Lutz Jermutus · Reto Kolly · Zeno Földes-Papp
Jozef Hanes · Rudolf Rigler · Andreas Plückthun

Ligand binding of a ribosome-displayed protein detected in solution at the single molecule level by fluorescence correlation spectroscopy

Received: 11 September 2001 / Revised: 5 December 2001 / Accepted: 6 December 2001 / Published online: 30 January 2002

Abstract Interaction of a single-chain antibody fragment (scFv) with its cognate antigen while still attached to the ribosome was studied by fluorescence correlation spectroscopy (FCS). In experiments with purified scFv, FCS was capable of resolving the difference in diffusion time between free and antibody-bound labelled antigen. Ribosome-displayed antibody fragments generated by in vitro translation, in which neither the protein nor the mRNA leaves the ribosome owing to the absence of a stop codon and stabilizing buffer conditions, could be shown to specifically bind the antigen. The antibody-antigen interaction was specific, as shown by inhibition or displacement with unlabelled antigen and by control experiments with a non-cognate antibody fragment.

Keywords Fluorescence correlation spectroscopy · Single molecule analysis · Ribosome display · Single-chain antibody fragment · scFv

Introduction

Recent advances in fluorescence and micro-mechanical technology have decreased the detection limit down to the level of single molecules. Fluorescence correlation spectroscopy (FCS), based on the detection of single molecules in a confocal volume element (Eigen and Rigler 1994; Nie et al. 1994; Rigler 1995), provides a highly sensitive way to study biomolecular interactions (Rigler and Elson 2001). In FCS, a mixture of soluble fluorescent molecules with different molecular weights can be analyzed by a single measurement. Up to three species with different diffusion times can be resolved and their amounts quantitated using a single fluorophore. Therefore, the amount of ligand being in the free state (fast diffusion) and the receptor-bound state (slow diffusion of the complex) can be quantitated and measured as a function of concentration and, thereby, affinity and interaction kinetics can be determined. Moreover, measurements can be performed in small volumes (down to 1 µL) within a short experimental time (1–60 s). During this time period the fluorescence intensity of molecules in a small volume element (0.2 fL) is recorded and correlated in time. By using such a small measurement volume, background fluorescence can be minimized since contaminating fluorescence molecules will only rarely enter the measurement volume. The fluorescence fluctuations in the measurement volume are due to the variations in concentration of the fluorescent dye by Brownian diffusion of single molecules. From the autocorrelation function the average number of molecules and their respective diffusion coefficients can be calculated.

Since the detection method is so sensitive, it would be attractive to study the protein directly after in vitro translation. Moreover, upon in vitro translation of mRNA, a ternary mRNA-ribosome-polypeptide complex can be formed by omitting a stop codon and by using stabilizing buffer conditions, such that the nascent protein remains attached to the ribosome translating the encoding mRNA. Formation of this complex, in which the genetic information and the protein encoded by that gene are linked, allows evolution and selection of binding proteins (Amstutz et al. 2001).

Several previous reports on FCS described the interaction of ligands with their receptor embedded in the cell
membrane (Hasler et al. 1999; Rigler et al. 1999; Schuler et al. 1999; Van Craenenbroeck and Engelborghs 1999; Wohland et al. 1999) or in solution (Boldicke et al. 2000; Hink et al. 2000). In this study we report for the first time the detection by FCS of the specific interaction of both purified and in vitro translated, ribosome-bound antibody fragments with their labelled cognate antigen.

Ribosome-displayed antibody fragments are detected, characterized and quantified at subnanomolar concentrations. In particular, it is shown that the ribosome-displayed antibody fragments specifically bind the antigen in solution. These experiments prove that cell-free translation provides sufficient material to study protein-protein interactions by FCS, provided that one partner can be fluorescently labelled.

Materials and methods

Peptide synthesis

The GCN4 (7P14P) peptide (NH2-RMKQLEPKVEELL-)partner can be fluorescently labelled. protein-protein interactions by FCS, provided that one cell-free translation provides sufficient material to study the antigen in solution. These experiments prove that antibody fragments with their labelled cognate antigen. Ribosome-displayed antibody fragments are detected, characterized and quantified at subnanomolar concentrations. In particular, it is shown that the ribosome-displayed antibody fragments specifically bind the antigen in solution. These experiments prove that cell-free translation provides sufficient material to study protein-protein interactions by FCS, provided that one partner can be fluorescently labelled.

Protein purification and ELISA

The single-chain Fv antibody fragment (scFv) c11 was purified from Escherichia coli as described previously (Hanes et al. 1998). Purified protein at 10 nM concentration was assayed for functionality by ELISA according to published protocols (Hanes et al. 1998). For inhibition, the protein was equilibrated for 2 h with 0, 1 and 100 nM of labelled or unlabelled GCN4-7P14P peptide and then allowed to bind to immobilized antigen on a microtiter plate.

Fluorescence correlation spectroscopy

The FCS instrument used is a prototype built by Carl Zeiss. It is based on the ConfoCor device of Zeiss/Evotec and has been described previously (Rigler et al. 1998). Samples of 20 μL total volume were placed on a chambered coverglass (sterile borosilicate, Nunc) and measured at ~18 °C. The detection branch of the optical system (emission-measuring system) was focused in the object plane. Fluorescence emissions were collected by the objective (numerical aperture 1.2) and passed through a dichroic mirror. The transmitted light was focused by a lens on the pinhole. Since the excitation and emission optical pathways overlapped, out-of-focus emissions and scattered light were efficiently blocked by the pinhole. A pinhole size of 30 nm was used throughout the study. The emissions were focused by a lens and measured by an actively quenched avalanche photodiode (photon counting mode, SPCM 131-AQ, EG&G). The electronic output signals were autocorrelated by a two-channel ALV5000 correlator on a PC board. Data analysis was performed using in-house developed software based on the Marquardt non-linear least-squares parameterization for calculating the normalized mean square deviation between data and model (Marquardt 1963). Each sample was measured at least twice and each experiment was carried out at least three times.

In FCS the fluctuations δ of the emitted intensity around its mean value <I> are measured and correlated. The autocorrelation function G(τ) is defined as:

\[ G(\tau) = \langle I(t) + I(t + \tau) \rangle = \langle I \rangle^2 + \langle \delta I(\tau) \delta I(t + \tau) \rangle \]

where the brackets indicate the time average.

The thermal motion (diffusion) through a Gaussian confocal volume element G'(τ) is given as (Rigler et al. 1993):

\[ G'(\tau) = 1 + \frac{1}{N} \left( 1 + \frac{x}{\sigma} \right) \left( 1 + \left( \frac{z}{\sigma_a} \right)^2 \right)^{-1/2} \]

with:

\[ \tau_D = \frac{\omega^2}{4D} \]

where N is the mean number of fluorescent molecules in the volume element, τD the characteristic diffusion time, D the translational diffusion coefficient of the fluorescing species, and ω and z are the half axes of the cylindrical volume element. The parameters ω/z were determined from measurements using only the fluorescent dye rhodamine green. Equation (2) or its expansion to two or three components (molecular species) was fitted to the data of the FCS experiments.

In vitro translation for ribosome display

Ternary ribosomal complexes (mRNA-ribosome-scFv) were prepared as described, except that heparin was omitted (Hanes et al. 1998, 1999, 2000). We used the scFv c11 recognized by a monoclonal antibody (c11) as previously described (Hanes and Plückthun 1997; Hanes et al. 1998, 1999, 2000). We used the scFv c11 recognizing the 7P14P variant of the yeast GCN4 transcription factor with a dissociation constant (KD) of 40 pM (Hanes et al. 1998). The peptide antigen was