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Kinetics of the Reaction between Oxygen and Haemoglobin Bound to Haptoglobin

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The kinetics of the reaction between O_2 and haemoglobin bound to haptoglobin 1–1 were investigated by the stopped-flow and temperature-jump techniques. The reaction, which follows second-order kinetics in the lower concentration range, becomes independent of O_2 concentration above about 150μM-O_2.

The structural and functional properties of the complexes between haptoglobin and haemoglobin have been the object of considerable interest (Pintera, 1971); however, the reaction between O_2 and haemoglobin bound to haptoglobin has received comparatively limited attention, since only equilibrium properties have been investigated. The available data show that the O_2 affinity of haemoglobin bound to haptoglobin is much higher than that of the free protein, and that both homotropic and heterotropic interactions have disappeared (Nagel *et al.*, 1965).

We now report experiments performed to measure the kinetics of O_2 combination and dissociation with haemoglobin bound to haptoglobin. The kinetics of the reaction between O_2 and tetrameric haemoglobins lacking haem–haem interactions have not been carefully investigated so far.

Human haemoglobin and human haptoglobin 1–1 (three specimens) were prepared according to previously published procedures (Waks & Alfsen, 1966; Antonini & Brunori, 1971). Most of the experiments were performed with a 2:1 molar ratio of haptoglobin (mol.wt. 85000) to haemoglobin (mol.wt. 64500). Under these conditions the ligand (CO) binding and the redox properties of the mixture correspond to those of a single reacting species, although two types of complex exist in which either a haemoglobin tetramer ($\alpha_2\beta_2$) or a dimer ($\alpha\beta$) is bound to one haptoglobin molecule (Nagel & Gibson, 1966; Brunori *et al.*, 1968; Alfsen *et al.*, 1970). In some experiments, however, a 1:1 molar ratio of haptoglobin to haemoglobin was used. Under these conditions the combination with CO and the redox titration reflect heterogeneity in the system, although only one type of complex, made by one haemoglobin tetramer and one haptoglobin molecule, appears to be present (Brunori *et al.*, 1968; Alfsen *et al.*, 1970). O_2-equilibria, stopped-flow and temperature-jump experiments were performed according to standard procedures (for references see Antonini & Brunori, 1971).

The O_2-equilibrium properties of the samples with a 2:1 molar ratio of haptoglobin to haemoglobin, in agreement with the results obtained by Nagel *et al.* (1965), yielded values of n of 1.00–1.05 and $p_{\frac{1}{2}}$ of 59–61 Pa (0.44–0.46 mmHg) at 20°C and at haemoglobin concentrations of approx. 4 mg/ml (see Table 1). For the same specimen, also, the reaction with CO was followed by the stopped-flow technique. In accordance with expectations based on previous work (Alfsen *et al.*, 1970), the combination of CO with the bound haemoglobin followed a second-order time-course, with a rate constant similar to the previously reported value (4.7×10^6 M$^{-1}\cdot$s^{-1}).

Temperature-jump experiments, performed on the same haptoglobin–haemoglobin mixture (2:1 molar ratio) in equilibrium with O_2, show that, within the accuracy of the experiments, a single exponential process is sufficient to describe the approach to equilibrium. The relaxation time is dependent on O_2 concentration, indicating that the observed process reflects a ligand-binding step. Under all conditions the concentration of free ligand, $\overline{O_2}$, largely exceeds the equilibrium concentration of free sites, $\overline{Fe}$; therefore in Fig. 1 the reciprocal of the relaxation time, i.e. $1/\tau$, is plotted as a function of $\overline{O_2}$. Up to an O_2 concentration of approx. 0.1 mM the relationship between the two parameters follows approximately the behaviour expected for a simple second-order reaction, when $\overline{O_2} \gg \overline{Fe}$:

$$1/\tau = k + k' \cdot \overline{O_2} \qquad (1)$$

From the linear increase of $1/\tau$ over this O_2 concentration range the second-order combination velocity constant, k', can be calculated by applying eqn. (1). The observed value is $k' = 5\times10^7$ M$^{-1}\cdot$s^{-1} at 25°C. This value is indeed very close to that characteristic of rapidly reacting forms of haemoglobin (Brunori & Antonini, 1972), and this in turn is similar to that of the isolated α- and β-chains of human haemoglobin (Antonini & Brunori, 1971). However, at higher O_2

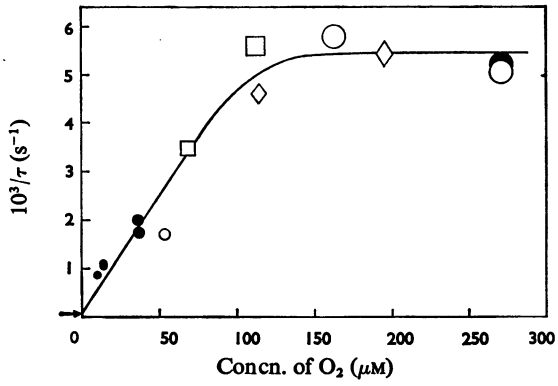

Fig. 1. *Dependence on O_2 concentration of the reciprocal of the relaxation time in the reaction of O_2 with haemoglobin bound to haptoglobin (haptoglobin/haemoglobin molar ratio* 2:1)

The haemoglobin concentration ranged from 1.5 to 15 μM (with respect to $\alpha_2\beta_2$ tetramer). Different symbols refer to different experiments; their size gives an indication of the approximate experimental error. The arrow indicates the overall value of the dissociation velocity constant on the basis of stopped-flow experiments.

concentrations (above 0.1 mM) the observed relaxation time appears to level off at a value of about 5000–6000 s^{-1}.

The reaction of simple monomeric haemoproteins with O_2 follows second-order kinetics up to very high first-order rates, and in general even at high O_2 concentrations shows no sign of the appearance of rate-limiting steps. As a recent example of this may be quoted the reaction of *Chironomus* haemoglobin with O_2, for which $1/\tau$ is linear with respect to O_2 concentration up to rates of more than 8000 s^{-1} (Amiconi *et al.*, 1972). Therefore, although a molecular interpretation for the presence of a rate-limiting step is not available, the system is one where the interaction with haptoglobin may impose constraints on the conformational flexibility of haemoglobin. Parkhurst & Gibson (1967) have provided evidence that rates of conformational decays are slowed down when the protein is in the crystalline state.

With a 1:1 molar ratio of haptoglobin to haemoglobin more than one exponential process was observed in temperature-jump experiments. Qualitatively this finding is in agreement with the heterogeneity displayed in the CO-combination kinetics (Alfsen *et al.*, 1970).

The dissociation of O_2 was measured in a stopped-flow apparatus by the dithionite method. The time-course of O_2 dissociation shows two distinct components, with relative percentages of about 40% for the faster one and about 60% for the slower, the distribution being independent of observation wavelength (from 416 to 445 nm). This finding was reproduced with two preparations of haptoglobin, the molar ratio of haptoglobin to a haemoglobin always being kept at 2:1. However, somewhat different values of the rate constant were measured for the two preparations. The observed values are reported in Table 1. The interpretation of the heterogeneity in the O_2-dissociation process cannot be explained in terms of the two types of complex existing in mixtures containing a 2:1 molar ratio of haptoglobin to haemoglobin, since the relative amounts of the complexes are about 85% for haptoglobin–haemoglobin $\alpha_2\beta_2$ tetramer and about 15% for haptoglobin–haemoglobin $\alpha\beta$ dimer (Waks *et al.*, 1969). It may possibly be related, in the light of previous work (Olson *et al.*, 1971), to a difference in the intrinsic k_{off} for the α- and β-chains in the complex, although no dependence of the relative amounts of the two components with wavelength was noticed. Since a single value of τ was observed in temperature-jump experiments, and in view of the dominant contribution made by the second-order term $k' \cdot \overline{O_2}$ to the speed of approach to equilibrium, differences in the 'on' constant between the two types of chains may not exceed a factor of 2–3.

Table 1. *Equilibrium and kinetic constants for the reaction between O_2 and haemoglobin bound to haptoglobin (haptoglobin/haemoglobin molar ratio* 2:1)

The reaction was studied in 0.1 M-potassium phosphate buffer, pH 7, at 20°C.

$K_{eq.}$ (M^{-1})	1.4×10^6	Experimental
k' ($M^{-1} \cdot s^{-1}$)	4.5×10^7*	Experimental
k (s^{-1})	30†	Calculated
k (s^{-1})	8–14 (slow component)	Experimental
	54–65 (fast component)	Experimental

* Calculated from the value at 25°C ($5 \times 10^7 M^{-1} \cdot s^{-1}$) on the basis of an activation energy of about 17 kJ/mol (4 kcal/mol).

† Calculated from $K_{eq.}$ and k', and therefore to be taken as an average value.

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