Kinetics of the Reaction with Oxygen of Mixtures of Oxy- and Carbon Monoxide Hemoglobin

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SUMMARY

The paper reports rapid mixing and relaxation experiments performed on mixtures of oxy- (HbO₂) and carbon monoxide (HbCO) human hemoglobin. On the (well justified) assumption that the two ligands will distribute at random between the available sites, intermediates containing different proportions of O₂ and CO will be formed.

In the stopped flow experiments mixtures containing different proportions of the two ligands have been mixed with sodium dithionite (Na₂S₂O₅), which rapidly reduces to zero the O₂ concentration in the system. The apparent dissociation velocity constant for O₂ (k_diss) measured under these conditions decreases progressively as the fraction of HbCO in the mixture increases, in agreement with previous observations on sheep hemoglobin.

Temperature jump experiments performed on mixtures of HbCO and HbO₂ show that the amplitude of the faster relaxation time (τ_F) relative to that of the slower one (τ_S) increases as the percentage of HbCO in the mixture progressively increased. At high enough percentage of HbCO (≥70%), the amplitude of the faster relaxation time becomes dominant. The reciprocal relaxation time (τ_F⁻¹), measured under these conditions, is linearly dependent on oxygen concentration, while it is independent of protein concentration (so long as O₂ is buffered). The apparent second order velocity constant is k_m = 4.8 × 10⁹ M⁻¹ s⁻¹ at 25°C.

Simple considerations indicate that the kinetics of the reaction with oxygen of mixtures containing high enough percentages of HbCO should represent the combination and dissociation velocity constants of high affinity forms of hemoglobin.

In view of the well recognized difficulty of describing quantitatively and completely the kinetics of the reaction of hemoglobin with ligands, special efforts have been made to devise experimental methods that would allow a direct determination of the kinetic constants pertaining to individual reaction steps. Within this conceptual framework, different types of "artificial" intermediates have been prepared and characterized from the structural and functional standpoints, with the idea that the resulting information may be usefully applied to the "naturally occurring" intermediates (for a review see Reference 1).

In general, however, kinetic investigations on ligand binding have been performed using carbon monoxide, since studies on the reaction with oxygen, the physiological ligand, have been hindered by technical difficulties due to the very high rates involved. Thus, while the conventional rapid mixing methods may be used to follow rates of dissociation of oxygen from the ligand-bound species (1, 2), experiments on combination of deoxyhemoglobin with the ligand are only possible under suitably restricted conditions (3), and some information on the oxygen combination at high ligand concentration was obtained employing a specially devised stopped flow apparatus (4). Relaxation methods, on the other hand, should be devoid of this difficulty in view of their very high time resolution, and this was shown to be the case by a temperature jump study of the hemoglobin-oxygen reaction (5). In summary this work indicated that, contrary to the simple behavior characteristic of the isolated α and β chains, the relaxation spectrum in the reaction of hemoglobin with oxygen is complex, and at high protein concentration two exponential processes are necessary (and sufficient) to describe the over-all approach to equilibrium. The slower relaxation time (τ_S) was shown to be dependent upon the concentration of the reagents, and an apparent combination rate constant similar to the value estimated by rapid mixing methods (i.e., 1 to 2 × 10⁹ M⁻¹ s⁻¹ for sheep hemoglobin at pH 9.1 and 20°C) was obtained by treating the data on the simple assumption of obedience to bimolecular behavior. The other relaxation time (τ_F), which is faster by a factor of 10 to 20, was also found to be dependent upon O₂ concentration (5); according to Schuster and Ilgenfritz (6) the apparent combination velocity constant reckoned from the O₂ dependence of τ_F at very high O₂ saturations is 5 × 10⁸ M⁻¹ s⁻¹ for sheep hemoglobin (at pH 0.1 and 10°C). However, while, on the whole, the experimental findings obtained by temperature jump appear fairly clear, their detailed interpretation is still to be settled.

In this note, the results of rapid mixing and temperature
RESULTS

As well known, the kinetics of dissociation of oxygen from hemoglobin can be followed by mixing (in a stopped flow apparatus) the oxygenated derivative with sodium dithionite (1, 2). When the reaction of dithionite with free O₂ is not rate-limiting, the time course of the observed process obeys first order kinetics, and the measured rate constant represents an over-all dissociation of the ligand from completely saturated hemoglobin. When the same experiment is repeated using different equilibrium mixtures of HbO₂ and HbCO, the over-all velocity constant decreases monotonically as the fraction of HbCO in the mixture is progressively increased (Fig. 1); in the limit, the first order rate constant tends to a value similar to that obtained by the replacement method, which for human hemoglobin in phosphate buffer at pH 7 and 20°C is about 15 s⁻¹.³ The decrease in the observed dissociation velocity constant when CO is present appears in agreement with previous results obtained by Gibson and Roughton (7) using sheep hemoglobin.

Fig. 2 reports the oscilloscope traces of temperature jump experiments performed either on oxyhemoglobin or on mixtures of oxy- and carbon monoxide hemoglobin. In the absence of carbon monoxide (Fig. 2a) the relaxation spectrum consists of two processes, in agreement with previous observations (5, 6) (see “Introduction”). The relative amplitude of the faster process (A₁) corresponds to about 15 to 25% of the total change over a fairly large saturation range (from ~20 to ~95% saturation) (Table I). On the other hand, when the experiment is performed with mixtures of oxy- and carbon monoxide hemoglobin (Fig. 2, b and c), the relative amplitude of the faster relaxation phase becomes larger as the ratio (HbCO:HbO₂) in the original mixtures is increased, until finally it becomes prominent. The dominance of A₁ under these conditions is noticeable even at comparable total saturation with the ligand. The absolute amplitude of the observed effect of course becomes smaller and...
smaller as the percentage of HbCO in the mixture increases, and
tends to zero for HbCO approaching 100% (see "Discussion").
The faster relaxation time ($\tau_F$), which is the only observable
effect in mixtures containing more than 70% HbCO, has been
followed at different $O_2$ concentrations. It may be noticed that,
since in these experiments a large fraction of the total heme is
saturated with either CO or $O_2$, the concentration of the free
ligand ($O_2$) exceeds the concentration of the free sites ($Fe$).
Therefore, being ($O_2$) $> ($Fe$)$, the reciprocal relaxation time in-
creases linearly with the concentration of free $O_2$ (Fig. 3), follow-
ing the behavior expected for a simple bimolecular process:

$$1/\tau_F = k_{a} + k_{a}(O_2)$$

(1)
The measured rates are essentially independent of the total pro-
tein concentration (from 0.15 to 12.4 $\times$ $10^{-5}$ M in heme) and of
the percentage of HbCO in the original mixture. The intercept at
($O_2$) = 0 is consistent with the value of the dissociation ve-
locity constant determined by rapid mixing experiments with
dithionite on the same mixtures (i.e., $\approx 30$ s$^{-1}$ at 25°). The
bimolecular combination velocity constant calculated from the
slope of the plot in Fig. 3 is $4.8 \times 10^3$ M$^{-1}$ s$^{-1}$ (at 25° and pH 7).
Table I, showing a comparison of the observed kinetic para-
eters with the constants obtained for the isolated $\alpha$ and $\beta$
chains, brings out the similarities between the two sets of values.

**DISCUSSION**

On the assumption that, given enough time, $O_2$ and CO will
runate at random between the available sites of tetrameric
hemoglobin, it is easy to calculate the relative percentage of
the possible species under conditions in which all the sites are
occupied. In a mixture containing 90% of HbCO and 10% of
Hb$O_2$, Hb$4$($CO_3$)$O_2$ represents the predominant intermediate
(29.2%), while other molecules containing both ligands in the
same unit (i.e., Hb$4$($O_2)3$($O_2$) and Hb$4$($CO)1$($O_2$)2) are present
in much smaller amounts. Therefore, if the kinetic experiments
are devised to follow exclusively the oxygen reaction before
re-equilibration of the system can occur, the measured rate con-
stants should reflect events associated with the reaction of the
last ligand molecule with hemoglobin. According to allosteric
models (1, 8, 9), they represent the combination and dissociation
of oxygen from the high affinity conformation of hemoglobin,
i.e. the unconstained (R) state:

$$\text{Hb}_4^R(CO_3) + O_2 \overset{k_{on}}{\underset{k_{off}}{\rightleftharpoons}} \text{Hb}_4^R(CO_3)O_2$$

(2)

In rapid mixing experiments with dithionite, the observed
first order rate constant tends to approach a value very similar
to that obtained by the replacement method, i.e. the dissoci-
ation rate constant of oxygen from hemoglobin maintained fully
saturated during the dissociation. Therefore the value obtained
by dithionite at high enough proportions of HbCO can be identi-
fied with $k_{off}$ in Scheme 2.

In temperature jump experiments, the sites combined with
CO should be essentially "frozen" and relaxation effects pertain-
ing to these sites should be, and indeed are, experimentally unob-
servable for two reasons: (a) the high affinity constant for CO
does not allow a significant dissociation of the ligand under the
conditions used, as shown by the fact that $\Delta A$ (absorbance) $\rightarrow 0$
when $[\text{HbCO}] / [\text{HbO}_2]$ $\rightarrow \infty$; and (b) any possible re-equili-
bration would be much too slow to be observed in a temperature

**Table I**

<table>
<thead>
<tr>
<th>Hb concentration</th>
<th>$A_F$</th>
<th>Range of $\bar{V}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb heme %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.9 $\times 10^{-5}$</td>
<td>15</td>
<td>0.60-0.95</td>
</tr>
<tr>
<td>9.2 $\times 10^{-5}$</td>
<td>25</td>
<td>0.20-0.93</td>
</tr>
<tr>
<td>2.1 $\times 10^{-5}$</td>
<td>25</td>
<td>0.65-0.88</td>
</tr>
<tr>
<td>4.1 $\times 10^{-5}$</td>
<td>20</td>
<td>0.45-0.95</td>
</tr>
</tbody>
</table>

- The contribution of the protein concentration-dependent
  relaxation time (see Reference 6 and "Introduction") has been
taken into consideration.

**Figure 3.** Dependence of the reciprocal relaxation time for the
fast process ($1/\tau_F$) on the $O_2$ concentration. Conditions: pH 7,
0.2 M phosphate buffer, and 25° (after the jump). Different per-
centages of Hb$O_2$ in the mixture are as follows: 30% (X, +, O,
$\Delta$); 20% (●, ▼); 10% (●). Different total protein concentra-
tions (in heme) are as follows: 3 $\times$ 10$^{-5}$ M (●, X, +, O,
$\Delta$); 4 $\times$ 10$^{-5}$ M (●, ▼); 0.5 $\times$ 10$^{-5}$ M (○,
$\Delta$); 0.15 $\times$ 10$^{-5}$ M (●).

**Table II.**

**Equilibrium and kinetic constants for $O_2$ binding to isolated $\alpha$
and $\beta$ chains (1) and to Hb$CO$.Hb$O_2$ mixtures**

<table>
<thead>
<tr>
<th>Mixture$^a$ (35°)</th>
<th>$k_{on} \text{ (m}^2 \text{s}^{-1})$</th>
<th>$k_{off} \text{ (s}^{-1})$</th>
<th>$K_{eq} \text{ (m}^3)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>α chain (20°)</td>
<td>$4.8 \times 10^7$</td>
<td>$5 \times 10^7$</td>
<td>$5 \times 10^7$</td>
</tr>
<tr>
<td>β chain (20°)</td>
<td>$1.6 \times 10^8$</td>
<td>$1.2 \times 10^8$</td>
<td>$1.4 \times 10^8$</td>
</tr>
</tbody>
</table>

$^a$ See Scheme 2.
$^b$ Calculated from the kinetic constants.

The findings reported in this paper, which are consistent with
the simple expectations outlined above, deserve some further comments. The monotonic increase of the relative amplitude of the faster relaxation phase \(A_1\) with increase in the proportion of HbCO in the mixture indicates that the species responsible for the slower relaxation phase \(A_2\) can be made to be present in vanishingly small concentrations under proper conditions. At high percentages of HbCO, the observable relaxation time is dependent upon \(O_2\) concentration in a simple fashion in accordance with Equation 1, and this shows that it represents a ligand-binding step. The observed second order rate constant \(4.8 \times 10^7 M^{-1} s^{-1}\) may be identified, most probably, with the combination velocity constant in Scheme 2, i.e. \(k_{on}\). Although there are no measurements of the same type available to us with which this figure may be compared, its value is similar to that reported by Schuster and Ilgenfritz (6) for sheep hemoglobin at pH 9.1 and 10, following the reaction at very high \(O_2\) saturation (see “Introduction”). Moreover it is consistent with the value of \(k^* \approx 3 \times 10^4 M^{-1} s^{-1}\) obtained in partial photodissociation experiments (2, 3, 10). This point is of particular interest because it emphasizes once more that kinetic information obtained by flash photolysis cannot be underestimated on the grounds that perturbation of the system is obtained by light.

It may be relevant to consider the results reported here in conjunction with other experiments on intermediate forms. The behavior of HbCO-HbO\(_2\) mixtures resembles, in some respects, that of artificial intermediates in which either the \(\alpha\) or the \(B\) chains are frozen in the ligand bound form (11, 12), and may be correlated with the behavior of hemoglobin in partial photodissociation experiments (13). A conclusion reached from these studies was that the fast combination with ligands cannot be uniquely ascribed to the last (fourth) step in the sequence of reactions leading to saturation of hemoglobin, but that fast rates may appear also at other stages due to conformational transitions which may become kinetically relevant.

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REFERENCES


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3 Temperature jump experiments on the artificial CN-met intermediates have shown that more than one relaxation phase is observable, although they are predominantly fast (E. Antonini, M. Brunori, and K. H. Winterhalter, unpublished observations).
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