceeds at a high rate due to the high concentration of reactants in the RBC. This study uses a much faster dye laser with a 300 ns pulse width to examine both CO and O_2 recombination kinetics in the RBC.

2. Experimental

2.1. Methods and materials

Photolysis of ligand bound to Hb was achieved with a Phase-R model 2100-B dye laser (3 J maximum output at 578 nm with Rhodamine 590 dye in absolute methanol) with a pulse width of 300 ns. The photolyzing laser light was incident on the reaction cell at 90° to the monitoring light. A 1 cm path length jacketed reaction cell was used to contain the RBC suspensions and the solutions of low [Hb]. The temperature was maintained to ± 0.5° and was measured with a thermocouple inserted directly in the reaction cell. For experiments at high [Hb] a short path length (15 μm) reaction cell was constructed of two micros-

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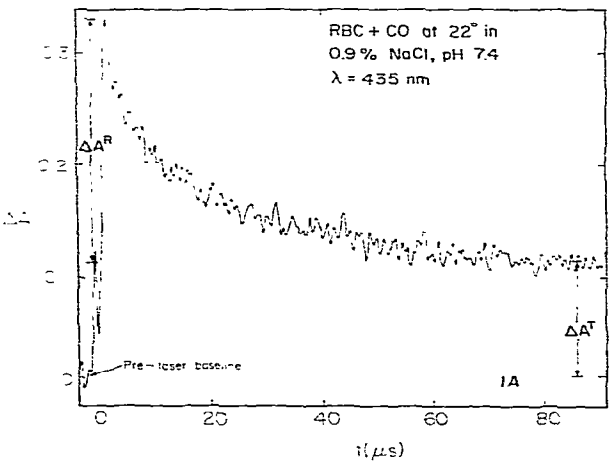
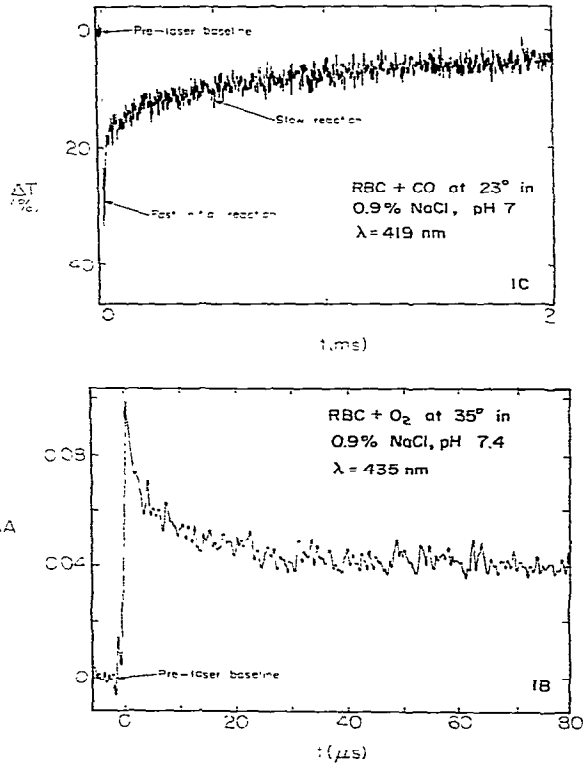
cope slides pressed together and sealed with a heavy grease. This cell was placed at 45° to both the laser and observation light beams. Changes in light transmission in the reaction solution were detected with a 50 MHz bandpass photomultiplier and recorded digitally on a Biomation 805 transient recorder with a minimum sampling rate of 200 ns per data point. The rest of the spectrophotometric detection and computer-linked data acquisition system has been described [12]. Due to the high degree of light scattering ($\%T \sim 1\%$) of the RBC suspensions it was necessary to use a monochromator band pass of 5 nm in order to obtain a satisfactory signal-to-noise ratio. The reactions studied in solution, however, used a 1 nm band pass. The 256 voltage-time digitized data points were computer fitted by weighted linear least squares analyses. Since absorbance changes near the completion (~40 μs) of the fast initial reaction had a small contribution from the slower reaction (with a $t_{1/2} \sim 5$ ms) the value of the infinity absorbance change for the initial fast process was systematically varied in order to minimize the sum of the squares of the residuals and further refine the rate constants. Absorbance *changes* (total of 0.1 for O_2 and 0.3 for CO, fig. 1) were measured from a base-line average of 20 data points obtained just *prior* to the laser pulse. Typically 45–65% of the total absorbance change was due to the initial fast reaction. Kinetic measurements of O_2 binding to Hb in RBC were more difficult than the CO binding measurements due to the lower quantum yield for HbO_2 dissociation and the higher rate for the O_2 binding reaction [6].



Human blood was collected over EDTA and used within several hours. Reaction solutions were prepared only minutes before their use. Generally dilutions of 1.5 μL of blood per mL of reaction solution gave acceptable kinetic traces. The CO or air-saturated reaction solution in which the RBC were suspended was isotonic to blood plasma and contained 0.9% NaCl along with 1 mM KH_2PO_4-NaOH buffer. Examination of the descending RBC in the suspension with an inverted phase-contrast microscope revealed that approximately 10% of the cells had undergone a transformation from the normal biconcave disc to the echinocyte (sphere covered with crenations) shape as has been noted [13]. There was no rouleaux (stacking) forma-



Figs. 1A, B, C. Reaction records for the recombination of ligand with Hb in red blood cell suspensions after photolysis with a 300 ns laser pulse. Traces are chart recorder records of the digital-to-analog conversion of data stored in the transient recorder.

tion seen in the suspensions. The CO saturated reaction solution was checked for hemolysis after 30–40 laser pulses by spectrophotometrically measuring the supernatant after centrifugation. The results indicated that only 1.6% of the observed absorbance change could thus be attributed to Hb in solution which in any event would react at a much slower rate than Hb in the RBC. Samples for kinetic studies at high [Hb] in the short path length cell were prepared by washing and centrifuging CO saturated blood which was then frozen in liquid N_2 to cause hemolysis. This resulted in a solution with [HbCO] of 15 mM or 73% of that in the red blood cell. Kinetic studies under pseudo first order conditions were also carried out on the unstripped hemolysate with a [HbCO] of 11 μM and a $[CO]_0$ of 0.96 mM in the 1 cm reaction cell. Conditions of partial photolysis were used for the reactions of Hb^R and complete photolysis for the reaction of Hb^T [7].

3. Results and discussion

3.1. Titration of RBC suspensions

Spectrophotometric titrations of air-saturated RBC suspensions were carried out by introducing μL amounts of CO-saturated RBC suspensions; or CO-saturated buffer or gaseous CO into a serum cap sealed spectrophotometer cell. After a consistent mixing procedure the Soret absorption spectra was repeatedly recorded. While there was significant drift in recorded absorbance values due to inhomogeneities in the RBC suspensions over the time ($\sim$ 1 min) that the measurements were carried out plots of *absorbance changes* versus amounts of added CO were sufficiently linear to justify the assumption made in the kinetic analysis that *absorbance changes* are proportional to Hb *concentration changes*.

3.2. Kinetic model

While the kinetics of ligand binding to Hb after photodissociation have been well studied [3–8,14] there is no single satisfactory detailed kinetic model. The simple Monod-Wyman-Changeux (MWC) allosteric model and the Adair model are generally used in providing a simple qualitative description of the kinetics [14]. Upon photolysis of HbCO or HbO_2, partially or fully deligated Hb is produced in its fast reacting "R-state" or "oxy" conformation (Hb^R) and its slower reacting "T-state" or "deoxy" conformation (Hb^T). The Hb^T and Hb^R forms are kinetically intraconvertible but at rates that probably depend upon the number of bound ligands [7,8,14]. The kinetic scheme used then is

$$2HbL \xrightarrow{h\nu} Hb^R + Hb^T + 2L,$$

$$Hb^R + L \xrightarrow{k^R} HbL,$$

$$Hb^T + L \xrightarrow{k^T} HbL.$$

Under reaction conditions in the RBC and at high [Hb] the initial fast rate is

$$-d[Hb^R]/dt = k^R[Hb^R][L]$$

and from the integrated second order rate equation for unequal starting reactant concentrations.

$$k^R t = \frac{1}{([L]_0 - [Hb^R]_0)} \times \ln \frac{[Hb^R]_0}{[L]_0} \left(\frac{[L]_0 - [Hb^R]_0 + [Hb^R]}{[Hb^R]} \right), \quad (1)$$

where $[L]_0$, $[Hb^R]_0$ are concentrations present at $t = 0$ and $[Hb^R]$ that at time t. In terms of absorbance changes indicated in fig. 1A with

$$[Hb]_0 = [Hb^R]_0 + [Hb^T]_0,$$

$$[Hb^R] = \left(\frac{\Delta A - \Delta A^T}{\Delta A^R} \right) [Hb^R]_0 = \left(\frac{\Delta A - \Delta A^T}{\Delta A^R + \Delta A^T} \right) [Hb]_0$$

$$[Hb^R]_0 = \left(\frac{\Delta A^R}{\Delta A^R + \Delta A^T} \right) [Hb]_0$$

and $[L]_0 = [Hb]_0$ at $t = 0$, eq. (1) becomes

$$\log \left(\frac{\Delta A}{\Delta A - \Delta A^T} \right) = \frac{k^R [Hb]_0}{2.3} \left(\frac{\Delta A^T}{\Delta A^R + \Delta A^T} \right) t + \log \left(\frac{\Delta A^R + \Delta A^T}{\Delta A^R} \right), \quad (2)$$

where ΔA is the absorbance change at time t measured from the pre-laser flash photolysis base line; ΔA^R is the absorbance change at $t = 0$ due to the fast Hb^R

Table 1
Comparison of reactions of human Hb with CO and O_2 in blood cell suspensions and in solution by laser flash photolysis [a]

Reaction	Rate constant ($\mu M^{-1}\ s^{-1}$)	Medium	$[Hb]_0$ (mM)	pH	E_a (kcal/mol)
$Hb^R + CO$	6	RBC suspension	21	7.4	1.0 ± 0.6
$Hb^R + CO$	3	RBC suspension [b]	26	7	1.3 ± 0.2
$Hb^R + CO$	6	solution	15	7	–
$Hb^R + CO$	9 [c]	solution	0.011	7.4	3.4 ± 0.2
$Hb^T + CO$	0.06 [d]	RBC suspension	21	7	6 ± 1
$Hb^T + CO$	~0.3 [e]	solution	15	7	–
$Hb^T + CO$	0.22 [f]	solution	0.011	7.4	–
$Hb + O_2$	11	RBC suspension	21	7.4	3 ± 3

a) Unless otherwise indicated at 20° in 0.9% NaCl and 1 mM KH_2PO_4-NaOH buffer at an observation wavelength of 435 or 436 nm. Errors on E_a are fitting errors only from the linear squares analyses.
b) In 1.8% NaCl, 2 mM KH_2PO_4-NaOH buffer and at 419 nm. $[Hb]_0$ estimated from data of ref. [19].
c) Determined under pseudo first order conditions with $[CO]_0 = 0.96$ mM. Compares to: 8 $\mu M^{-1}\ s^{-1}$ [20] (HbA hemolysate at 20°, pH 7.4, 100 mM phosphate) or 6 $\mu M^{-1}\ s^{-1}$ [20] at pH 7; and also 4.6 $\mu M^{-1}\ s^{-1}$ [18] (stripped HbA at 20°, pH 7, 50 mM phosphate).
d) An apparent rate only as diffusion of CO away from RBC contributes to the CO binding rate. The half-time for this process is 5 ms.
e) Estimated from a measured value of 0.6 $\mu M^{-1}\ s^{-1}$ at 31° and the temperature dependence of ref. [7].
f) At 17°, determined under pseudo first order conditions with $[CO]_0 = 0.96$ mM. Compares to 0.15 $\mu M^{-1}\ s^{-1}$ [18] (stripped HbA at 20°, pH 7, 50 mM phosphate).

binding reaction; and ΔA^T is the absorbance change at $t = 0$ due to the slow Hb^T binding reaction and any other slow binding such as that due to diffusion. A plot of the left hand side of eq. (2) versus time should be linear (fig. 2). Combining the slope and intercept with ΔA^T and $[Hb]_0$, values for k^R are obtained (table 1). Typically the standard errors on k^R from the linear fits to eq. (2) were about 10% for CO binding and 20% for O_2 binding in RBC suspensions and 5% for CO binding in solution. Arrhenius plots are given in fig. 3.

Eq. (2) is based on a simple model for the kinetics and its use necessarily involves several assumptions. Primarily these are: (1) that the intraconversion reaction

$$Hb^R \underset{k^{TR}}{\overset{k^{RT}}{\rightleftharpoons}} Hb^T$$

is kinetically insignificant over about 40 μs that the fast Hb^R reaction occurs; (2) that $k^R \gg k^T$; (3) that full photolysis is achieved so that $[Hb]_0$ is known at $t = 0$; (4) the dissociation reaction $HbL \rightarrow Hb + L$ can

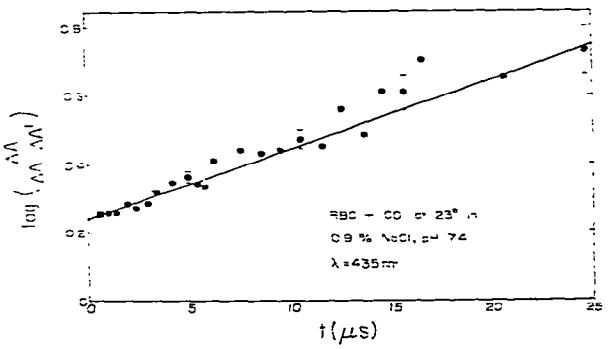


Fig. 2. Plot of $\log[\Delta A/(\Delta A - \Delta A^T 0]$ versus t for the recombination of CO with Hb after laser flash photolysis. The straight line is weighted linear least-squares calculated from about 200 data points, only a fraction of which are plotted. Some representative standard errors are plotted. From eq. (2) the slope and intercept yields k^R.

be neglected; and (5) Hb dimer concentration is small.

Concerning assumption (1) Sawicki and Gibson

[7,8], also using a simplified MWC model, found that at pH 9 for ligand-free Hb, k^{RT} varied from 1080 s^{-1} to 17000 s^{-1} from 3° to 30° irrespective of whether CO or O_2 was the binding ligand. At 20°, k^{RT} decreased by about a factor of 2 [7] for each additional ligand bound. Since the fast reaction observed in RBC suspensions and at high [Hb] is decaying at a rate of about 150000 s^{-1} the $Hb^R \rightarrow Hb^T$ conversion occurs on a much slower time scale. At pH 7 at 20°, k^{RT} is still only 8000 s^{-1} [8]. At pH 9 and 20° the value for ligand-free k^{TR} was 1 s^{-1} [9] to 2 s^{-1} [8]. These values increase from 50 to 130 fold [8] for each additional ligand bound. Even so these rates should also be well separated in time from the faster rates of this study particularly under conditions of full photolysis. Various fast relaxations [14–16] observed by ns laser photolysis on a time scale even faster than that used in this study have now almost certainly been identified with an initial ultra-fast caged geminate recombination [15,16].

Assumption (2) that $k^R \gg k^T$ for the CO reaction is very good with k^R/k^T being 22 at pH 9.4 [7] and 46 at pH 7 [14]. This assumption is less satisfactory for the O_2 reaction with k^R/k^T being about 10 for the α chain and 3 for the β chain [14,17].

Assumption (3) that there was full photolysis so that $[Hb]_0$ could be set equal to 21 mM in eq. (2) was achieved experimentally for the CO binding reaction by gradually increasing the laser energy output until the total absorbance excursion reached a maximum. This occurred at about 50% of the maximum output of 3 J. For the O_2 reaction there was substantially less than full photolysis even at the full laser output due primarily to the low quantum yield for HbO_2 photolysis [6]. Since a value of $[Hb]_0$ is required for a determination of k^R it was estimated by comparing the total absorbance change ($\Delta A^R + \Delta A^T$) at $t = 0$ to that observed for CO photolysis. With use of the known molar absorptivity changes [6–8,17] of the various Hb species it was estimated that about 35% photodissociation of HbO_2 was observed in RBC suspensions. Because of the approximate nature of this estimation of $[Hb]_0$ the O_2 binding data must be considered as less reliable than the CO binding data.

Assumption (4) that the dissociation rate HbL → Hb + L can be neglected is very good for both the O_2 and CO reactions [14,17] owing to the high [Hb] in the RBC.

Assumption (5) that the dimer concentration is small and kinetically insignificant is probably also very good. It can be estimated from a dimer dissociation constant of 5 μM for HbO_2 or HbCO [6] that only about 1.5% of the hemes in the RBC are present as dimers.

Determinations of the rate of the slow Hb^T reaction with CO in solution at high [Hb] and in RBC suspensions were also made on the assumption that

$$-d[Hb^T]/dt = k^T[Hb^T][CO],$$

and that the remaining unreacted [CO] was equal to $[Hb^T]_0$. Rough estimates of k^T were made from weighted linear least squares fitting of the integrated second order rate equation which predicts that plots of ΔA^{-1} versus t should be linear. The value of k^T in table 1 for the RBC suspension is an apparent rate constant only as there is probably a very significant contribution to $-d[Hb^T]/dt$ from diffusion of CO away from the RBC.

3.3. Comparison of RBC and solution kinetics

The fast Hb^R reaction with CO, as can be seen from table 1, proceeds in the RBC at rates and with activation energies not significantly different than in solution. This occurs in spite of the fact that conditions in the RBC are considerably different than they are in solution. This is somewhat surprising when it is

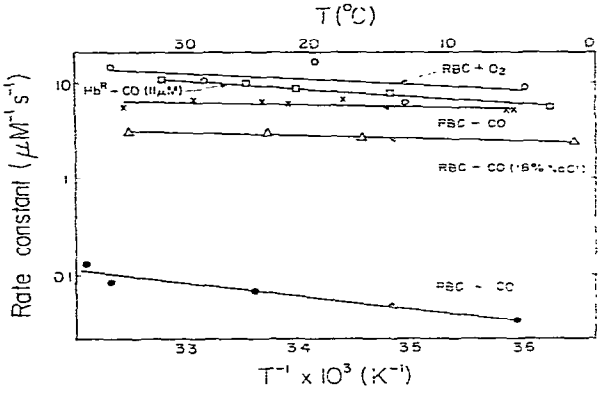


Fig. 3. Arrhenius plots of ligand binding to Hb in red blood cell suspensions and in solution. The straight lines are least squares calculated.

considered that a considerable fraction of the RBC volume is occupied by Hb and various salts with their bound water. From a consideration of Hb- and salt-water protein interactions it has been estimated that only about 60% of the volume of the isotonic RBC is "free" or "unbound" water [21]. On the supposition that the photodissociated ligand were confined to "free" water only the effective concentration of the ligand would be some 1.7 times higher than if the whole volume were considered. The value of k^R for the reaction of CO would thus become 4 μM^{-1} s^{-1} which is, however, still not significantly less than the rate found at the low [Hb]. This type of correction may not be valid, as it has been shown [22] from the fluorescence quenching of buried tryptophan that O_2 is freely permeable to the protein. The results of a previous study [11] of the reaction of CO with Hb in horse RBC with use of a much slower flash lamp also indicated that the rate of reaction of Hb^R is similar to that found in solution.

The fast reaction of O_2 with Hb in the RBC is about twice that found for CO. However, the interpretation of this value is not as clear. This value can be compared to previous studies at low [Hb]. In a laser study at pH 7 and 23° in 0.1 M phosphate McCray [23] observed biphasic O_2 binding kinetics with a slow rate of 7 μM^{-1} s^{-1} and a fast rate of 50 μM^{-1} s^{-1}. A ruby laser study [24] at pH 7 and 25° in 0.1 M phosphate gave a fast rate of 32 μM^{-1} s^{-1} by flashing off CO in the presence of O_2. However a more recent study [8] found for fast O_2 binding k^R = 55 μM^{-1} s^{-1} [8] at 20° in pH 7, 50 mM bis-tris buffer. Under conditions of partial O_2 saturation of Hb and with use of a model that considered kinetic heterogeneity of binding to the Hb chains, values of k^T (α chain) = 3 μM^{-1} s^{-1} and k^T (β chain) = 12 μM^{-1} s^{-1} were determined at 20° in pH 7, 50 mM phosphate. The rate constant for O_2 binding in the RBC of 11 μM^{-1} s^{-1} thus is only intermediate in value between the various slow and fast reacting forms of Hb and likely consists of contributions from each. Experimental limitations mitigate against using a more sophisticated model to resolve the separate rate constants. If the reaction of Hb^R with O_2 were preceeding in the RBC at its rate in solution of 55 μM^{-1} s^{-1} [8] the half-time for reaction would be about 1 μs and would not be well resolved from the 300 ns laser pulse.

3.4. Diffusion effects on the slow Hb binding in the RBC

The slow reaction of Hb with a $t_{1/2}$ of 3—5 ms is about 4 times less than that observed in solution. This reaction is probably slowed because a fraction of the CO is able to diffuse away from the RBC after photolysis before recombination can occur. The kinetic analysis is considerably complicated. In a previous study [11] the kinetics of this slow reaction in horse RBC suspensions were simulated by analog computer using a diffusion and chemical reaction model of the RBC as a 1.4 μm infinite thin film of Hb adjacent to an unstirred buffer. Thus when diffusion and chemical reaction are considered together [11]

$$\left(\frac{\partial[CO]}{\partial t}\right)_x = D\frac{\partial^2[CO]}{\partial x^2} - k[Hb]_x[CO]_x$$

where D is a diffusion coefficient, x a distance and k is the rate constant for a simplified model of the reaction. Thus over the several milliseconds that the slow Hb^T reaction occurs significant amounts of photolyzed CO diffuse away from the RBC [3], significantly slowing the CO rebinding rate. More recent calculations [2] on the RBC have indicated that the time scale over which the CO diffusion layer about the RBC expands appreciably (greater than the thickness of the RBC) is about 5 ms, which is on a time scale similar to the slow CO recombination reaction in RBC suspensions of this study.

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