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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- (HbO_2) 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 O_2 and CO will be formed.

In the stopped flow experiments mixtures containing different proportions of the two ligands have been mixed with sodium dithionite ($Na_2S_2O_4$), which rapidly reduces to zero the O_2 concentration in the system. The apparent dissociation velocity constant for O_2 (k_{off}) 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 HbO_2 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 is progressively increased. At high enough percentage of HbCO ($\geq$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 O_2 is buffered). The apparent second order velocity constant is $k_{on} = 4.8 \times 10^7$ M^{-1} s^{-1} at 25°.

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.[1] 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 $\times 10^6$ M^{-1} s^{-1} for sheep hemoglobin at pH 9.1 and 20°) 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 O_2 concentration (5); according to Schuster and Ilgenfritz (6) the apparent combination velocity constant reckoned from the O_2 dependence of τ_F at very high O_2 saturations is 5×10^7 M^{-1} s^{-1} for sheep hemoglobin (at pH 9.1 and 10°). 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

[1] As shown by Schuster and Ilgenfritz (6), when experiments are performed at low protein concentrations, a third relaxation effect is evident. The protein concentration dependence of both the amplitude and the relaxation time of this process indicates that it reflects a ligand-linked association-dissociation equilibrium, probably the dimer-tetramer equilibrium.

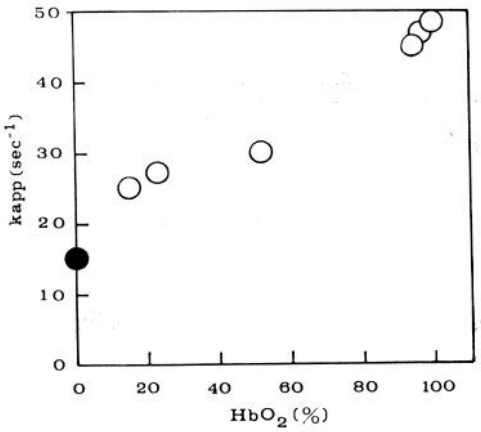

FIG. 1. Dependence of the apparent dissociation velocity constant for O_2, measured by the dithionite method, on the percentage of oxyhemoglobin present in the HbO_2-HbCO mixture. Conditions: pH 7, 0.2 M phosphate buffer, and 20°. Total protein concentration = 3×10^{-5} M in heme (after mixing). The value on the *intercept* indicates the apparent dissociation velocity constant obtained by replacement of O_2 with CO (results of R. W. Noble, personal communication).

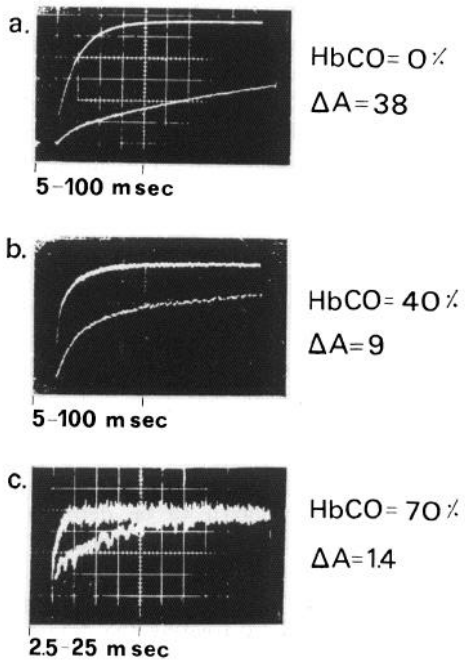

FIG. 2. Temperature jump experiments on the reaction of hemoglobin with O_2. From *top* to *bottom*, the percentage of HbCO in the original material is increased from 0% to 70% (indicated). The observed absorbance changes (ΔA) for a ~5° jump are also reported, in 10^{-3} O.D. units (1 cm observation cell). Conditions: pH 7, 0.2 M phosphate buffer and 25° (after the jump). Total protein concentration = 3×10^{-5} M in heme; oxygen concentration = 1.35×10^{-5} M (in heme). λ = 435 nm.

jump experiments on the reaction of mixtures of oxy- and carbon monoxide hemoglobin with oxygen will be reported. They have been undertaken with the idea of gaining direct information on the reaction kinetics of intermediate forms.

MATERIALS AND METHODS

Human hemoglobin, prepared by the ammonium sulfate procedure (1), was deionized using a mixed bed ion exchange column. Its concentration was determined using published values for the extinction coefficients (1). Oxy- and carbon monoxide hemoglobin were mixed in the appropriate proportions to yield the desired fractional saturation, all manipulations being performed with the aid of syringes in the absence of a gas phase. After appropriate equilibration time, the fraction of hemes occupied by CO was checked spectrophotometrically and found to be equal to the calculated one within a few per cent.

All experiments were performed in 0.2 M potassium phosphate buffer at pH 7.

Stopped flow measurements were performed using a Gibson-Durrum instrument equipped with a 2-cm observation tube.

Temperature jump results were obtained using an instrument built by Messanlagen (Göttingen) through the courtesy of Prof. L. de Maeyer. In the relaxation experiments the output of the photomultiplier was displayed on a dual beam oscilloscope (Tektronix 565), the time basis of which was set at different sweep times. Therefore, it was possible to obtain, *in the same experiment*, an accurate determination of both the critical part of the event and the base-line, a fact which was found essential for an accurate and reproducible analysis of the data.

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 O_2 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 HbO_2 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° is about 15 s^{-1}.[2] 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. 2*a*) the relaxation spectrum consists of two processes, in agreement with previous observations (5, 6) (see "Introduction"). The relative amplitude of the faster process (A_F) 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:HbO_2) in the original mixtures is increased, until finally it becomes prominent. The dominance of A_F 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

[2] R. W. Noble, personal communication.

smaller as the percentage of HbCO in the mixture increases, and tends to zero for HbCO approaching 100% (see "Discussion").

The faster relaxation time (τ_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 ($\overline{O_2}$) exceeds the concentration of the free sites ($\overline{Fe}$). Therefore, being ($\overline{O_2}$) $\gg$ ($\overline{Fe}$), the reciprocal relaxation time increases linearly with the concentration of free O_2 (Fig. 3), following the behavior expected for a simple bimolecular process:

$$(1/\tau_F) = k_{off} + k_{on}(\overline{O_2}) \quad (1)$$

The measured rates are essentially independent of the total protein 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 ($\overline{O_2}$) = 0 is consistent with the value of the dissociation velocity 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 $\times$ 10^7 M^{-1} s^{-1} (at 25° and pH 7). Table II, showing a comparison of the observed kinetic parameters with the constants obtained for the isolated α and β chains, brings out the similarities between the two sets of values.

DISCUSSION

On the assumption that, given enough time, O_2 and CO will distribute 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 HbO_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.*, $Hb_4(CO)_2(O_2)_2$ and $Hb_4(CO)_1(O_2)_3$) 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 constants 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 unconstrained (R) state:

$$Hb_4^R(CO)_3 + O_2 \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} Hb_4^R(CO)_3(O_2) \quad (2)$$

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 dissociation 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 identified with k_{off} in Scheme 2.

In temperature jump experiments, the sites combined with CO should be essentially "frozen" and relaxation effects pertaining to these sites should be, and indeed are, experimentally unobservable 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 ΔA (absorbance) $\rightarrow$ 0 when [(HbCO)/(HbO_2)] $\rightarrow$ ∞; and (*b*) any possible re-equilibration would be much too slow to be observed in a temperature jump instrument. Therefore, at high enough concentrations of CO only one relaxation time is observed, in accordance with Equation 2.

The findings reported in this paper, which are consistent with

TABLE I

Relative amplitude of fast relaxation time (A_F) in the reaction of human hemoglobin with oxygen

Conditions: pH 7, 0.2 M phosphate buffer, 25°.

Hb concentration	A_F[a]	Range of $\bar{Y}$
M heme	%	
9.8×10^{-5}	15	0.60–0.95
9.2×10^{-5}	25	0.20–0.95
2.1×10^{-6}	25	0.65–0.88
4.1×10^{-6}	20	0.45–0.95

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

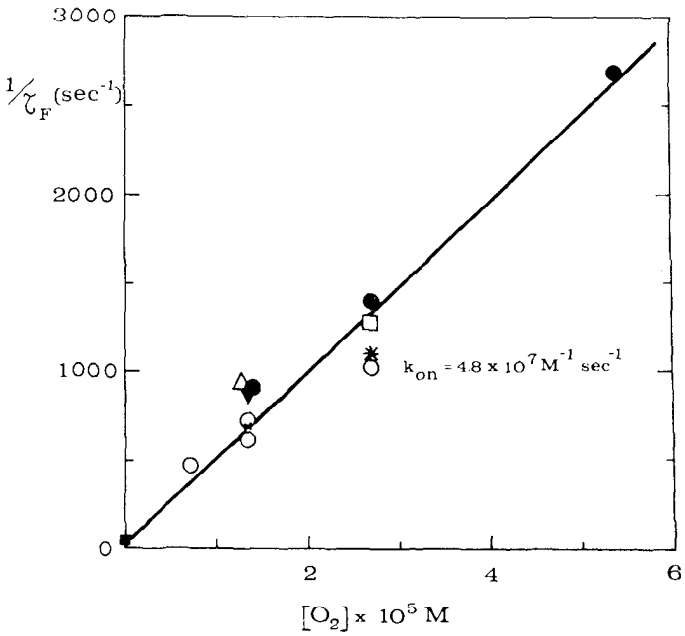

FIG. 3. Dependence of the reciprocal relaxation time for the fast process (τ_F^{-1}) on the O_2 concentration. Conditions = pH 7, 0.2 M phosphate buffer, and 25° (after the jump). Different percentages of HbO_2 in the mixture are as follows: 30% (×, □, ○, △); 20% (●, ▼); 10% (✳). Different total protein concentrations (in heme) are as follows: 3 to 12.4 $\times$ 10^{-5} M (●); 0.8 to 3 $\times$ 10^{-5} M (○); 1.5 $\times$ 10^{-5} M (▼, ×); 0.75 $\times$ 10^{-5} M (□); 0.5 $\times$ 10^{-5} M (△); 0.15 $\times$ 10^{-5} M (✳).

TABLE II

Equilibrium and kinetic constants for O_2 binding to isolated α and β chains (1) and to HbCO-HbO_2 mixtures

	Mixture[a] (25°)	α chain (20°)	β chain (20°)
k_{on} (M^{-1} s^{-1})	4.8 $\times$ 10^7	5 $\times$ 10^7	6 $\times$ 10^7
k_{off} (s^{-1})	30	28	16
K_{eq} (M^{-1})	1.6 $\times$ 10^{6}[b]	1.2 $\times$ 10^6	1.4 $\times$ 10^6

[a] See Scheme 2.

[b] Calculated from the kinetic constants.

the simple expectations outlined above, deserve some further comments. The monotonic increase of the relative amplitude of the faster relaxation phase (A_F) with increase in the proportion of HbCO in the mixture indicates that the species responsible for the slower relaxation phase (τ_S) 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 (4.8×10^7 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 $k^* \simeq 3 \times 10^7$ 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 α or the β chains are frozen in the ligand bound form (11, 12),[3] 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.

[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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