Kinetics of the reaction of intraerythrocytic haemoglobin by single cell microspectroscopy: effect of shape and osmolarity

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Received 22 July 1985

The kinetics of the reaction of CO with intraerythrocytic haemoglobin has been studied in single red blood cells (RBC) using a scanning microspectrophotometer and a photochemical perturbation method. Measurements have been carried out using red blood cells from man and camel *(Camelus dromedarius)*, the latter at different osmotic pressures. Camel RBC, which are smaller and different in shape compared to human RBC, are known to remain intact even at an osmolarity 6-times lower than physiological (280–290 mosm/l), swelling up to twice their normal volume. The results show that the recombination time course is affected by diffusion of CO through a stagnant layer of solvent around the cell membrane, but that it is also influenced by other parameters such as intracellular diffusion of ligand and haemoglobin.

Microspectroscopy *Camel hemoglobin* *Red blood cell* *Diffusion* *Osmolarity* *Cell volume*

1. INTRODUCTION

The reaction of gaseous ligands, such as O_2 and CO, with intracellular human haemoglobin (Hb) was studied by Hartridge and Roughton [1] long ago. This work has been recently reinvestigated in both red blood cell suspensions [2] and single erythrocytes [3,4]. Kinetic studies in single red blood cells (RBC) have indicated that rebinding of a ligand is characterized, in the case of both O_2 and CO [3,4], by zeroth-order kinetic behaviour, which led to the hypothesis that the observed recombination process is rate limited by ligand diffusion into the RBC (see also [2]). However, calculations have indicated that the barrier to diffusion is not likely to be represented by passage through the cell membrane [2,3] and therefore it was suggested that ligand diffusion occurs through a stagnant, unstirred extracellular layer of solvent, whose thickness may be as much as 5–10 μm in stationary RBC [3].

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Since the observation volume is much smaller than an RBC (i.e. 1–2 μm^3), the possible role of intracellular Hb concentration on the recombination rate process may be important, and therefore we have attempted to probe the effect of this variable. To achieve a change in the RBC volume through changes in the osmolarity of the extracellular medium, we have extended the investigation to low osmotic pressure, and thus have used RBC from *Camelus dromedarius*, which are known to remain intact even at an osmolarity as low as 50 mosm/l, that is 6-times below the iso-osmotic pressure (280–290 mosm/l), swelling in volume up to twice the normal value [5].

2. MATERIALS AND METHODS

Human and camel RBC were washed 3 times (dilution 0.1%) with iso-osmotic buffer (0.12 M NaCl, 2.4 mM KCl, 15 mM Na_2HPO_4, 2.5 mM NaH_2PO_4, 5.5 mM dextrose and 1 g/l of bovine serum albumin), brought to pH 7.4 ± 0.02 and the suspension centrifuged at 3000 rpm. After

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00145793/85/$3.30

washing, the RBC suspension was kept in the cold (4°C) in this buffer until suspended in the final buffer used for the experiment. In such a final buffer, equilibrated with CO at different pressures, the concentrations of KCl and NaCl were inverted to compensate for the slight potassium leakage through the membrane due to the addition of sodium dithionite at a final concentration of 10 mM, necessary to ensure complete deoxygenation. Low osmolarity buffer (50 mosm/l before the addition of sodium dithionite) was prepared by decreasing the concentration of KCl; the camel RBC, once suspended in the low osmolarity medium, were left to equilibrate for about 45 min before the experiment.

3. RESULTS AND DISCUSSION

Observations within a single RBC have been made possible by the use of a microspectrophotometer equipped with a computer-controlled fast-moving substage condenser [6] and a continuous light source to photolyse the CO bound to Hb [3]. As photolysis begins, the intracellular concentration of CO becomes higher than the highest extracellular concentration at 1 atm partial pressure (i.e. 1 mM CO), and therefore, in a short time ($t_{1/2} \simeq 1$ s) Hb becomes completely deoxygenated. In view of the volume ratio between the inside and outside, when the system has attained a steady state (i.e. when the intracellular and extracellular concentration of CO are the same) and the light is switched off, recombination of Hb demands diffusion of extracellular CO into the red RBC for binding to occur. Since the chemical reaction between intracellular Hb and CO is much faster than the observed recombination in a single RBC [7,8], the steady-state concentration of free ligand approaches zero at the internal boundary of the RBC and passage through the layer would represent an important step in determining the overall rate.

As reported [3], irradiation with a 100 W high-pressure mercury lamp leads to rapid and complete photodissociation of intracellular Hb; the time course of the photodissociation is not a single exponential and is not analyzed here in detail.

When the photodissociating light is switched off, recombination of CO with intracellular Hb in camel RBC follows zeroth-order behaviour,

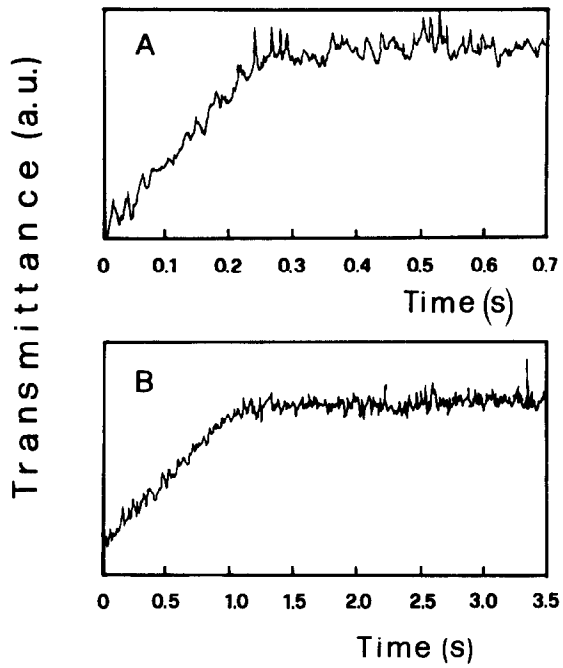

Fig.1. Time course of binding of CO to Hb in a single RBC from camel. [CO] = 0.5 mM. Temperature, 25°C. λ = 430 nm. Note the difference in time scale between (A) 50 mosm/l and (B) 280 mosm/l.

similar to that previously observed in human erythrocytes [3]; this feature is maintained at every osmotic pressure examined (see fig.1) and at all CO concentrations.

Experiments carried out at iso-osmotic pressure indicate that, at [CO] = 0.5 mM, the ligand binds to human RBC with an overall halftime similar to that of camel RBC. Since the diffusion constant for CO in the extracellular layer is always 2×10^{-5} cm^2/s and camel RBC display a smaller volume than human RBC (see fig.2 and [5]), a faster recombination process is expected for camel RBC when the unstirred layer is the only parameter affecting the recombination process. This need not be true if a larger value is postulated for the thickness of the layer; however, if one considers the similarity of the CO binding process and the different volume of the two different erythrocytes, this yields an unreasonable value for the thickness of the layer (~50–100 μm) which, in turn, suggests that other factors come into play to characterize the observed rate constant of the binding process. A likely candidate is the intracellular Hb concen-

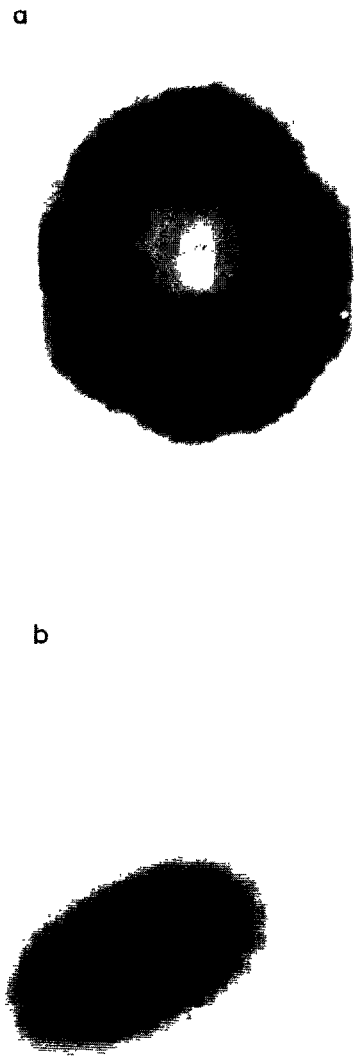

Fig.2. Images of human (a) and camel (b) red blood cells as obtained by a computer-controlled point-by-point measurement of the light transmittance at 436 nm. The difference between the two red cells in terms of shape, volume and intracellular distribution of Hb is evident.

tration, which is higher in camel RBC than in human RBC [5], and which is known to modulate the diffusion rate constant for CO [9]. This role of the intracellular Hb concentration seems to be also supported by the observation that, at [CO] $\leqslant$ 0.5 mM, a decrease of the medium osmolarity (and thus a swelling of the cell volume and a decrease of the intracellular Hb concentration) is associated with a shorter recombination halftime (see table 1). It should be noted that the relatively large deviation (apparent from table 1) for any set of experimental conditions is likely to be due to the variability in the Hb content among individual RBC, which, for a zeroth-order process, will be reflected in the halftime.

Furthermore, if the progress curves are analyzed in terms of the slope (i.e. the absorbance change

Table 1

CO dependence of $1/t_{1/2}$ (s^{-1}) for CO recombination to intracellular Hb at different osmotic pressures

[CO] (mM)	Iso-osmotic pressure (280 mosm/l)	Hypo-osmotic pressure (50 mosm/l)
Camel RBC		
0.2	1.26 ± 0.21	3.27 ± 0.33
0.5	1.70 ± 0.48	5.02 ± 0.77
1.0	9.20 ± 2.17	8.59 ± 0.88
Human RBC		
0.5	2.53 ± 0.45	

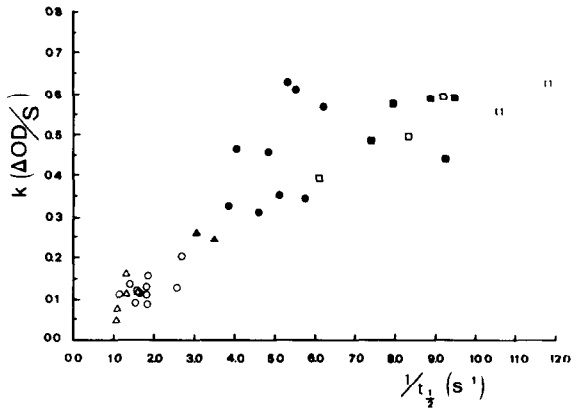

Fig.3. Correlation between k ($\Delta A/s$) and $1/t_{1/2}$ of the CO recombination time course in camel RBC. Open symbols refer to erythrocytes suspended at iso-osmotic pressure and closed symbols at hypo-osmotic pressure (50 mosm/l). (△, ▲) 0.2 mM CO, (○, ●) 0.5 mM CO, (□, ■) 1.0 mM CO.

per unit time interval) according to a zeroth-order equation:

$$- dA_{Hb}/dt = k \quad (1)$$

(where A_{Hb} is the absorbance of deoxyHb at 430 nm, in our case) k yields, independent of the total amount of intracellular Hb, a measure of the diffusion velocity of CO from the extracellular medium to the observation spot (expressed as the ratio of the absorbance change per s ($\Delta A/s$).

From fig.3 it is evident that k also depends on osmotic pressure, at least at [CO] $\leqslant$ 0.5 mM and, although for a constant ligand concentration the effect is not very large (approx. 3-fold), is clearly outside the uncertainty limits. Thus, the faster time course seen at lower osmolarity suggests that the observed diffusion rate is indeed also affected by some intracellular parameter (which further limits the intracellular CO diffusion at iso-osmotic pressure) and it seems reasonable to relate it to a decrease in the intracellular Hb concentration. However, this interpretation is complicated by the fact that halftimes and slopes are independent of osmolarity at 1 mM CO, which may indicate that under these conditions other factors contribute more than intracellular Hb concentration to the observed rate.

Such a similarity at 1 mM extracellular CO also rules out the possibility that the increased cell surface, related to the swelling at lower osmolarity, might play a major role in contributing to the enhanced rate of ligand diffusion.

In human RBC the physiological ligand, O_2, displays a combination halftime shorter than CO [2–4], although the reaction time course is close to zeroth order. This phenomenon has been related to the facilitated diffusion [10,11] of O_2 through an Hb solution, which is absent in the case of CO and which plays an important physiological role (especially for myoglobin).

These results open the way to a more extended investigation of the parameters which contribute to the governing of reaction rates at or through membranes near unstirred layers of solvent, a problem of general significance in cellular biophysics. Understanding the contributions of intracellular protein concentration should allow a closer to the 'in vivo' situation and to a more quantitative approach for the description of the role played by diffusion on the kinetic parameters which characterize O_2 transport in RBC.

ACKNOWLEDGEMENT

A special grant from the Scientific Commission of the University of Rome La Sapienza, which was given to M.B. to build a fast-scanning computerized microspectrophotometer, is gratefully acknowledged.

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