Biochimica et Biophysica Acta, 433 (1976) 215—222

BBA Report

BBA 71251

MEASUREMENT OF THE TRANSLATIONAL MOBILITY OF CON-CANAVALIN A IN GLYCEROL-SALINE SOLUTIONS AND ON THE CELL SURFACE BY FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING

K. JACOBSON, E. WU* and G. POSTE

Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, New York, N.Y. 14263 (U.S.A.)

(Received December 9th, 1975)

Summary

The fluorescence recovery kinetics of succinyl-fluorescein Concanavalin A (S-F-ConA) in glycerol-physiological saline solutions of high viscosity and when bound to the surface of mouse fibroblasts were measured following brief photobleaching using a laser excited fluorescence microscope. In the high viscosity solutions, the recovery kinetics, interpreted on the basis of a simple diffusion model, yielded a diffusion coefficient in close agreement with the values predicted by the Stokes-Einstein equation. Recovery kinetics for S-F-ConA bound to the surface of mouse 3T3 and SV3T3 cells cultured in vitro yielded diffusion coefficients in the range of $5—10 \cdot 10^{-11}$ cm^2/s, values considerably lower than those reported previously for membrane proteins. These measurements indicated that a considerable fraction of the S-F-ConA molecules bound to the cell surface are immobilized. These results are discussed in relation to current concepts of lateral motion of protein components within natural membranes.

Evidence obtained in many laboratories has shown that several classes of cell surface proteins and glycoproteins can undergo lateral movements within the plane of the membrane [1,2] and that such movement may be of considerable importance in determining the functional properties of the cell surface [3—6]. To date, only a limited number of direct experimental measurements have been made on the diffusion coefficients for natural membrane proteins in situ. These have yielded estimates ranging from $5 \cdot 10^{-9}$ cm^2/s for rhodopsin in photoreceptor membranes [7,8] to less than 10^{-12} cm^2/s for fluorescein-labeled surface proteins in the human erythrocyte [9].

*Present address: Department of Physics, University of Maryland, Baltimore County, Baltimore, Md. 14228 (U.S.A.).

Peters and his colleagues [9] recently described a method for the measurement of lateral diffusion of fluorescein-labeled macromolecules on the cell surface by photobleaching. In this communication, we report the use of similar photobleaching methods to study the diffusion of fluorescein-conjugated succinyl Concanavalin A (S-F-ConA) in both simple high viscosity solvents and on the surface of mammalian cells cultured in vitro. Our results indicate that experimentally determined values for the diffusion coefficient of this macromolecule in high viscosity glycerol-phosphate buffered saline solutions agree with the values expected from the Stokes-Einstein equation. In contrast, the translational diffusion of S-F-ConA when bound to specific receptors on the surface of cultured cells is significantly slower than reported values for other classes of plasma membrane proteins [1], suggesting that the lateral diffusion of lectin-receptor complexes within the cellular plasma membrane may be influenced by factors other than the viscosity of the lipid matrix of the membrane.

Photobleaching technique. The principles underlying the use of photobleaching to monitor molecular diffusion have been discussed elsewhere [9]. In this study, photobleaching measurements were made to determine the diffusion coefficient of succinylated fluorescein isothiocyanate-labeled Concanavalin A (S-F-ConA) in simple glycerol–phosphate-buffered saline solutions and when the same lectin was bound to specific receptors on the surface of cultured cells. Samples were photobleached over a small selected area to induce loss of specific fluorescence after which a coincident, but much less intense measuring beam, was used to monitor the recovery of specific fluorescence in the bleached area. The kinetics of recovery of fluorescence are governed by the rate of diffusion of unbleached fluorophores into the bleached region and can, therefore, be used to calculate the diffusion coefficient of the fluorophore.

Photobleaching and fluorescence recovery measurements were made using a Leitz MPV-2 microscope photometer equipped with a vertical fluorescence illuminator. A He-Cd laser (Liconix, Model 403, Mountain View, Calif.) with a 20 mW emission at 441.6 nm was used for both the photobleaching and measuring beams. The spatially filtered laser beam has a Gaussian intensity distribution. The excitation beam was reflected off a Letiz TK510 dichroic beam splitting mirror in the vertical illuminator and the fluorescence measured after passing through a Leitz K515 suppression filter. The diameter of the laser beam after focussing by the microscope objective was determined as described elsewhere [10]. The intensity of the incident laser beam was recorded on the second channel in order to correct the recovery curve for occasional drifts and fluctuations in laser output.

The fluorescence intensity of samples before bleaching (I_0') was measured by brief exposure ($\approx$1.5 s) to the measuring beam which was produced by a 2000- to 20 000-fold attenuation of the full laser beam using neutral density filters. (Throughout the text, primed values represent the experimental intensities which include unbleached S-F-ConA fluorescence and background emission (autofluorescence).) The filter was then withdrawn to permit photobleaching of the sample by exposure to the unattenuated laser beam. The filter was then reinserted after 300—500 ms and the shutter to the phototube

opened simultaneously. This time was designated as time zero and this latter procedure required approximately 200 ms. The fluorescence at time 0 was designated F_0'. The recovery of specific fluorescence in the bleached area of the sample was then determined at intervals by brief exposure to the measuring beam. This procedure served to minimize photobleaching by the measuring beam.

For analysis, the measuring conditions have been considered as approximate to the following cylindrical symmetric model. Photobleaching was assumed to create an empty cylinder of diameter $2a$ in the concentration of the fluorescent compound; a was assumed to be equivalent to the $1/e^2$ radius of the laser beam in the specimen plane. (This is an approximation because of the Gaussian distribution of intensity.) The concentration c of the fluorophore at position r and at time t after photobleaching is given by [11]:

$$c(r,t) = c_0 \left[1 - (2Dt)^{-1} \exp - r^2/4Dt \int_0^a (\exp - r^2/4Dt)\tilde{I}_0(rr'/2Dt)r' \, dr'\right] \quad (1)$$

where: D is the diffusion coefficient of the fluorophore; r is the distance from the optical axis; t is time; c_0 is the initial concentration for all $r > a$; and $\tilde{I}_0$ is the modified Bessel function of the first kind of order zero.

To determine the fluorescence at time t, the concentration of the fluorophore, $c(r,t)$, must be multiplied by the laser intensity $A\ (r) = A_0 \exp - 2r^2/a^2$ and integrated over all area. This integration, with infinite integration limits, yields the expression from [12]:

$$F_t/F_\infty = \exp - 2/(1 + 8Dt/a^2) \quad (2)$$

where F_t is the fluorescence at any time, t; and F_∞ is the fluorescence after recovery is complete.

The empty cylinder initial conditions predict a fast initial diffusive flux due to the steep concentration gradient at $r = a$. As an alternate initial condition, therefore, we considered the diffusion driven by an initial Gaussian concentration distribution having width, a, equal to the $1/e^2$ radius of the excitation beam. The time and spatial behavior of the concentration is from [11]:

$$c(r,t) = c_0 \{1 - [a^2/(a^2 + 8Dt)]\exp - 2r^2/(a^2 + 8Dt)\} \quad (3)$$

The corresponding fluorescent intensity is:

$$F_t/F_\infty = \tfrac{1}{2}[1 + (4Dt/a^2)/(1 + 4Dt/a^2)] \quad (4)$$

In the cell surface measurements, the 20 mW laser and ×100 objective provide fairly complete initial bleaching. Therefore, the empty cylinder is a reasonable approximation of the measuring condition at $t = 0$. In the glycerol phosphate-buffering saline mixture, however, the initial bleaching is only partially complete because of the relatively high fluorophore concentration and the low power (× 20) of the objective. For these experiments an initial condition in between the two extremes, namely the empty cylinder and the Gaussian profile conditions, would be a better model for our system. (An exact calculation of the initial concentration profile obtained from a Gaussian bleaching beam and subsequent fluorescence recovery has recently been

performed (Axelrod, D., Cornell Univ., personal communication)). Nevertheless, in the region of interest ($D \leqslant 10^{-9}$ cm^2/s) the diffusion coefficients obtained using either the empty cylinder or Gaussian model differ by less than 25%, which is well within the experimental error estimate. On this basis we fit all our recovery data to Eqn. 2 to obtain the diffusion coefficient.

Fitting was accomplished in the following way: A rough estimate for D can be obtained from the experimental data by finding the time at which recovery is 67% complete since this condition corresponds to $D = a^2/2t$ in Eqn. 2. Recovery curves were then plotted by computer using Eqn. 2 for D values in the range of the original estimate. The quoted diffusion coefficient is the D value giving a recovery curve which provided the best visual fit to the experimental data (See Figs. 1 and 2). The value of F_∞ was chosen as the

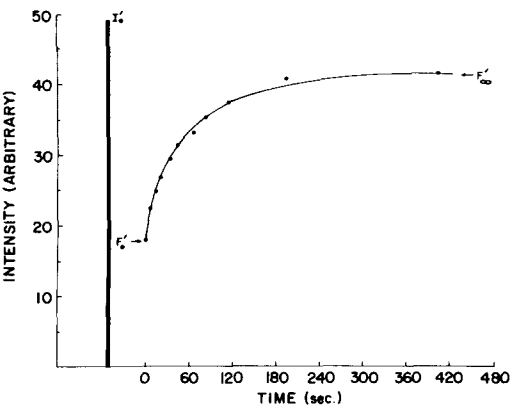

Fig. 1. Recovery kinetics of 2 μm S-F-ConA in 98% glycerol-2% phosphate-buffered saline (v/v) at 22°C. Points represent experimental data with I_0' denoted by the broad bar to the left of the origin. The plotted line is the theoretical curve obtained from Eqn. 2 using $D = 8.9 \cdot 10^{-10}$ cm^2/s and $a = 2.9$ μm; F_∞ was chosen as 41.5 units (see text for details of fitting procedure) and experimental values are indicated by the symbols. X 20 objective was employed and photobleaching beam was attenuated by 20 000 for measuring.

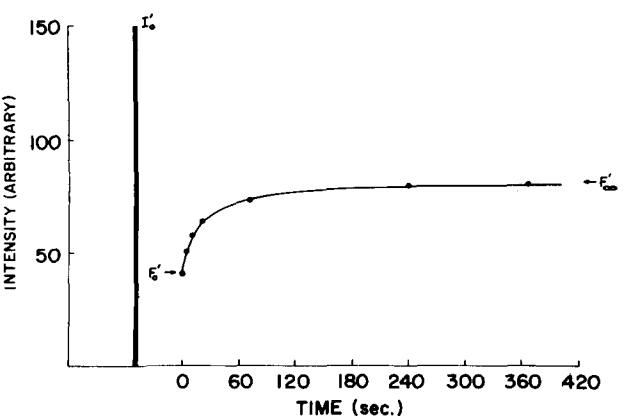

Fig. 2. Recovery kinetics of S-F-ConA bound to surfaces of SV3T3 cells in monolayer culture (cell density, 5—5.5·10^4 cells/cm^2). Cells were incubated with S-F-ConA (50 μg/ml) as described in text. The plotted line is the theoretical curve obtained from Eqn. 2 using $D = 8.7 \cdot 10^{-11}$ cm^2/s, $a = 0.65$ μm; F_∞ was chosen as 81.5 units. X 100 oil objective was employed and photobleaching beam was attenuated by 2000 for measuring.

reference point for all manipulations of the experimental data. Then $F_\infty = (F'_\infty - F'_0)/(1 - e^{-2})$ by evaluation of Eqn. 2 at time = 0 assuming the same contribution of background emission to F_∞ and F_0; determination of F_∞ allows calculation of $F_\infty - F_t$ at any time t and comparison with the experimental difference $F'_\infty - F'_t$.

For experiments on the diffusion of S-F-ConA in glycerol-phosphate-buffered saline mixtures, the laser beam was focussed through a × 20 objective to the center of a 50 μm path length microcuvette (Vitro Dynamics, Rockaway, N.J.) containing the test solution. The focussed Gaussian beam had an approximately constant diameter ($2/e^2$ diameter ≈ 6 μm) over the entire path length. For experiments on S-F-ConA-labeled cell cultures, the beam was focussed through a × 100 oil immersion objective to provide a $1/e^2$ beam diameter of about 1.3 μm. Cells were observed using a dark field transmission condenser with a tungsten light source. By defocussing slightly from the image achieved with bright field illumination, it was possible to maximize the fluorescence collected from the cell surface and reduce the contribution of fluorescence from the inside of the cell. I'_0 was measured at this focus position. Cells exhibited both diffuse pale green and punctate granular yellow-orange background fluorescence, the latter perhaps due to mitochondrial flavins. Background fluorescence was measured from preparations in the absence of S-F-ConA. The intensity before photobleaching in such control preparations was usually less than 20% of that in preparations containing S-F-ConA. Furthermore, the level of background fluorescence did not increase after treatment of cells with S-F-ConA which had been premixed with the haptenic Con A inhibitor, α-methylmannoside (0.1 M) indicating that little non-specific binding of S-F-ConA was occurring. The backgrounc fluorescence exhibited by cell populations could be bleached and recovery kinetics determined. Data from such experiments indicated that the contribution of background to recovery kinetics in the experiments with S-F-ConA would result in a reduction of the measured diffusion coefficient for S-F-ConA of no more than 15%. Peters et al. [9] established that spontaneous regeneration of fluorescein isothiocyanate without lateral diffusion is negligible and this was confirmed for cells in monolayer culture by us and by others [17].

Reagents, cell cultures and labelling procedure. Fluorescein isothiocyanate labeled Concanavalin A was purchased from Miles-Yeda (Laporte, Ind.) and succinylated by the method of Gunther et al. [13]. The resulting S-F-ConA preparation was presumed to be predominantly dimeric Con A and had sedimentation coefficient ($s_{20,w}$) of 4.3 S, a value in agreement with previous data on dimeric Con A [13]. Glycerol was purchased from Fisher (Rochester, N.Y.), succinic anhydride and α-methylmannoside from Sigma (St. Louis, Mo.).

BALB/c mouse 3T3 cells were grown on 1 mm thick glass coverslips in Dulbecco's modification of Eagles Medium supplemented with 10% fetal calf serum as described before [6]. Similar 3T3 cells transformed by simian virus 40 (SV3T3) were obtained from Dr. C. Borek and cultured under identical conditions to the above 3T3 cells. Coverslip cell cultures were washed three times with PBS and incubated with S-F-ConA (50 μg/ml) in phosphate-buffered saline for 15 min at 37°C after which they were washed three times

with phosphate-buffered saline and wet-mounted and paraffin sealed on microscope slides for the fluorescence recovery experiments.

Measurement of the translational diffusion coefficients of S-F-ConA in glycerol-phosphate-buffered saline mixtures. Diffusion coefficients for S-F-ConA in various mixtures of glycerol and are given in Table I and typical kinetics of fluorescence recovery after photobleaching are shown in Fig. 1. The solid line in Fig. 1 indicates the fit of Eqn. 2 with D as an adjustable parameter.

Table I indicates that in high viscosity mixtures, the values of D determined from the experimental recovery curve are in reasonable agreement with those predicted from the Stokes-Einstein equation. This agreement can be taken to indicate that the recovery kinetics are diffusion controlled. Further, D increases monotonically as the glycerol content and viscosity decreases. However, the quantitative agreement of experimental values with the Stokes-Einstein equation becomes progressively poorer as viscosity decreases. We interpret this to mean that the steep concentration gradient assumed to exist at $t = 0$ for our diffusion model is dissipated by fast diffusion during the finite photobleaching interval. This would produce slower recovery kinetics than predicted by the model for a given D. In this regard, the ratio of F_0/I_0 is seen to increase as viscosity decreases reflecting the diffusion into the beam during the photobleaching period. Also, the instrumental "dead time" of about 200 ms between the end of photobleach and the first measurement becomes important for faster diffusion. Indeed, a correction for the recovery which occurs during the "dead time" improves the agreement between experiment and theory.

Finally, the extent of recovery, R, (defined as $(F'_\infty - F'_0)/(I'_0 - F'_0)$ is over 80%. This is in marked contrast to the much lower R values obtained in cellular experiments (see below). The reasons for incomplete recovery in the glycerol-phosphate-buffered saline system are not clear, but may be related to imperfect mixing.

TABLE I

DIFFUSION OF S-F-ConA IN GLYCEROL-PHOSPHATE-BUFFERED SALINE MIXTURES
The concentration of S-F-ConA in all experiments was 20 μM or less.

% Glycerol by volume	No. samples measured	F'_0/I'_0 (%)	D (cm^2/sec)	D (corrected for dead time)*	Predicted D ($= K_B T/\sigma\pi\eta a'$)**
98	3	37	$6.7 \pm 3 \cdot 10^{-10}$		$7 \cdot 10^{-10}$
89.3	4	37.5	$1.4 \pm 0.5 \cdot 10^{-9}$		$3 \cdot 10^{-9}$
76	4	50.5	$3.7 \pm 1.2 \cdot 10^{-9}$	$4.5 \cdot 10^{-9}$	$1.6 \cdot 10^{-8}$
55	3	59	$6.4 \pm 1.0 \cdot 10^{-9}$	$1.7 \cdot 10^{-8}$	$7.5 \cdot 10^{-8}$
26	2	65	$1.2 \pm 0.2 \cdot 10^{-8}$	$5.6 \cdot 10^{-8}$	$2.7 \cdot 10^{-7}$

*For faster diffusion, the time between the end of bleach and the first measurement, the "dead time", becomes important. Dead time is estimated to be 200 ms. The correction is neglible for the first two rows.

**For calculation of D by the Stokes-Einstein equation, T was taken as 295°K and viscosity (η) obtained from Handbook of Chemistry and Physics, 28th edn., The Chemical Rubber Co., Cleveland, Ohio. η at 22°C was obtained by linear interpolation. a', the particle radius was taken as 40 Å [16]. The application of the Stokes-Einstein equation is admittedly approximate and intended only to demonstrate that fluorescence recovery is diffusion controlled. First, η is quite temperature sensitive especially in the higher viscosity glycerol-water mixtures. Secondly, the choice of a' assumes dimer

Measurement of the diffusion coefficient of S-F-ConA bound to the surface of mammalian cells. The recovery of fluorescence of S-F-ConA bound to specific receptors on the surface of SV3T3 cells is shown in Fig. 2. The data in Fig. 2 refer to cells treated with 50 μg/ml S-F-ConA although similar results were obtained at lower doses of S-F-ConA ($\geqslant$10 μg/ml). Recovery is presumed to commence from the indicated level. The residual fluorescence in the system is caused by unbleached background fluorescence contributed by cellular organelles and by the spatial extent of the measuring beam (see Methods). The results in Fig. 2 indicate that in contrast to the behavior of S-F-ConA in the glycerol-phosphate-buffered saline model system, the recovery of S-F-ConA fluorescence on bleached regions of the cell surface is always incomplete (R = 36% in Fig. 2). These curves could be fitted by Eqn. 2 with D values in the range of 10^{-10} to 10^{-11} cm^2/s. It is well to note that these values represent the mean diffusion coefficients obtained by averaging over one or possibly more ConA binding sites and various degrees of aggregation of the individual ConA-receptor complexes. In the present study, no significant difference was detected in the kinetics of fluorescence recovery in untransformed and transformed cell populations and recovery kinetics were not strongly dependent on the cell denisty (results not shown). However, it is conceivable that lower doses of S-F-ConA ($\approx$1 μg/ml) and analysis of larger numbers of cells will reveal mobility differences dependent on cell density, phase in the cell cycle or malignant transformation.

The present experiments have demonstrated the feasibility of using photobleaching techniques to measure diffusion of macromolecules of biological importance in both model systems and in living cells. The good agreement obtained between experimental and theoretical values for the diffusion of S-F-ConA ($D \leqslant 10^{-9}$ cm^2/s) in high viscosity glycerol-phosphate-buffered saline solutions serves to strengthen confidence in the results obtained with this technique in monitoring mobility of ConA receptor-ligand complexes on the cell surface.

The apparent lateral diffusion coefficient for S-F-ConA bound to the surface of 3T3 and SV3T3 cells is less than 10^{-10} cm^2/s. These values are almost two orders of magnitude lower than the values reported for other natural membrane proteins (for review, see ref. 1). Although our cell recovery kinetics can be fitted by the diffusion model, we have employed the term apparent because the possible contribution of flow mechanisms in recovery have not been rigorously excluded. However, for the purposes of discussion, we will assume the recovery is diffusion controlled.

Several non-exclusive mechanisms might contribute to the slow diffusion of S-F-ConA receptor complexes. Firstly, S-F-ConA is divalent and thus, able to cross-link saccharide receptors on adjacent membrane glycoproteins. Such cross-linking would create considerably larger S-F-ConA-receptor complexes which would diffuse more slowly than S-F-ConA bound to a single membrane glycoprotein. It is also clear from recent studies in several laboratories that materials on the outer and inner faces of plasma membrane may modify the opportunities for lateral diffusion within the membrane. For example, a high proportion, if not all, Con A receptors may be linked to cytoskeletal elements associated with the inner face of the plasma membrane which may restrict

the mobility of ConA-receptor complexes within the membrane [3,5,6,14]. The possibility also exists that the high concentration of sulfated glycosaminoglycans associated with the outer face of the plasma membrane could further restrict the lateral diffusion of ConA-receptor complexes and other molecules within the membrane (see refs. 2, 15). Consequently, the lateral mobility of certain classes of plasma membrane components cannot be considered solely in terms of their ability to diffuse within a lipid bilayer and other membrane components together with membrane-associated structures could exert significant effects on their translational mobility. Indeed, such additional restraints may be of considerable importance in maintaining the spatial organization and topographic distribution of different classes of molecules on the cell surface (see refs. 3,5,6,14,16). The present observation that the recovery of S-F-ConA fluorescence in bleached areas of the cell surface is never 100% provides further evidence to support the above proposal that a certain fraction of ConA-receptor complexes may be immobilized within the plasma membrane, though the nature of the surface components responsible for restriction of lateral diffusion in the present system remains to be identified.

Two other recent photobleaching studies of ConA mobility on a variety of cultured cells have yielded qualitatively similar results [17,18].

We thank H. Allen and research colleagues in membrane biophysics in the Department of Applied Physics and Chemistry at Cornell University for many helpful discussions and we also acknowledge Y. Hou, M.E. Conroy, A. MacKearnin, and J. Morey for their excellent technical assistance, and K. Paigen for the use of the fluorescence microscope. This work was supported by Grant No. CA-16743 (K.J.) and CA-13393 (G.P.) from the National Institutes of Health.

References

1 Edidin, M. (1974) Annu. Rev. Biophys. Bioeng. 3, 179—202.
2 Nicolson, G.L. (1974) Int. Rev. Cyt. 39, 89—190.
3 Edelman, G.M., Yahara, I. and Wang, J.L. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1442—1446.
4 Ukena, T.E., Borysenko, J.Z., Karnovsky, M.J. and Berlin, R.D. (1974) J. Cell Biol. 61, 70—82.
5 DePetris, S. (1975) J. Cell Biol. 65, 123—146.
6 Poste, G., Papahadjopoulos, D., Jacobson, K. and Vail, W. (1975) Biochim. Biophys. Acta 394, 520—539.
7 Poo, M.M. and Cone, R. (1974) Nature 247, 438—440.
8 Liebman, P. and Entine, G. (1974) Science 185, 457—459.
9 Peters, R., Peters, J., Tews, K. and Bahr, W. (1974) Biochim. Biophys. Acta 367, 282—294.
10 Kogelnik, H. and Li, T. (1966) Appl. Opt. 5, 1550—1567.
11 Carslaw, H. and Jaeger, J. (1959) Conduction of Heat in Solids, Chapt. 10, Clarendon Press, Oxford.
12 Masters, J. (1955) J. Chem. Phys. 23, 1865—1874.
13 Gunther, G., Wang, J., Yahara, I., Cunningham, B. and Edelman, G. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1012—1016.
14 Poste, G., Papahadjopoulos, D. and Nicolson, G.L. (1975) Proc. Natl. Acad. Sci. U.S. in the press.
15 Roblin, R., Chou, I.N., and Black, P.H. (1975) Adv. Cancer Res. 22, in the press.
16 Edelman, G.M., Cunningham, B.A., Reeke, Jr., G.N., Becker, J.W., Waxdahl, M. and Wang, J.L. (1972) Proc. Natl. Acad. Sci. U.S., 69, 2580—2584.
17 Zagyansky, Y., and Edidin, M. (1976) Biochim. Biophys. Acta 433, 209—214.
18 Schlessinger, J., Koppel, D., Axelrod, D., Jacobson, K., Elson, E. and Webb, W., in preparation.