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Single cell microspectroscopy reveals that erythrocytes containing hemoglobin S retain a 'memory' of previous sickling cycles

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Red blood cells from patients homozygotes for hemoglobin S (HbS) have been studied using a computer-controlled microspectrophotometer, which allows measurements of spectra and dynamics to be undertaken in a single erythrocyte. Complete photodissociation of HbCO results in polymerization of intracellular deoxyhemoglobin S and deformation of the cell. This is associated with a delayed optical change, which, for the same cell, was found to be highly reproducible between repeated cycles of sickling. Comparison of photographic records and absorbance time courses indicates that an erythrocyte, once having undergone a photochemically induced sickling event, always deforms along the same axis during subsequent cycles. This behaviour implies that the cell retains a 'memory' of its previous cycle(s), possibly via slow relaxations of the membrane. In addition, rebinding of CO to intracellular hemoglobin was found to be slower if measured after deformation of the cell, with possible important implications for the pathological mechanism of sickling.

Hemoglobin S; Sickle cell anemia; Single-cell microspectroscopy

1. INTRODUCTION

Sickle cell anemia is a hereditary disease characterized by occlusive crises in the capillaries due to the altered shape and rheological properties of the erythrocytes. This phenomenon is known to be caused by the polymerization of the abnormal hemoglobin S (HbS) ($\alpha_2\beta_2$ 6Glu → Val), which rapidly aggregates in the deoxygenated form [1,2] within the erythrocyte. Thus, during oxygen unloading to the peripheral tissues, the formation of polymers of HbS renders the cell much more rigid and may therefore impair its passage through the capillaries.

Kinetic studies of polymerization [3-5] and of cellular deformation [6-9] have made important contributions to the formulation of a molecular mechanism for the sickling process. These investigations showed that a nucleation mechanism [10] is operative not only in solution [4] but also in cells, and that polymerization is an early event taking place in < 1 s for most of the SS cells (i.e. from homozygotes) [5]. On the other hand, cell deformation occurs at somewhat longer times, possibly because sickling requires fiber formation through extensive polymer alignment.

However, since deoxygenation is generally not complete during the transit time in the capillary ($\simeq 1$ s [11]), most SS cells are usually able to escape and (if at all) they polymerize and sickle only in the veins. Melting of polymer and unsickling then occur in the lungs, where hemoglobin is reoxygenated. It is therefore of interest to study the functional dynamic behaviour of sickling cells to unravel possible effects related to multiple sickling cycles, which may occur in the veins.

In this paper we report observations on sickling of red blood cells (RBCs) from patients carrying predominantly HbS, studied by single cell microspectroscopy. Analysis of the time course of absorbance change and photographic records of cell shape indicate that an erythrocyte, once deformed,

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retains a 'memory' of its previous sickling cycles. Moreover, CO rebinding was found to be somewhat slower if measured after sickling. The results are discussed with reference to the pathological mechanism of sickling.

2. MATERIALS AND METHODS

We have followed the dynamics of sickling and unsickling in individual RBCs from various SS patients, making use of a microspectrophotometer which allows the kinetics of photodissociation and/or recombination of carbon monoxide with intracellular hemoglobin (HbCO) to be investigated. The instrument and the experimental approach have been previously described [9,12,13]; the instrument permits optical changes to be monitored inside a single cell with a monochromatic observation beam, whose size can be reduced to a limiting value of 1–2 μm^2. Complete photolysis is achieved by illumination of the whole RBC with a 150 W high pressure Hg lamp (Osram). It is important to mention the possibility offered by this instrument to record photographically the sickling event several times on the same cell, allowing a fairly complete morphological and dynamic documentation on each individual cell.

RBCs, withdrawn from individual patients, were washed with isotonic buffer at pH 7.4 (290–300 mOsm/l) (0.12 M sodium chloride, 2.4 mM potassium chloride, 15 mM disodium hydrogen phosphate, 2.5 mM sodium dihydrogen phosphate, 5 mM dextrose and 1 g/l of bovine serum albumin) and kept at 4°C. RBCs were then suspended in isotonic buffer (where the concentrations of sodium and potassium chloride were inverted), equilibrated to the desired carbon monoxide concentration, and sodium dithionite (up to a final concentration of 20 mM) was added to remove traces of oxygen. A small amount of this suspension was sealed between two coverglasses and experiments were carried out at 27°C.

Observations with RBCs from SS homozygotes (i.e. containing $\leqslant$10% HbF) clearly indicated reversible sickling under the microscope and showed that it was indeed possible to collect reproducible spectrophotometric time courses for photodissociation and recombination of CO, in spite of the shape change of the erythrocyte.

3. RESULTS AND DISCUSSION

Fig.1 reports photographic records of repeated sickling cycles induced in succession for the same cell by rapid ligand dissociation brought about by illumination. The records are documented for two erythrocytes, which are representative of the general behaviour observed. From the morphological standpoint, two features are of particular significance. First, each cell can undergo repeated cycles, each time reacquiring the normal discoid shape in the fully liganded state achieved in the dark. Second, each cell always follows the same pattern of deformation, insofar as the major axis of the sickled erythrocyte retains its orientation from cycle to cycle. Different individual cells display different preferential orientation axes, ruling out the possibility that the shape of the photolysing source (which covers an area of 100 μm^2) might influence this observation. The presence of a preferential axis of sickling, which was reproduced over and over again for all the cells that clearly sickled, suggests that the cell retains a memory of its first deformation, possibly related to a slow relaxation of membrane components distorted by growth of HbS fibers [14].

The time course of absorbance changes in a single cell occurring during its deformation cycle was followed by observations at a single wavelength (e.g. 430 nm where there is a maximum difference in the spectra of CO- and deoxyHb). The succession of these events is illustrated in fig.2, where they are compared to cell shape changes. Initially the cell displays the normal discoid shape containing HbCO, characterized by higher transmittance at 430 nm. Photodissociation of CO, induced by illumination of the whole RBC is as rapid as in normal RBCs, taking place in less than 1 s. As previously reported [9], light flux is sufficient to cause complete deoxygenation of intracellular hemoglobin and it may be noted that HbS polymerization, followed by light scattering, occurs over the same time range for most SS cells [5].

In general it was observed that cells that did not sickle displayed a time-independent plateau under steady photolysis, similar to normal RBCs [9]. However, when the cells sickled, after a lag of 1–2 s transmittance either decreased or increased with time, as indicated in two examples shown in fig.2. This observation was found to be very reproducible for a given cell, several cycles of photodissociation and recombination giving the same optical profile. Comparison with the time dependence of the shape change indicates that such delayed optical changes correspond to cell deformation, with either accumulation or depletion of hemoglobin molecules in the path of the observation beam. The possibility that the second process is due to further photodissociation of residual HbCO is made unlikely by the observation that for some of the sickling cells the initial event gives way to a decrease (rather than an increase) in absorbance at 430 nm (see fig.2).

In agreement with what has been reported for

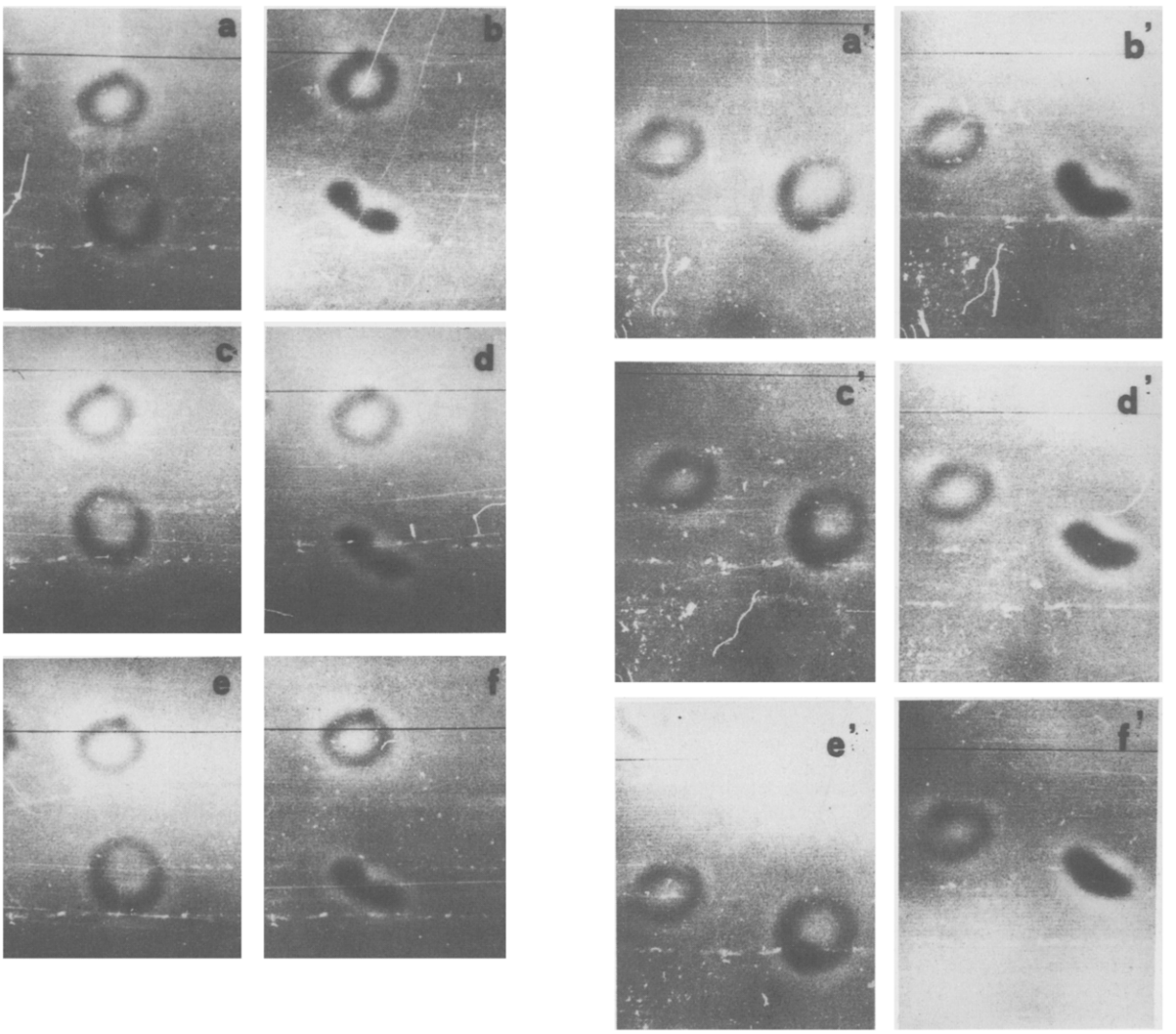

Fig.1. Photographic records of two representative red blood cells containing HbS either as CO-bound (frames a, a′, c, c′, e, and e′) or as deoxy following illumination (frames b, b′, d, d′, f, f′). The frames from a to f and from a′ to f′ depict three successive cycles of sickling for each cell. The time interval between successive frames is approx. 10 s. Conditions: [CO] = 0.5 mM, T = 27°C.

normal RBCs, the CO recombination, which occurs in the dark phase, follows a linear zeroth-order time course for sickling cells (fig.2). The interpretation previously offered [9] for this behaviour was that recombination of CO with intracellular deoxyHb is limited by diffusion of the ligand from the extracellular environment [15]; at this stage a similar explanation may be advanced also for recombination in sickling cells.

Since the total absorbance change following the cessation of photolysis corresponds, by-and-large, to the total absorbance increase due to photodissociation and cell deformation (see fig.2) it appears that depolymerization and cell relaxation are limited by CO diffusion into the erythrocyte.

The zeroth-order recombination time course has been followed for a number of sickling cells as a function of CO concentration. Although the combination is rate limited by diffusion, the greater concentration gradient prevailing at higher CO concentrations (say up to 1 mM) speeds up the overall recombination process. The results indicate that for sickling cells showing a clearcut slow optical change during steady photolysis (see fig.2),

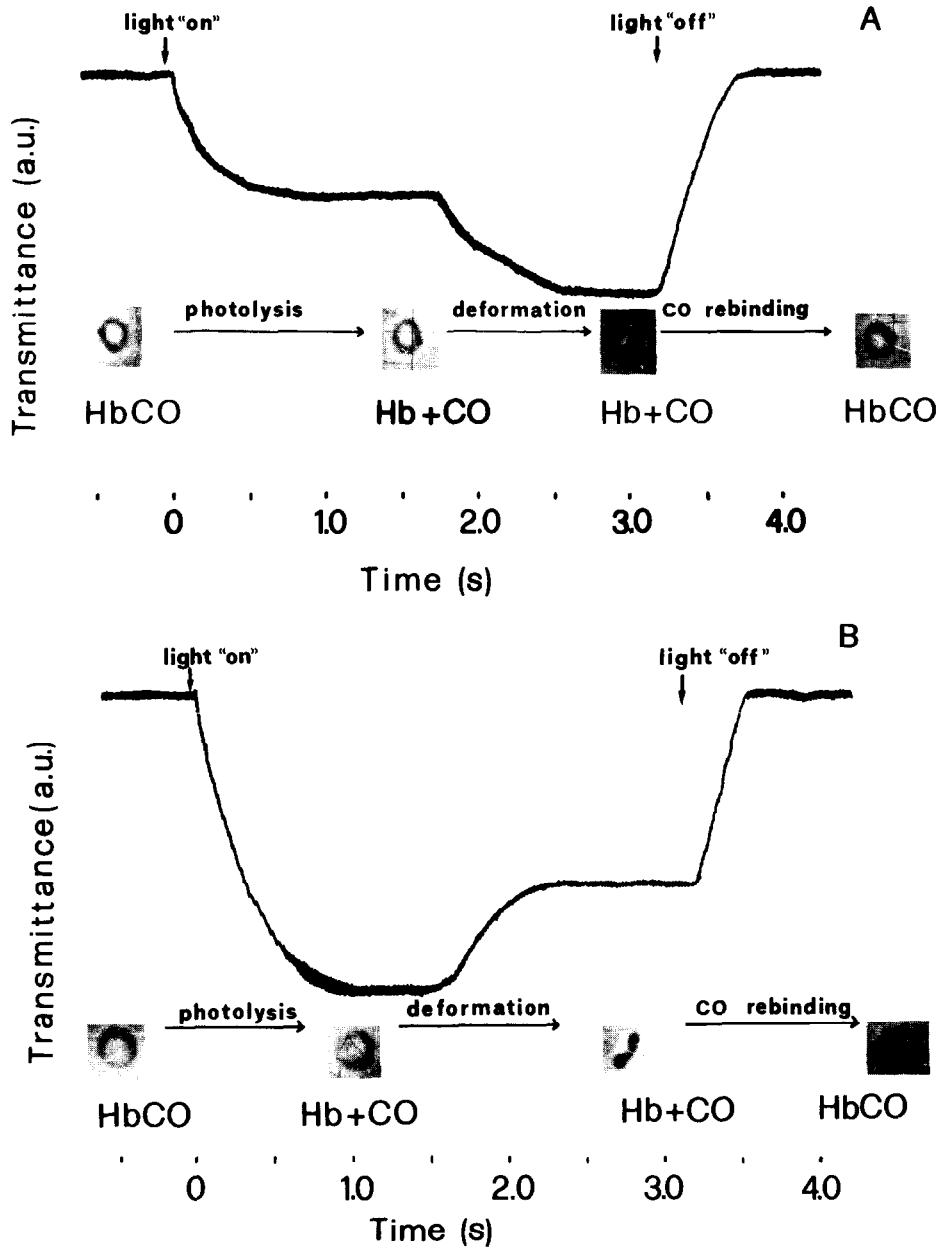

Fig.2. Records of CO photodissociation from hemoglobin S and subsequent recombination in the dark, followed at $\lambda = 430$ nm, T = 27°C, [CO] = 0.5 mM. A and B show the records for two representative erythrocytes from a patient homozygote for HbS. At time = 0 the photodissociating light is switched 'on' and data acquisition begins; the second arrow indicates the time at which light is turned 'off' and rebinding starts. (A) The total absorbance changes assigned to ligand dissociation and cell deformation are: $\Delta A = 0.03 \pm 0.003$ and 0.027 ± 0.008, respectively; (B) the corresponding values are: $\Delta A = 0.044 \pm 0.005$ and -0.02 ± 0.008, respectively.

the rate of recombination of CO was significantly different if photolysis was stopped before or after the delayed optical change (i.e. cell deformation).

In table 1 we report the zeroth-order rate constant (i.e. the slope $\Delta A/s$) at 0.5 mM CO for normal and SS RBCs; the slower CO recombination in

Table 1

Apparent rate constant (ΔA/s) for rebinding of CO to normal and SS erythrocytes, before and after the sickling at [CO] = 0.5 mM, 27°C and λ = 430 nm

RBC type	Slope (ΔA/s)
Normal human erythrocyte	0.275 ± 0.065
SS carrier (before deformation)	0.177 ± 0.026
SS carrier (after deformation)	0.109 ± 0.029

deformed cells is clearcut. In addition, CO recombination to SS cells, even before deformation, appears somewhat slower than that observed in normal erythrocytes (experiments carried out in parallel on the same instrument). This may possibly be explained by a reduced diffusion rate constant in the deformed cell due to the increased intracellular hemoglobin concentration after sickling; in fact, it is known that SS RBCs have a decreased water and potassium content [16] and that sickling itself brings about a further loss of intracellular water [17]. As a consequence, the increased hemoglobin concentration could make the intracellular environment more viscous, thus reducing the diffusion constant of CO.

The slower ligand rebinding in sickled cells and the memory of previous sickling cycles reported above, may have a pathological significance in triggering occlusive crises in sickle cell anemia. Thus, after several cycles of sickling and unsickling a RBC that would escape from the capillaries on a single pass may enter a vicious cycle, which would bring the delay time of the cell below the mean transit time in the capillary system [18]. The cell's spatial memory of previous sickling cycles may be involved in the processes leading to irreversible sickling. In addition, if associated with a decrease of elasticity slowing down the effective transit time [19], it may contribute to the onset of a crisis.

It is possible therefore that erythrocytes, which apparently deform too slowly to induce occlusive crises, might become potentially dangerous after several cycles of sickling and unsickling in the veins.

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