Eur Biophys J (1987) 14:257–261

European Biophysics Journal

Fluorescence correlation spectroscopy in the nanosecond time range: rotational diffusion of bovine carbonic anhydrase B

P. Kask*, P. Piksarv, Ü. Mets, M. Pooga, and E. Lippmaa

Institute of Chemical Physics and Biophysics, Estonian Academy of Sciences, Lenini puiestee 10, Tallinn 200001, USSR

Received March 19, 1986/Accepted in revised form August 1, 1986

Abstract. A fluorescence correlation experiment for measurement of rotational diffusion in the nanosecond time scale is described. Using this method, the rotational diffusion coefficient of bovine carbonic anhydrase B labelled with tetramethylrhodamine isothiocyanate was estimated to be $D_r = (1.14 \pm 0.15) \times 10^7\ s^{-1}$ at 22 °C. The experiment is based on a cw argon ion laser, a microfluorimeter with local solution flow inside the sample cell, and two photon detectors. The fluorescence intensity autocorrelation function in the nanosecond time range is computed with the help of a time-to-amplitude converter and a multichannel pulse-amplitude analyser.

Key words: Carbonic anhydrase, fluorescence correlation spectroscopy, fluorescence intensity fluctuations, photon correlation spectroscopy, rotational diffusion

1. Introduction

Fluorescence correlation spectroscopy (FCS; Magde et al. 1972) is a method for studying the dynamics of molecular number fluctuations under the conditions of a small mean number of fluorescent molecules per sample volume, usually between 10 and 10^6. It has mostly been applied to translational diffusion of labelled molecules in different media: in solutions (Magde et al. 1974; Rigler et al. 1979), on membranes (Fahey et al. 1977), and in isolated cell nuclei (Sorscher et al. 1980). The typical time range of FCS is between a tenth and hundreds of milliseconds. If photostable labels are used, FCS permits a good signal-to-noise ratio in this time range.

Recently the extension of FCS into the nanosecond time range was demonstrated, observing the photon antibunching in dye fluorescence (Kask et al. 1985). The present work has been undertaken to clarify the possibilities of the method in rotational diffusion studies. Unlike fluorescence dynamic depolarization, FCS is expected to be useful in the studies of rotational motion in longer time ranges than the lifetime of the excited state of the chromophore (Ehrenberg and Rigler 1974).

2. Theoretical background

In an FCS experiment the measured fluorescence intensity autocorrelation function can be treated as the conditional probability density of detecting photons, provided one photon is detected at zero time. The condition that a photon is detected at zero time gives a certain amount of information about the initial state of the molecule responsible for emitting the zero-time photon, namely information about the position, orientation and electronic state. The measured function describes the time behaviour of this single molecule.

In the case of a rigid spherical macromolecule and fast solution flow, when

$$4D_t/w^2 \ll V/w \ll 6D_r \ll 1/\tau\,, \tag{1}$$

(where D_t is the translational diffusion coefficient; w is the radius of the exciting laser beam; V is the solution flow velocity; D_r is the rotational diffusion coefficient of a rigid spherical molecule; and τ is the lifetime of the singlet excited state of the label), the formula for the normalized fluorescence intensity autocorrelation function $g(t)$ takes the form:

$$\begin{aligned} g(t) = 1 + N^{-1}\{&-(1+a+b)\exp(-t/\tau) \\ &+ a\exp(-6D_r t) + b\exp(-20D_r t) \\ &+ \exp[-(Vt/w)^2]\}\,. \end{aligned} \tag{2}$$

N is the mean number of labelled molecules per sample volume. The values of the coefficients a and b depend on the geometry of experiment (Aragon and Pecora 1976). Equation (2) represents a syn-

thesis of the formulae derived by Ehrenberg and Rigler (1974), Aragon and Pecora (1975), and Magde et al. (1978). The first term inside the curly brackets is for photon antibunching and describes the relaxation of the molecule, responsible for emitting the zero-time photon, from the ground electronic state towards the equilibrium in occupation probabilities of the singlet ground and excited states. Relaxation of the orientation of the molecule is described by the second and the third terms which reflect rotational diffusion. For certain experimental geometries of considerable practical importance, the coefficient b becomes zero (Ehrenberg and Rigler 1974; Aragon and Pecora 1976). The last term describes solution flow, which forces the molecule to leave the observation domain. Equation (2) was derived assuming low excitation intensity, i.e. for conditions under which the ground-state occupation probability for a labelled molecule at any position and orientation is close to unity.

In designing the fluorescence correlation experiments in the nanosecond time range, it is very important to consider the signal-to-noise ratio (S/N). Under conditions where the number of photons detected per characteristic time per molecule is much below unity, S/N is proportional to the fluorescence intensity per molecule (Koppel 1974). Therefore, this intensity should be enhanced as much as possible. However, there is a danger in using excessive excitation light intensity. At too high an excitation intensity the ground state occupation probability may fall substantially below unity. A decrease in the ratio of fluorescence to excitation light intensity is symptomatic of this effect, which can result in unwanted intensity-dependent distortions of the fluorescence intensity autocorrelation function. If high excitation light power is nevertheless used, an extended theory or additional test experiments are needed to delineate the dependences of the quantities under estimation on excitation light intensity.

3. Apparatus

The apparatus for the experiment (Fig. 1) is similar to that previously described for the photon antibunching experiment (Kask et al. 1985). The beam from a cw argon ion laser at 514.5 nm is focused into a cuvette using an optical system consisting of a long focal length lens and a microscope objective with a numerical aperture of 0.11. The diameter of the beam at its focus is about 3.5 μm. The electric vector of the laser beam is horizontal and the polarizer is usually oriented to transmit emission with the electric vector parallel to that of the incident beam.

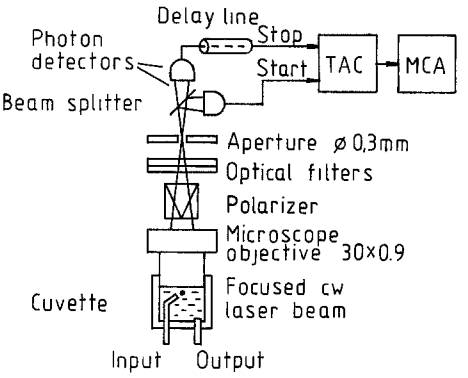

Fig. 1. The principal elements of the experiment. TAC – time-to amplitude converter; MCA – multichannel pulse-amplitude analyser. The focused laser beam is marked by a spot. Its direction is perpendicular to the plane of the figure

The solution enters the cuvette through a syringe needle, which is accurately aimed at the focus of observation. Fast local solution flow, perpendicular to the exciting laser beam, is created in the sample cell by connecting the input and the output of the filled cuvette to two vessels at different solution levels. Depending on the level difference, the flow velocity can be chosen to be between 0 and 200 mm/s. The flow velocity is estimated by fluorescence correlation experiments in the microsecond time range (see the last term in Eq. (2)) using a 32-channel clip-correlator (Sirk et al. 1979). The flow exchanges the bleached molecules for fresh ones. This process is faster and more complete than exchange by translational diffusion.

The fluorescence emission is observed using a water-immersion objective with a numerical aperture of 0.9. The output light is spectrally filtered by glass filters to cut off the scattered light at 514.5 nm and to remove also the most intense lines of the Raman scattering from water. An aperture is positioned in the image plane of the observation objective to separate a short segment of the image of the fluorescent trace of the laser beam. To observe a segment shorter than 15 μm is impractical because of the defocussing of the image caused mostly by the depth of the sample position (Kask et al. 1985). The roughly estimated value of the sample volume of our experiment is 150 μm^3. The true value of this quantity depends on the quality of optical adjustment and, hence, should not be taken as a constant.

The light emerging from the aperture is divided into two approximately equal parts by a beam-splitter for observation by the two photon detectors.

The electronic part of the apparatus (Kask et al. 1985) consists of two photon detectors, a time-to-amplitude converter (type 1701, Polon), and a multichannel pulse-amplitude analyser (type NTA 1024,

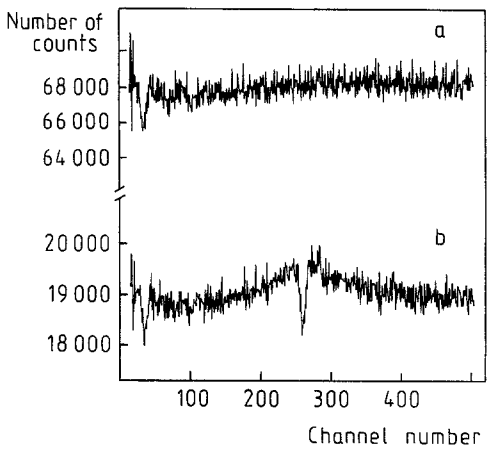

Fig. 2a and b. The memory content of the multichannel analyser, **a** after a 35-h experiment on light from incandescent lamp, **b** after a 10-h experiment on fluorescence of BCA-TRITC solution at 22 °C at a flow rate of 50 mm/s, using 24 mW excitation

EMG). The photon detectors consist of a photomultiplier (FEU-79) whose output is fed to a constant fraction discriminator via a preamplifier. The response function of the system has a FWHM of 1.2 ns.

To test the apparatus, the response of the system to uncorrelated photons from an incandescent lamp was measured. The expected result of this measurement is

$$R_n = UTi_1 i_2 \exp(-nTi_2)/(1+Si_1),$$

where R_n is the expected content of the n-th MCA memory channel; U is the total duration of the measurement; T is the time equivalent of one MCA memory channel (the channel width); i_1 and i_2 are the average counting rates in the start and stop photon detectors, respectively; and S is the dead time of the system with respect to the following start pulses. For $i_2 = 45{,}000\ s^{-1}$, $T = 0.337$ ns, the expected result is a straight line with a small negative slope of -0.7% per 500 channels. Evidently, the experimental result (Fig. 2a) deviates systematically from expectation. The origin of these deviations lies in our apparatus and is treated as a slight dependence of the channel width on the channel number. A simple procedure to eliminate this instrumental distortion will be described below.

4. Materials and experimental procedures

Bovine carbonic anhydrase *B* (BCA) was prepared by R. Aguraiuja (Lippmaa et al. 1983). Tetramethylrhodamine isothiocyanate (TRITC) was purchased from Fluka, tris from Sigma Chemical Company. Sephadex G-15 and Sephadex G-100 superfine were obtained from Pharmacia Fine Chemicals.

For labelling, approximately tenfold molar excess of dry TRITC powder was added to the 2–5 mg/ml solution of BCA in 0.15 *M* sodium carbonate buffer, pH 9.0, at 4 °C. The mixture was left stirring in the dark at 4 °C. After 12–16 hours of conjugation the reaction mixture was passed through a Sephadex G-15 column equilibrated with 5 m*M* sodium carbonate, pH 9.0. The main fraction containing labelled protein BCA-TRITC was chromatographed on a Sephadex G-75 superfine column equilibrated with 25 m*M* *tris*-HCl, pH 7.5, and the second coloured fraction was collected. The dye-to-protein ratio was estimated by light absorbtion measurements to be near unity. The catalytic activity of BCA-TRITC has been determined to be equivalent to that of pure BCA.

250 ml of about 4×10^{-10} M solution of BCA-TRITC in 50 m*M* *tris*-HCl, pH 7.0, was prepared for each fluorescence correlation experiment, an amount which flows through our cuvette in 10 h at a flow velocity of 50 mm/s. Flow speed values of about 20 mm/s were also used in some experiments. Laser beam powers of 16, 24 and 33 mW were employed. Further increase of excitation light intensity produces little gain in fluorescence intensity, an empirical sign of approaching saturation of the singlet excited state. According to simple calculations, the lifetime of the ground state is about 7 ns in the centre of the focused 24 mW laser beam for the most favourable orientation of the label. The mean photon counting rate in both the start and the stop channels varied from 28,000 to 100,000 s^{-1} depending on the excitation intensity and on the concentration of the labelled protein. Pure water would give a counting rate from 5,500 to 12,000 s^{-1} depending on excitation intensity.

The direct result of every experiment, the content of the memory of the analyser (Fig. 2b), is subjected to further corrections. To eliminate the instrumental distortion mentioned above, the results of the fluorescence correlation experiments are divided, channel by channel, by the result of a subsequent measurement of light from the incandescent lamp. At the same time the most significant dead-time effect, described by a factor of $\exp(-nTi_2)$, is also automatically corrected. We note that the dead-time corrections are small under the conditions of our experiments, usually amounting to less than 1%.

After this correction, the result is the estimated fluorescence intensity autocorrelation function over a segment of its arguments around the zero value. (The zero argument is positioned at the centre of the scale by choosing a delay cable of suitable length in

the stop channel, see Fig. 1). Since the autocorrelation function is symmetrical, the two values of the correlation function, corresponding to the same absolute value of the argument, can be averaged. This procedure reduces the noise and, in addition, is also a good compensator for any residual dead-time distortion.

5. Results and discussion

A fluorescence intensity autocorrelation function calculated from a typical experiment on BCA-TRITC is represented in Fig. 3. The polarizer was oriented to transmit emission with the electric vector parallel to that of the incident beam. The total duration of this experiment at 22 °C was 10 h, the excitation light power was 24 mW, the flow velocity was about 50 mm/s and the mean photon count rate was 42,000 s^{-1}.

The experimental result should be fitted by the convolution of the formula given in Eq. (2) and the response function of the apparatus. Instead of this we have used a simplified fitting formula:

$$g(t) = 1 + N^{-1}[-(1+a)\exp(-t/\tau) + a\exp(-6D_r t) + 1], \quad (4)$$

equivalent to Eq. (2) with $b = 0$ since only a small b value is expected from our experimental geometry ($b/a = 0.09$) and the last term of Eq. (2) is practically indistinguishable from unity in the time range under observation, while the effect of convolution was not taken into account, because the width of the time response function is much smaller than the rotational correlation time.

The fit curve is represented by a smooth line in Fig. 3. The values of the estimated parameters are $N = 24$, $a = 1.22$, $\tau = 1.7$ ns and $1/(6D_r) = 14.7 \pm 2.0$ ns. The expected deviation of the last value is determined from the limit of reproducibility; the estimated D_r values were independent of both the excitation power and the flow rate. On the other hand, the estimated N values depend slightly but noticeably on excitation power. The estimate of τ is biased because the width of the time response function is comparable with the τ value. Consequently, the estimates of N and a are also biased. The estimated τ, N and a values are probably true with the precision of a factor of 1.5.

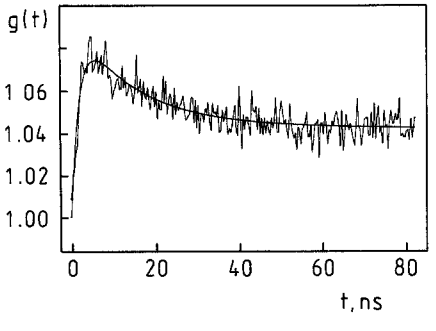

Fig. 3. The measured fluorescence intensity autocorrelation function for the BCA-TRITC solution

Indeed, the apparent N values are in fair agreement with the number of labelled molecules per sample volume, $N = 32$, calculated from the values of the label concentration and the sample volume. $a = 1.78$ would be expected from the theory if the transition moments for excitation and emission of the label were parallel. The estimated a value is considerably lower, probably on account of partial rotational freedom of the label. However, the results of the present experiments are too noisy to set up a more complicated model.

A few experiments have been run without the polarizer inside the observation microscope. The normalized fluorescence intensity autocorrelation function measured without the polarizer differs from that of Fig. 3. The amplitude of the slow term is smaller by a factor of about two (i.e., a smaller a value). This difference is direct evidence for the rotational nature of the motion responsible for the measured correlation decay.

The estimated $1/(6D_r)$ value of carbonic anhydrase can be compared with the value of 12.2 ns, measured by the fluorescence dynamic depolarization method and adjusted for temperature and water viscosity at 22 °C (Yguerabide et al. 1970).

Further improvement of S/N in the fluorescence correlation experiment, resulting in principle from higher efficiencies of fluorescence light collection, filtering and photon detection, will certainly increase the usefulness of FCS for studies of molecular rotations in the nanosecond and longer time ranges.

References

Aragon SR, Pecora R (1975) Fluorescence correlation spectroscopy and Brownian rotational diffusion. Biopolymers 14:119–138

Aragon SR, Pecora R (1976) Fluorescence correlation spectroscopy as a probe of molecular dynamics. J Chem Phys 64:1791–1803

Ehrenberg M, Rigler R (1974) Rotational brownian motion and fluorescence intensity fluctuations. Chem Phys 4:390–401

Fahey PF, Koppel DE, Barak LS, Wolf DE, Elson EL, Webb WW (1977) Lateral diffusion in planar lipid bilayers. Science 195:305–306

Kask P, Piksarv P, Mets Ü (1985) Fluorescence correlation spectroscopy in the nanosecond time range: photon antibunching in dye fluorescence. Eur Biophys J 12: 163–166

Koppel D (1974) Statistical accuracy in fluorescence correlation spectroscopy. Phys Rev A 10:1938–1945

Lippmaa ET, Olivson AI, Jarvet JI-H, Aguraiuja RK (1983) Study on bovine carbonic anhydrase B by [^{13}C] NMR spectroscopy. Mol Biol (U.S.S.R.) 17:484–491

Magde D, Elson E, Webb WW (1972) Thermodynamic fluctuations in a reacting system – measurement by fluorescence correlation spectroscopy. Phys Rev Lett 29:704–708

Magde D, Elson EL, Webb WW (1974) Fluorescence correlation spectroscopy. II. An experimental realization. Biopolymers 13:29–61

Magde D, Webb WW, Elson EL (1978) Fluorescence correlation spectroscopy. III. Uniform translation and laminar flow. Biopolymers 17:361–376

Rigler R, Grasselli P, Ehrenberg M (1979) Fluorescence correlation spectroscopy and application to the study of brownian motion of biopolymers. Phys Scr 19:486–490

Sirk A, Kask P, Kändler T, Karu T, Puskar J, Lippmaa E (1979) Clip-correlator for fluorescence correlation experiments. Proc Acad Sci Estonian SSR Phys Math 28:227–232

Sorscher SM, Bartholomew JC, Klein MP (1980) The use of fluorescence correlation spectroscopy to probe chromatin in the cell nucleus. Biochim Biophys Acta 610:28–46

Yguerabide J, Epstein HF, Stryer L (1970) Segmental flexibility in an antibody molecule. J Mol Biol 51:573–590