Deoxygenation Affects Fluorescence Photobleaching Recovery Measurements of Red Cell Membrane Protein Lateral Mobility

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ABSTRACT We have used the fluorescence photobleaching recovery technique to study the dependence on oxygen tension of the lateral mobility of fluorescently labeled band 3, the phospholipid analogue fluorescein phosphatidylethanolamine, and glycophorins in normal red blood cell membranes. Band 3 protein and sialic acid moieties on glycophorins were labeled specifically with eosin maleimide and fluorescein thiosemicarbazide, respectively. The band 3 diffusion rate increased from 1.7×10^{-11} cm^2 s^{-1} to 6.0×10^{-11} cm^2 s^{-1} as oxygen tension was decreased from 156 to 2 torr, and a further increase to 17×10^{-11} cm^2 s^{-1} occurred as oxygen tension was decreased from 2 to 0 torr. The fractional mobility of band 3 decreased from 58 to 32% as oxygen tension was decreased from 156 to 0 torr. The phospholipid diffusion coefficient remained constant as oxygen tension was decreased from 156 to 20 torr, but increased from 2.3×10^{-9} cm^2 s^{-1} to 7.1×10^{-9} cm^2 s^{-1} as oxygen tension was decreased from 20 to 0 torr. Neither the diffusion coefficient nor the fractional mobility of glycophorins changed significantly at low oxygen tension. Under non-bleaching excitation conditions, intensities of fluorescence emission were identical for oxygenated and deoxygenated eosin-labeled RBCs. Deoxygenated eosin-labeled RBCs required 160-fold greater laser intensities than did oxygenated RBCs to achieve comparable extents of photobleaching, however. Oxygen seems to act as a facilitator of fluorophore photobleaching and may thereby protect the fluorescently labeled red cell membrane from photodamage. Removal of oxygen may allow excited state fluorophores in close proximity to the plasma membrane to react with neighboring proteins or lipids during photobleaching. This effect has important implications for the ability of the fluorescence photobleaching recovery technique to report accurate lateral mobilities of cell membrane molecules under hypoxic conditions.

INTRODUCTION

Studies of protein and lipid lateral mobility in biological membranes have aided in elucidating the molecular organization of cell membranes and molecular details of cellular function, regulation, and communication. The fluorescence photobleaching recovery (FPR) technique has been used extensively to quantify the lateral mobility of a number of membrane proteins including red blood cell (RBC) band 3 (Golan and Veatch, 1980; Sheetz et al., 1980; Tsuji and Ohnishi, 1986; Golan, 1989; Weaver et al., 1990) and glycophorins (Golan et al., 1986; Knowles et al., 1990; Weaver et al., 1990). Proteins are labeled for FPR experiments using directly coupled fluorophores (Golan and Veatch, 1980; Golan et al., 1986; Tsuji and Ohnishi, 1986; Golan, 1989; Weaver et al., 1990) or fluorescently labeled antibodies (Edidin and Zuniga, 1984; Bierer et al., 1987; Edidin, 1989; Chan et al., 1991). Biological systems are fragile, however, placing stringent requirements on the conditions under which physical techniques can measure their properties accurately. It is important, therefore, to determine whether or not FPR measurements of lateral mobility are valid under all experimental conditions.

Recent work in our laboratory has uncovered a condition under which lateral diffusion measurements seem not to represent true protein and lipid diffusion. Band 3, glycophorins, and phospholipids in normal RBC membranes were specifically labeled with fluorescent probes, and the lateral mobility of these molecules in the plane of the membrane was measured by FPR. Reduced oxygen levels in the sample were found to affect the apparent lateral mobility of band 3 and of phospholipids in these cells. Removal of oxygen seems to be responsible for the abnormal mobilities seen under hypoxic conditions. These results suggest that the FPR technique may not report accurate lateral mobilities of membrane molecules in cells that are experimentally subjected to hypoxic conditions.

MATERIALS AND METHODS

Fluorescein phosphatidylethanolamine (Fl-PE) was obtained from Avanti Polar Lipids (Alabaster, AL). Eosin maleimide (EMA) and fluorescein thiosemicarbazide (FTSC) were purchased from Molecular Probes (Eugene, OR). Glucose oxidase and catalase were obtained from Sigma Chemical Co. (St. Louis, MO). After informed consent was obtained, fresh blood was collected by venipuncture into heparinized tubes. The buffy coat was immediately removed by aspiration, and RBCs were washed three times and stored at 4°C in KPBS (140 mM KCl, 15 mM $NaPO_4$, 10 mM glucose, pH 7.4). High potassium buffers were used to prevent possible cellular dehydration associated with deoxygenation and fluorescent labeling.

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Abbreviations used: FPR, fluorescence photobleaching recovery; BSA, bovine serum albumin; EMA, eosin maleimide; Fl-PE, fluorescein phosphatidylethanolamine; FTSC, fluorescein thiosemicarbazide; KPBS, high potassium phosphate buffered saline; RBC, red blood cell.

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Labeling of RBC band 3

100 μl of freshly washed packed RBCs were incubated with 40 μl of EMA; 0.25 mg/ml in KPBS, at room temperature for 12 min. Cells were then washed three times in KPBS with 1% bovine serum albumin (BSA).

Labeling of RBC glycophorins

100 μl of freshly washed packed RBCs were incubated with 100 μl of $NaIO_4$, 2 mM in KPBS without glucose, at 4°C for 15 min. Cells were then washed twice in KPBS with 0.1 M glycerol and once with KPBS. 100 μl of oxidized RBCs were added to 100 μl of FTSC, 0.5 mg/ml in KPBS, at 4°C for 1 h. Labeled cells were washed three times in KPBS with 1% BSA.

Labeling of RBCs with a fluorescent phospholipid analogue

20 μl of Fl-PE, 1 mg/ml in chloroform, was dried, resuspended in 0.5 ml of KPBS, and sonicated for 20 min in a low power bath sonicator. 100 μl of freshly washed packed RBCs were incubated with the Fl-PE solution at room temperature for 30 min. Cells were then washed three times in KPBS with 1% BSA.

Deoxygenation of RBCs

Fluorescently labeled RBCs were deoxygenated by incubation in a glove box (818GB; Plas-labs, Lansing, MI) set to the desired oxygen tension, or by using an enzyme oxygen scavenging system. The enzyme scavenging system, which consisted of 50 U/ml glucose oxidase, 20 mM glucose, and 10^4 U/ml catalase, served to reduce O_2 to H_2O_2 and H_2O_2 to H_2O (Johnson and Garland, 1981). The excess of catalase reduced significantly the lifetime of unwanted peroxides. To decrease further the potential for peroxide-mediated membrane damage, samples were first deoxygenated to 0.2% O_2 at room temperature for 60 min in the glove box and then treated with the enzyme oxygen scavenging system. Oxygen tension in the glove box was measured by using an oxygen meter (OM-2; Microelectrodes, Londonderry, NH). Slides were prepared for FPR experiments by placing 3.2 μl of a 10% RBC suspension on a BSA-coated glass slide, and using vacuum grease to seal a BSA-coated cover slip over the sample, all in the glove box. Lateral mobility measurements were performed immediately after slide preparation, and within 2 h of fluorescent labeling. Results of FPR experiments using the enzyme scavenging system were identical to those using a more efficient glove box (Braun MB150 M; courtesy of Dr. C. T. Walsh, Harvard Medical School) to deoxygenate samples to 1 ppm O_2 without the use of scavenging enzymes.

Preparation of CO-oxy and CO-deoxy RBCs

Carbon monoxide saturated RBCs were prepared in either an oxygen-free or an oxygen-rich environment. CO-deoxy cells were produced by sealing a suspension of labeled RBCs in a Wheaton vial, passing humidified N_2 gas into the vial (without foaming) for 1 h, passing humidified CO gas over the cells for 1 h, and adding the enzyme scavenging system (described above) to complete the removal of oxygen. CO-oxy cells were produced by passing a humidified 1:1 mixture of CO/O_2 gases over the cells for 1 h. Because the affinity of CO for hemoglobin is much greater than that of O_2, both protocols yielded RBCs in which virtually all of the hemoglobin was liganded by CO and therefore in the R conformation.

FPR

The optics and electronics are reported in detail elsewhere (Corbett and Golan, 1993). Briefly, a 5-watt argon laser (164–08; Spectra-Physics Inc., Mountain View, CA) tuned to 488 nm was intensity-modulated by two acousto-optic modulators (N-35083–3; Newport Electro-Optics, Melbourne, FL) to produce coincident photobleaching and monitoring beams. The sealed sample slide was mounted on a fluorescence microscope (Orthoplan/MPV-3; E. Leitz Inc., Rockleigh, NJ). The beams were focused to a waist at the secondary image plane of the microscope by a 500-mm planoconvex lens and to another waist at the specimen plane by a 100 × objective. Emitted light was collected by the objective, directed through a filter set (Omega Optical Inc., Brattleboro, VT) consisting of a 563 DF 55-nm dichroic and a 520-nm long pass filter, and detected by a thermionically cooled (TE-104RF; Products for Research Inc., Danvers, MA) photon-counting photomultiplier (9658RA; Thorn-EMI, Rockaway, NJ) driven by a high voltage power supply (1109; EG&G Princeton Applied Research, Princeton, NJ). Pulses from the photomultiplier were amplified and discriminated to 100 mV (1121A; EG&G Princeton Applied Research), and the resulting TTL pulses were fed into a multichannel scaler (370; Nicolet Instrument Corp., Anal. Instrs. Div., Madison, WI). After each experiment data were transmitted to a Sun 386i/250 workstation for processing. Experimental timing was controlled by a Micro Linear Controls MLC-1A dedicated timing computer. During the intense bleaching pulse, the photomultiplier was protected by an electromechanical shutter (SD-122B; Vincent Associates, Rochester, NY).

Experimental parameters were adjusted to produce photobleaching of 50–70% of fluorophores during the intense bleaching pulse, and photobleaching of < 5% of fluorophores during the fluorescence recovery phase of the experiment. Photobleaching beam power at the sample was approximately 10 μW for oxygenated samples and 1 mW for deoxygenated samples. Photobleaching pulse duration was 20–200 ms for protein and 40–70 ms for lipid mobility measurements. Measuring beam power at the sample was 0.1 μW. Measuring pulse duration was 80–800 ms for protein and 20–200 ms for lipid mobility measurements. The duration of the recovery phase of the experiment was 40–400 s for protein and 10–40 s for lipid mobility measurements. All FPR experiments were performed at 37°C. The local temperature rise during the bleaching pulse because of laser-induced heating of surface fluorophores was calculated (Yoshida and Barisas, 1986) to be < 0.02°C, and the maximum local temperature rise because of laser-induced heating of hemoglobin was calculated (Yoshida and Barisas, 1986) to be 3.3°C.

The diffusion coefficient (D), representing the rate of translational diffusion in the plane of the membrane, and the fractional mobility (f), representing the fraction of molecules free to diffuse on the time scale of the experiment, were calculated from FPR data by nonlinear least squares analysis (Bevington, 1969). Each data set represents the mean of 8–12 independent measurements on individual cells; data are expressed as mean ± SD.

RESULTS

The lateral mobility of band 3 in membranes of intact normal RBCs was markedly altered upon deoxygenation. Reduction of oxygen tension from 156 (atmospheric) to 0 torr caused the fraction of mobile band 3 molecules to decrease and the diffusivity of the mobile molecules to increase. These changes were especially marked at oxygen tensions less than 7 torr. The f decreased from 58 to 32% as oxygen tension was reduced from 156 to 0 torr. The band 3 diffusion rate was constant at 1.5–3.2 × 10^{-11} cm^2 s^{-1} as oxygen tension was decreased from 156 to 7 torr, but the diffusivity increased progressively to 6.0 × 10^{-11} cm^2 s^{-1} and 17 × 10^{-11} cm^2 s^{-1} at oxygen tensions of 2 torr and 0 torr, respectively (Fig. 1, *A* and *B*).

The lateral mobility of the phospholipid analogue Fl-PE was also perturbed upon deoxygenation, especially at oxygen tensions less than 2 torr. The fractional mobility increased from 84 to 96% as oxygen tension was reduced from 156 to 0 torr. The Fl-PE diffusion rate was constant

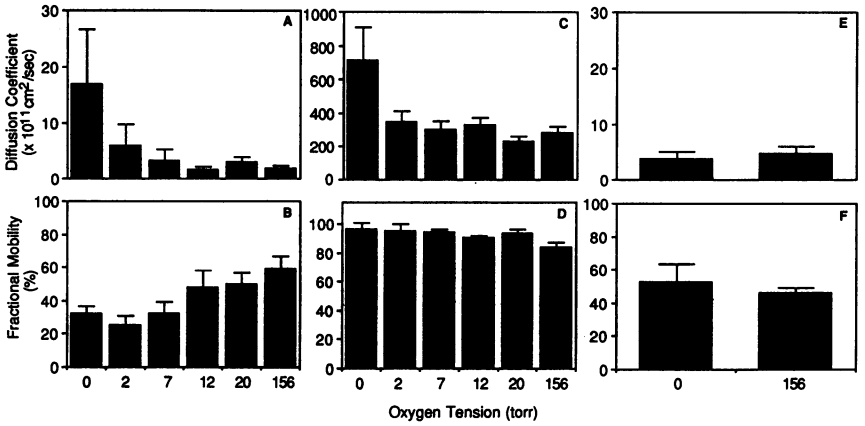

FIGURE 1 Lateral mobility of eosin-labeled band 3 (*A* and *B*), fluorescein-labeled phosphatidylethanolamine (*C* and *D*), and fluorescein-labeled glycophorins (*E* and *F*) in membranes of intact normal RBCs at various oxygen tensions. Each bar represents the mean ± SD of 16–84 independent measurements on 3–7 RBC samples from 2–5 different individuals. (*A*, *C*, *E*) Effect of oxygen tension (torr) on lateral diffusion coefficients ($cm^2\ s^{-1}$). (*B*, *D*, *F*) Effect of oxygen tension (torr) on fractional mobilities (%).

at 2.3–3.5 × 10^{-9} $cm^2\ s^{-1}$ as oxygen tension was decreased from 156 to 2 torr, but the diffusivity increased to 7.1 × 10^{-9} $cm^2\ s^{-1}$ at an oxygen tension of 0 torr (Fig. 1, *C* and *D*).

In contrast to band 3 and Fl-PE mobility, glycophorin lateral mobility was unaffected by deoxygenation. Neither the D nor the f of glycophorins changed significantly at an oxygen tension of 0 torr (Fig. 1, *E* and *F*).

Deoxygenated hemoglobin binds more tightly to the cytoplasmic domain of band 3 than does oxygenated hemoglobin (Salhany and Shaklai, 1979; Cassoly, 1983; Low et al., 1984; Walder et al., 1984; Chetrite and Cassoly, 1985; Premachandra, 1986). CO binding to deoxyhemoglobin converts hemoglobin from the "deoxy" (T) state to the "oxy" (R) conformation. If binding of deoxygenated hemoglobin to band 3 were primarily responsible for changes in RBC membrane organization and in band 3 and Fl-PE lateral mobility, then CO-saturated cells should manifest normal mobilities under deoxygenated conditions. The D and f of eosin-labeled band 3 in deoxygenated RBCs were similar to those of band 3 in deoxygenated, CO-saturated RBCs, however. Further, band 3 mobility in CO-saturated oxygenated RBCs was similar to that in oxygenated cells (Table 1). The band 3 mobility differences associated with RBC deoxygenation seem, therefore, to be a function of the presence or absence of oxygen in the medium, rather than of the oxygen-sensitive conformational state of hemoglobin.

TABLE 1 Effect of deoxygenation and CO treatment on lateral mobility of eosin-labeled band 3 in normal RBC membranes*

Sample	Diffusion Coefficient ($\times 10^{11}$ $cm^2\ s^{-1}$)	Fractional Mobility (%)	*N*
Oxygenated RBCs	1.5 ± 0.8	49 ± 8	29
Oxygenated RBCs + CO	1.2 ± 0.6	42 ± 7	20
Deoxygenated RBCs	20 ± 10	30 ± 8	125
Deoxygenated RBCs + CO	23 ± 15	32 ± 8	30

* The FPR technique was used to measure the lateral mobility of eosin-labeled band 3 in normal RBC membranes at various O_2 and CO tensions, as described in Materials and Methods. Diffusion coefficient and fractional mobility values are reported as mean ± SD of *N* independent measurements.

In an FPR experiment, the prebleach fluorescence intensity is sensitive to the fluorophore microenvironment, whereas the photon density required for photobleaching is a function of both the microenvironment of the fluorophore and the chemical mechanism of photobleaching. Using a nonbleaching laser beam under identical excitation conditions, fluorescence emission intensities were similar for oxygenated and deoxygenated eosin-labeled RBCs. Deoxygenated eosin-labeled RBCs required 160-fold greater laser intensities than did oxygenated RBCs to achieve comparable extents of photobleaching, however (Table 2). These data suggest that the microenvironments of the eosin moiety are similar in oxygenated and deoxygenated samples, but that the mechanisms of photobleaching are different for the two samples.

DISCUSSION

At least three molecular mechanisms could underlie the changes in band 3 and Fl-PE mobility observed at low oxygen tensions. First, deoxyhemoglobin could bind

TABLE 2 Effect of deoxygenation on photobleaching of eosin conjugated to band 3 in normal RBC membranes

Sample	Prebleach Fluorescence Intensity* (counts)	Photobleaching Photon Density‡ ($\times 10^{11}$ μm^{-2})
Oxygenated RBCs	650 ± 30	13
Deoxygenated RBCs	580 ± 80	2065

* Prebleach fluorescence counts were recorded using a nonbleaching laser beam, as described in Materials and Methods. The beam power at the sample was 0.1 μW, the Gaussian beam radius was 0.7 μm, and the measuring duration was 800 ms for both oxygenated and deoxygenated RBC samples. Values represent mean ± SD of 10 independent measurements per sample.

‡ Photon density required to achieve photobleaching of 65 ± 3% (mean ± SD) of the eosin molecules in a 0.7-μm radius Gaussian spot, using a bleaching duration of 100 ms for both oxygenated and deoxygenated RBC samples. Photobleaching beam power at the sample was approximately 10 μW for oxygenated samples and 1 mW for deoxygenated samples. Photon density, which was calculated using the Planck equation, was stable to ±1%.

preferentially to the cytoplasmic domain of band 3, causing clustering of band 3 molecules and changes in both protein and lipid lateral mobility. Second, the increased laser intensities required for photobleaching of fluorophores in deoxygenated samples could cause local heating of the photobleached area and thereby perturb protein and lipid lateral mobility. Third, the increased photobleaching intensities used in FPR experiments on deoxygenated samples could facilitate crosslinking of photoexcited dye species to nearby fluorophores or proteins, causing alterations in both protein and lipid lateral mobility.

Direct binding of deoxyhemoglobin to the cytoplasmic domain of band 3 is unlikely to be responsible for the observed alterations in band 3 and Fl-PE mobility in deoxygenated samples, inasmuch as CO treatment does not alter the effects of deoxygenation on band 3 lateral (the present study) or rotational (Corbett and Golan, 1993) mobility. Similarly, laser-induced heating is calculated to be negligible for oxygenated samples and minimal (about 3.3°C) for deoxygenated samples.

A deoxygenation-induced change in the photochemistry of the bleaching process could explain the experimental findings reported here. Molecular oxygen is likely to serve as the major facilitator of eosin and fluorescein photobleaching under oxygenated conditions. Excited state fluorophores can interact with oxygen to produce singlet oxygen and other reactive dye species, and such species can cause irreversible membrane damage under certain experimental conditions (Lepock et al., 1978; Nigg et al., 1979; Sheetz and Koppel, 1979). Studies using bleaching times of varying duration indicate, however, that little-to-no photodamage occurs during brief FPR photobleaching pulses such as those used in the present study (Sheetz and Koppel, 1979). Koppel and Sheetz (1981) showed that the lateral mobility of RBC membrane glycoproteins measured by FPR was equivalent to that determined by fluorescence redistribution after fusion in intact oxygenated cells. The fluorescence redistribution after fusion technique, unlike the FPR technique, does not involve an intense photobleaching pulse. Other photobleaching studies on oxygenated leukemic cells (Wolf et al., 1980), CHO cells, fibroblasts, and other cultured cells (Jacobson et al., 1978) provide further support for the contention that photobleaching under oxygenated conditions does not alter local membrane structure. Nigg et al. (1979) detected crosslinking of the RBC membrane protein acetylcholinesterase after photobleaching of oxygenated RBC *ghosts*. No protein crosslinking was detected using oxygenated *intact* cells under the same conditions, however, suggesting that the oxidoreduction systems used by intact cells to detoxify reactive oxygen species (e.g., the glutathione cycle) also prevent photobleaching-induced protein crosslinking.

The lifetime of photoexcited eosin or fluorescein is likely to be significantly increased in the absence of oxygen, allowing crosslinking of these dyes to nearby fluorophores or proteins. Brief, intense visible illumination in the presence of oxygen seems to cause molecules containing conjugated ring systems, similar to those found in eosin and fluorescein, to undergo photobleaching by autooxidation rather than by intermolecular adduction (Stevens and Algar, 1968; Koizumi and Usui, 1972; Saito and Matsuura, 1979; Bjarneson and Petersen, 1990; Bjarneson and Petersen, 1991). In contrast, intense illumination of fluorophore solutions in the absence of oxygen induces photobleaching by intermolecular crosslinking of fluorophores into higher order oligomers (Wei and Livingston, 1967; Fournie et al., 1972). If molecular oxygen is unavailable to quench or oxidize excited triplet state dye molecules, such molecules can also attack nearby proteins directly (Seliger and McElroy, 1965). Our data are consistent with a model in which, under oxygenated conditions, molecular oxygen facilitates the bleaching of protein- or lipid-linked eosin or fluorescein without damage to RBC membrane components (Koizumi and Usui, 1972; Britt and Moniz, 1973). In the absence of oxygen, however, direct attack of excited state dye on RBC membrane proteins and lipids causes macromolecular crosslinking during the photobleaching pulse. At an eosin surface density of 7×10^3 molecules per μm^2 (Golan, 1989) and a band 3 lateral D of 1×10^{-11} cm^2 s^{-1}, approximately 10 eosin-conjugated band 3 molecules are contacted by each photoexcited eosin molecule during a 100-ms photobleaching pulse.

Molecular crosslinking induced by excited state fluorophores could change significantly the organization of proteins and lipids in the RBC membrane, resulting in altered local mobilities of membrane components. Decreased band 3 fractional mobilities under deoxygenated conditions could indicate that a portion of band 3 molecules are clustered and thereby immobilized in the membrane, providing fewer obstacles to diffusion of the nonclustered (i.e., laterally mobile) band 3 molecules and therefore allowing the latter molecules to diffuse more rapidly (Saxton, 1982; Eisinger and Halperin, 1986; Saxton, 1987; Saxton, 1989). Because aggregated band 3 molecules would be localized preferentially to the membrane region exposed to the photobleaching laser pulse, unbleached laterally mobile band 3 molecules just outside the area of bleaching could diffuse more rapidly into these relatively protein-free areas. Alternately, fluorophore catalyzed protein crosslinking could modify the structure of the spectrin-based membrane skeleton in the photobleached area, reducing steric hindrance to band 3 lateral mobility (Corbett, J. D., P. Agre, J. Palek and D. E. Golan, submitted for publication). Changes in Fl-PE mobility under deoxygenated conditions could also be the result of fluorophore-induced crosslinking of neighboring proteins and consequent formation of relatively protein-free membrane domains in which lipids could diffuse more rapidly (Saxton, 1982; Eisinger and Halperin, 1986; Saxton, 1987; Saxton, 1989). In other studies, fluorescent lipid analogues were predicted (Saxton, 1982) and later observed (Golan et al., 1984) to diffuse fourfold faster in multilamellar liposomes formed from protein-free RBC membrane lipids than in native RBC ghost membranes. This degree of increase in diffusion rate is similar to that observed here upon deoxygenation of intact RBCs. Because the sialic acid-linked fluorescent labels on glycophorins are located a significant distance from the

membrane bilayer, unlike the labels on band 3 and Fl-PE, fluorophore-catalyzed protein crosslinking may be less likely to occur in FTSC-labeled red cells under deoxygenated conditions (i.e., an activated fluorophore conjugated to glycophorins could be less likely to attack nearby protein molecules). Intermolecular interactions of RBC membrane proteins may therefore be less affected by photobleaching of FTSC-labeled RBCs than by bleaching of EMA or Fl-PE labeled RBCs.

In conclusion, a number of studies in the literature indicate that FPR experiments on fluorescently labeled membranes under oxygenated conditions do not cause large scale membrane perturbation. In the absence of oxygen, however, membrane structure seems to be altered by the intense photobleaching beam. A molecule that mimics the ability of molecular oxygen to facilitate bleaching without molecular crosslinking or cellular toxicity would allow accurate measurements of RBC membrane protein and lipid lateral mobility under hypoxic conditions.

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