KINETICS OF THE REACTION WITH CO OF HUMAN ERYTHROCYTES

Observations by single cell spectroscopy

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1. Introduction

A study of the kinetics of ligand reactions of Hb within single red blood cells (RBC) is obviously of great interest to correlate molecular events to more general cellular phenomena, both physiological and pathological. In this note we report an investigation of the kinetics of reaction of intraerythrocytic Hb with CO followed in single RBC by the use of a photochemical technique coupled to microspectrophotometric observation.

Two lines of application are illustrated by the experiments reported below.

(i) We have re-investigated the problem of the relationships between diffusion and chemical reaction in erythrocytes. It is well known [1–3] that total resistance to gas exchange in the lung and tissues is related to those two phenomena [4]. The basic observation is that the rate of O_2 uptake by Hb in RBC is reduced as compared to the solution properties [1,5]. Among the possible explanations of this phenomenon, the analysis [5] excluded that a layer of unstirred fluid around the cell could be responsible for the drop in reaction rate, and attributed the effect to a high resistance of the erythrocytic membrane to O_2 diffusion. However the role of a possible layer of unstirred fluid around the RBC is not clarified, and is obviously of relevance to the control of the rate of the overall diffusion processes from the bulk of the solution to the inside of the erythrocyte.

(ii) We have deviced a new experimental method to follow the time relationships between deoxygenation of Hb and RBC sickling in erythrocytes of S–S carriers. This approach is of great interest in view of the important role played by the kinetics of sickling in the pathology of sickle cell anemia [6].

2. Experimental

The experiments described here are based on the reversible dissociation of the carbon-monoxide hemoglobin complex which may be induced by light:

$$HbCO \xrightarrow{\text{light}} Hb + CO$$

$$Hb + CO \xrightarrow{\text{dark}} HbCO$$

These reactions may be investigated in different regions of any one RBC under a variety of environmental conditions.

The measurements have been carried out using a modified version of the instrument [7,8] based on a fast condenser-scanning technique. Figure 1 shows a diagram of the apparatus. An auxiliary excitation source (Hg lamp using the 546 nm line) has been

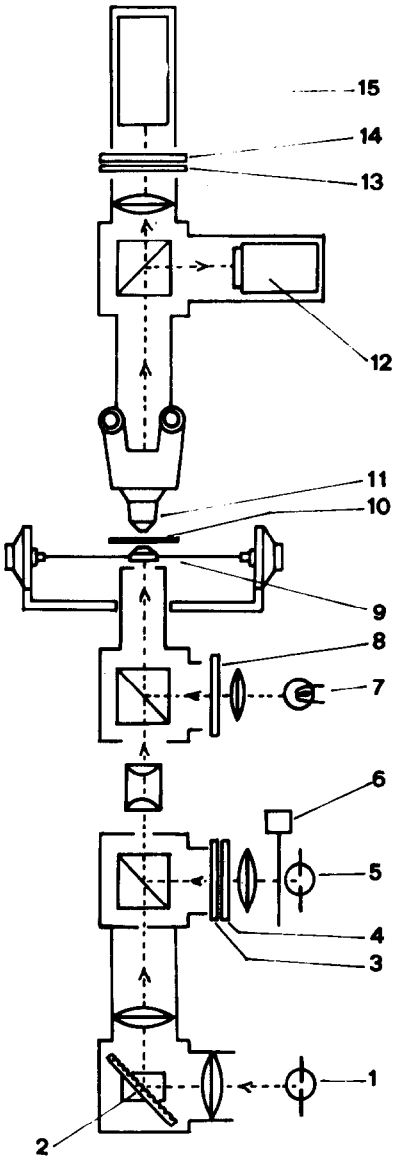

Fig.1. Configuration of the apparatus as used for the experiments described in this paper. (1) Xenon arc lamp XBO 75 W2-2) Grating monochromator. (2) Absorption filter (Schott, BG 38, 4 mm). (4) Interferential filter (Balzers, λ = 546 nm). (5) Mercury arc lamp, HBO 100 W2. (6) Electromechanical shutter. (7) Tungsten lamp. (8) Infrared filter. (9) Moving-condenser unit (70 X 0.90 dry). (10) Sample carrier. (11) Collection optics. (12) Infrared video camera. (13) Filter (Schott, BG 12, 3 mm). (14) Interference filter (Balzers FITC 3). (15) Photomultiplier tube (EMI 96588).

employed introducing a beam splitter and a suitable filter combination in order to eliminate interferences with the analyzing beam. An electromagnetic shutter ($t_{\frac{1}{2}} \sim 5$–10 ms) controls the photolyzing beam which illuminates the whole erythrocyte. Dual beam measurements of the absorption spectra, as well as density maps, can be taken in the range from 380–500 nm, while continuous infrared illumination allows to display the cells on a TV monitor by means of a silicon–vidicon telecamera (fig.1).

RBC suspensions were prepared in isotonic solutions of sodium chloride plus phosphate buffer at pH 7.2 equilibrated with 1 atm CO at 20°C. The CO concentration was changed by dilution of the RBC suspension with the same isotonic gas free solution. Removal of oxygen was assured by addition of a slight excess of sodium dithionite. The total heme content of the samples used was always $\leqslant 10^{-5}$ M.

3. Results

Figure 2 shows the density map, as obtained at λ = 415 nm, of a single erythrocyte fully saturated

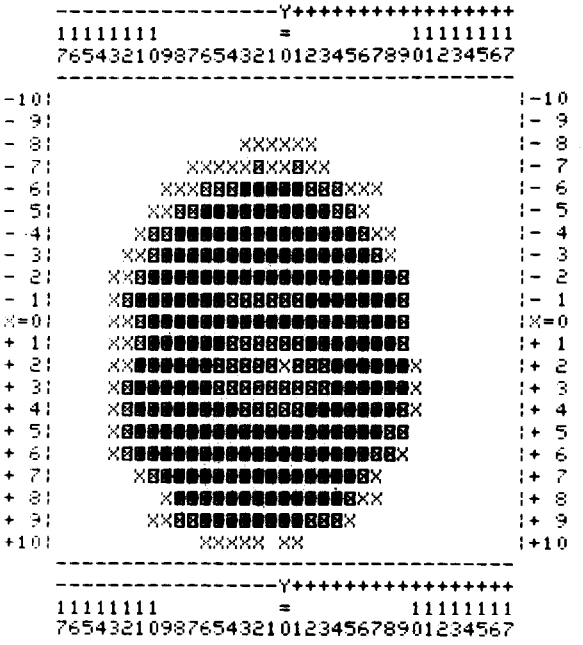

Fig.2. Optical density map of a single erythrocyte at pH 7.2 fully saturated with carbon monoxide ([CO] 10^{-3} M). Observation wavelength λ = 415 nm.

with carbon monoxide ([CO] 10^{-3} M). The scanning has been carried out over an area of 25 X 25 μM with a resolution corresponding to about 1 μm. The optical density, proportional to the hemoglobin content, is encoded in the map using four characters having different darkness. The biconcave disc form of the RBC is revealed by the lower optical density in the center of the cell.

The conditions used for the kinetic studies are such that each erythrocyte is separated from the others by a large distance, i.e., is immersed in a volume of fluid which is infinite as compared to the volume of the cell.

3.1. *Kinetics of CO binding in immobile RBC*

The kinetics of CO release and uptake going from dark to light and viceversa, was studied for a number of erythrocytes as a function of CO concentration ranging from 10^{-4}–10^{-3} M. In all cases photodissociation levels higher than 60% were achieved even at the highest CO concentration. Typical oscillograph traces of an experiment performed at λ = 430 nm, showing the two transitions (activated by light and by dark), are reported in fig.3.

No significant difference in the kinetic properties of several individual cells, and within any single cell among its different spatial regions (central or marginal positions), was observed. Experiments performed on aged red cells of spherical form gave essentially the same kinetic behavior.

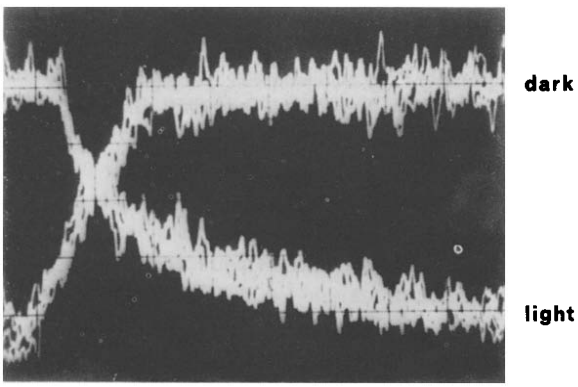

Fig.3. Typical oscillograph traces of an experiment performed at pH 7.2 and [CO] 10^{-3} M. Observation wavelength λ = 430 nm; $T \sim 25°C$; Sweep = 100 ms/grid division.

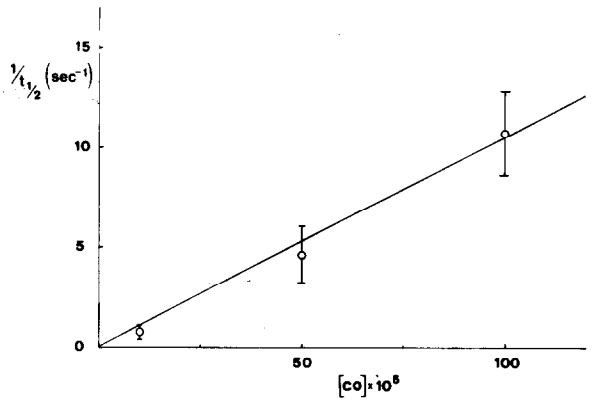

Fig.4. Reciprocal half-time of the light–dark transition as a function of CO concentration. Sodium chloride plus phosphate buffer pH 7.2. Total heme content ≤ 10^{-5} M. $T \sim 25°C$.

In the case of dark–light transitions, the approach to the steady state in the light cannot be accounted by a single exponential process and the half-time is independent, within a factor of 2, of CO concentration from 10^{-4}–10^{-3} M ($t_{\frac{1}{2}}$ = 150–80 ms)*.

The relaxation from the steady state in the light to the equilibrium in the dark, when the light is suddenly turned off, corresponds under all conditions explored to a zero order process. The reciprocal of the half-time, a measure of the zero-order rate constant, increases linearly with the CO concentration over the range examined (fig.4). It is crucial to realize that the data in fig.4 show that apparent combination of CO with Hb within immobile erythrocytes is about 10-fold slower than that measured in analogous experiments by rapid mixing methods ($t_{\frac{1}{2}}$ = 25 ms at [CO] = 5 X 10^{-4} M). In turn this rate of uptake was found to be smaller (by a factor of ~ 2–3) than that measured with hemoglobin in solution, essentially in agreement with [5]. Thus combination of CO with Hb in immobile RBC is rate limited by a diffusive process as indicated also by the zero-order time course of the observed absorbance change. Our interpretation of these findings is related to the presence of an unstirred layer of fluid around the erythrocyte. If this interpretation is correct it may be calculated on the basis of simple diffusion laws and of the diffusion coefficient of CO in water

that the thickness of this layer should be approx. 10 μM*.

The situation which prevails in the blood stream under physiological conditions is obviously represented neither by the violent stirring present in the mixing chamber of mixing experiments nor by the stagnancy of present ones; it should be emphasized again that the observed rate of reaction with CO differs in the two cases by about 10-fold. Thus if the situation 'in vivo' is intermediate between these two conditions, the hydrodynamic situation around the erythrocytes may be a major factor in determining the rate of equilibration of the cells with the respiratory gases.

3.2. *Kinetics of sickling of S–S RBC*

A set of preliminary experiments have been performed in RBC from patients with homozygous sickle cell disease (S–S cells). The method described here in fact allows us to follow RBC sickling by controlling with light the extent of carbon monoxide dissociation from hemoglobin within a single RBC. Apart from the estimate of the rate of RBC sickling following removal of the ligand ($\sim$ 3–5 s in all the cells examined) two main observations seem to be relevant at present to the pathology of sickling within the blood stream.

(1) S–S cells in which sickling was induced photochemically by stripping CO several times, show to have a preferential direction of sickling. In other words, the shape of the RBC is modified by the sickling always in the same manner. This fact may be due either to a certain degree of alteration in membrane elasticity or to the presence of residual polymers which could trigger the stacking simulating a preferential direction of sickling.

(2) Once sickled, the erythrocyte could be maintained in this 'status' by a light intensity several times lower than that which is necessary to induce the sickling.

Although still preliminary these observations should be taken to illustrate the potentialities of the approach in studying both the kinetics of sickling within single RBC and the effects of controlled variables, such as Hb concentration or specific drugs, on this process.

* It is of relevance to point out that J. T. Coin and J. S. Olson have reached a similar conclusion on the basis of extensive stopped-flow experiments with RBC suspension and computational analysis of the reaction with O_2. The authors in their paper (in press in the proceedings of the 'Conference on abnormal hemoglobins', Colorado State University, Pingree Park, Colorado, Oct. 1977) have estimated a layer of unstirred fluid at infinite time of approx. 3 μM which, though smaller, is of the same order of magnitude of our estimate. We thank J. S. Olson for providing his manuscript before publication

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