SINGLE CELL OBSERVATIONS OF GAS REACTIONS AND SHAPE CHANGES IN NORMAL AND SICKLING ERYTHROCYTES

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ABSTRACT Microspectrophotometry has been applied to single red blood cells to reinvestigate the linked processes of diffusion of gases inside the erythrocyte and their combination with hemoglobin. The experiments took advantage of the photosensitivity of the carbon monoxide derivative of hemoglobin, which allows ligand release from the CO-saturated red cells under strong illumination and recombination when the light is switched off. The photochemical method was also used to study the kinetics of sickling on ligand removal in single erythrocytes of Hb S carriers. The results give new information on the mechanism of the sickling process.

INTRODUCTION

Single cell spectroscopy represents a powerful tool to obtain information on the time and space distribution of a great number of intracellular events.

In the case of red blood cells, this technique has now been applied to reinvestigate the linked processes of diffusion of gases inside the cell and their combination with hemoglobin (1–5). Taking advantage of the photosensitivity of the carbon monoxide derivative of hemoglobin, our experimental approach is based on the direct spectrophotometric observation of single cells, within which the reversible dissociation of the HbCO complex may be induced by light: $\text{HbCO} \xrightarrow{h\nu} \text{Hb} + \text{CO}$; $\text{Hb} + \text{CO} \xrightarrow{\text{dark}} \text{HbCO}$.

Thus any single red cell may be subjected to ligand dissociation and binding, with simultaneous monitoring and recording of optical density changes within small areas and of overall morphological changes. This photochemical approach is particularly useful in studying the time relationships between ligand removal and sickling in erythrocytes of Hb S carriers.

Thus, the kinetics of the polymer formation may be studied within any single "*SS*" cell under a variety of environmental and intracellular conditions.

INSTRUMENTATION AND METHODS

Measurements were carried out with an instrument based on a fast condenser-scanning technique described elsewhere (6, 7).

Dual-beam measurements of the absorption spectra, as well as optical density maps, can be taken in the range from 380 to 700 nm, while continuous infrared illumination allows the display of the cells on a TV monitor by means of a silicon-vidicon telecamera.

Photodissociation of intraerythrocytic hemoglobin was obtained with a Mercury lamp (546-nm line) as excitation source and a suitable filter combination to eliminate interferences from the analyzing beam.

Red cell suspensions were prepared in isotonic solutions of sodium chloride plus phosphate buffer at pH = 7.2 equilibrated with 1 atm of CO at 20°C. The CO concentration was changed by dilution of the red cell 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 $\leq 10^{-5}$ M.

RESULTS

Kinetics of Ligand Photodissociation and Ligand Binding in Normal Erythrocytes

The photodissociation levels achieved were in all cases $\geq 60\%$, as shown from the analysis of the absorption spectra of the intraerythrocytic hemoglobin (Fig. 1). Fig. 2 reports typical oscillograph traces of experiments performed at two different CO concentrations, showing the light-activated transition and the dark recombination process, followed at $\lambda = 430$ nm.

DARK TO LIGHT RELAXATION The approach to the steady state in the light cannot be accounted for by a single exponential process and the half-time is independent, at least within a factor of about two, of CO concentration (10^{-4}M $\leq$ CO $\leq$ 10^{-3}M).

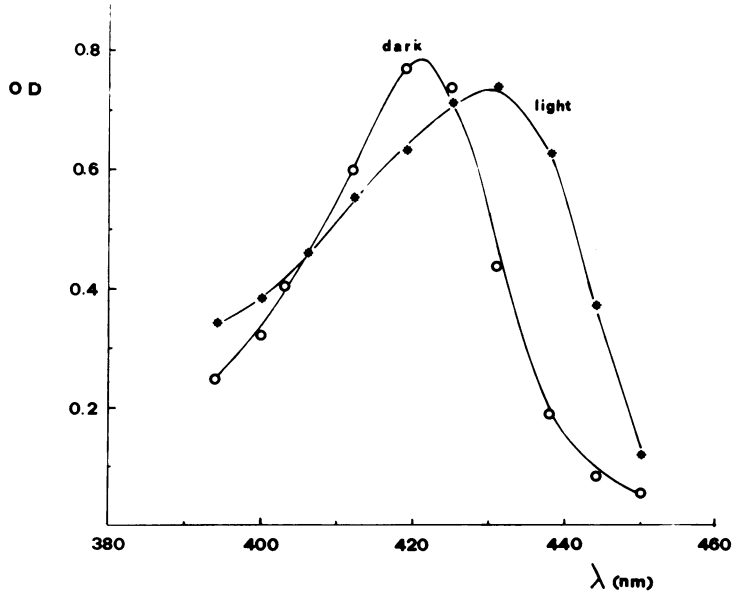

FIGURE 1 Soret absorption spectra of CO and deoxy Hb in a single human erythrocyte, as recorded in the dark and in the light. Conditions: $T \sim 25$°C; CO concentration = 10^{-3}M.

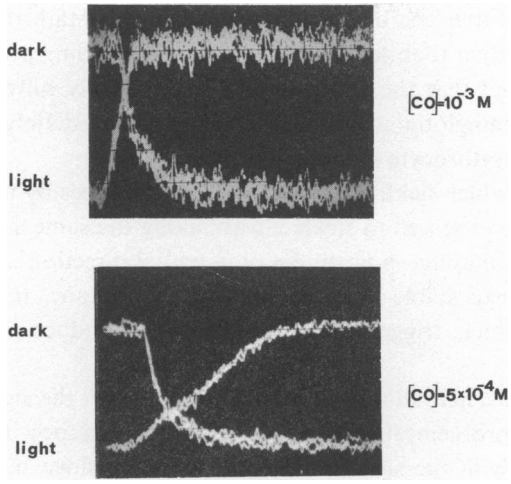

FIGURE 2 Typical oscillograph traces of experiments performed at pH = 7.2, λ = 430 nm, $T \sim$ 25°C, sweep = 100 ms per grid division; carbon monoxide concentration: 10^{-3} and 5×10^{-4}M.

LIGHT TO DARK RELAXATION The relaxation from the steady state in the light to the equilibrium in the dark corresponds under all the conditions explored to a zero-order process (Fig. 2). In the same range of CO concentration the reciprocal of the half-time is ligand concentration-dependent: i.e. increases linearly with carbon monoxide concentration (8). This body of results shows that combination of CO with hemoglobin within erythrocytes is about 30-fold slower than that measured with hemoglobin in solution, and an order of magnitude smaller than that obtained by stopped-flow experiments with red cells.

It should be pointed out that the present experiments are performed on erythrocytes under the limiting condition of complete stagnancy of the surrounding extracellular fluid. Under these conditions, it appears that a layer of unstirred solvent around the cells may become an important factor in determining the rate of equilibration of intraerythrocytic hemoglobin with respiratory gases. A more detailed analysis of data concerned with this particular problem is given elsewhere (8).

Hemoglobin S Aggregation

The photochemical approach described above may allow one, in principle, to obtain information on the time relationships between hemoglobin deoxygenation, polymer formation, and cell deformation within red blood cells of individuals carrying Hb S. It is, in fact, possible to investigate the sickling process by controlling with light the extent of carbon monoxide dissociation from hemoglobin within the *SS* cell, since sickling follows carbon monoxide dissociation, which is photochemically controlled. In general, the photochemical behavior of *SS* cells was similar to that reported above for normal cells. It was found that the time necessary for the cell to sickle completely, i.e., to undergo the full final deformation, ranged in all the cells examined from 3 to 5 s. By reducing the intensity of the photodissociating beam, once the sickling was ob-

tained, we observed that the deformation could be maintained by a light intensity several times lower than that necessary to induce the sickling process. In addition, it should be pointed out that the phenomenon is apparently fully reversible, since full recombination of hemoglobin with CO in the dark is immediately followed by acquisition of the original erythrocyte shape.

Single *SS* cells in which sickling was induced several times by repeatedly turning the light on and off were observed to sickle always along the same axis and with the same apparent rate. This finding, indicating a preferential direction of sickling, may be interpreted as due (*a*) to stable alterations in the membrane, (*b*) to the presence of residual polymers which, triggering the stacking, could induce a preferential direction of sickling.

The results reported here should be taken as examples of the significance of this type of approach to the problem of sickling, for instance in respect to the effect of drugs that may interfere with the sickling process. Finally, since human erythrocytes in heterozygotes contain both Hb A and Hb S, it may be feasible to study the distribution of the two hemoglobins among erythrocytes.

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DISCUSSION

SCHECHTER: A comment submitted by a referee: With regard to the ligand photolysis experiments on normal erythrocytes, the authors report only that there was greater than 60% photodissociation in the steady state, which, from Fig. 2, appears to require between 300 and 400 ms to attain. Is this the best guess possible for the fractional saturation of normal erythrocytes under the photolysis conditions used for these experiments? Furthermore, is this also the best estimate of the amount of photolysis in the experiments on *SS* erythrocytes?