Fluorescence anisotropy analysis of protein–antibody interaction

Nadia Barbero[a] 1, Lucia Napione[a]; Pierluigi Quagliotto[b], Simona Pavan[a], Claudia Barolo[b], Ermanno Barni[b], Federico Bussolino[a], Guido Viscardi[b].

Abstract

The interaction between glutathione S-transferase and its antibody α-glutathione S-transferase (B-14) was studied using fluorescence anisotropy, subsequent to glutathione S-transferase bioconjugation with fluorescein-5-maleimide, leading to the determination of the dissociation and association binding constants, Kd and Ka; good binding specificity was observed between glutathione S-transferase and the antibody B-14. The use of spectroscopic techniques, fluorescence anisotropy in particular, is a useful and favourable tool to study biochemical problems.

Keywords

Protein−antibody interaction;

Fluorescence anisotropy;

Bioconjugation;

Fluorescein-5-maleimide;

Glutathione S-transferase (GST)

1 Corresponding author. Tel.: +390116707598; fax: +390116707591.

Present address: Department of General Chemistry and Organic Chemistry and NIS, Interdepartmental Centre of Excellence, University of Torino, C.so M. d’ Azeglio 48, I-10125 Torino, Italy.

1. Introduction

Glutathione S-transferases (GSTs) [1] constitute a family of detoxification enzymes that are involved in the metabolism of endogenous and xenobiotic compounds [2], [3], [4] and [5]. All of these enzymes catalyze the conjugation of glutathione to the electrophilic center of a variety of substrates, resulting in a more water soluble product that can be further degraded or transported out of the cell. GSTs have been implicated in the development of anticancer drug resistance and have been found in elevated levels in tumors [6].

The Glutathione S-transferase Gene Fusion System is a versatile system for the expression, purification and detection of fusion proteins produced in Escherichia coli. The system is based on inducible, high-level expression of genes or gene fragments as fusions with Schistosoma japonicum GST [7]. Expression in E. coli yields fusion proteins with the GST moiety at the amino terminus and the protein of interest at the carboxyl terminus. The protein accumulates within the cell's cytoplasm.

The GST Gene Fusion System has been used successfully in many applications including molecular immunology [8], the production of vaccines [9] and [10] and studies involving protein−protein [11] and protein−DNA [12] interactions.

Interactions between antigen and antibody involve non-covalent binding of an antigenic determinant to the variable region of both the heavy and light immunoglobulin chains. These interactions are analogous to those observed in enzyme−substrate interactions and they can be defined similarly. To describe the strength of the antigen (Ag) antibody (Ab) interaction, one can define the affinity constant (K) as shown by equation (1):

equation(1)

$K=[Ab{-}Ag][Ab]\times[Ag]=10^{4}$ to $10^{12}M^{-1}$

Therefore, the greater the K, the stronger the affinity between antigen and antibody. These interactions are the result of complementarity in shapes, hydrophobic interactions, hydrogen bonds and Van der Waals forces.

Anisotropy measurements [13] are based on the molecular motion of fluorescent molecules in solution in the time window occurring between absorption and emission of light. According to equation (2), the fluorescence anisotropy (r) values were determined as:

equation(2)

$$r=\frac{I_{vv}-G\cdot I_{vh}}{I_{vv}+2\cdot G\cdot I_{vh}}$$

where I_{vv} and I_{vh} represent the vertically and horizontally polarized emission intensities, respectively, following instrumental excitation with vertically polarized light and G is a correction factor which detects the instrumental sensitivity of the polarization direction of emission. G is defined as $G = I_{hv}/I_{hh}$, where I_{hv} and I_{hh} represent the vertically and horizontally polarized emission intensities obtained by excitation with horizontally polarized light.

Molecules in solution rotate and tumble. In the case of small molecules, the movement is very rapid, but the movement of larger molecules becomes slower. When fluorescent-labelled small molecules in solution are excited with a plane polarized light (Fig.1, top), the emitted light is depolarized due to fast movement of the molecule. However, when the fast-moving small fluorescent-labelled molecule is bound to the receptor having a large molecular mass, the movement of the conjugate is restricted and becomes slow. When such a conjugate is irradiated with a polarized light, the emitted light remains obviously polarized (Fig.1, bottom).

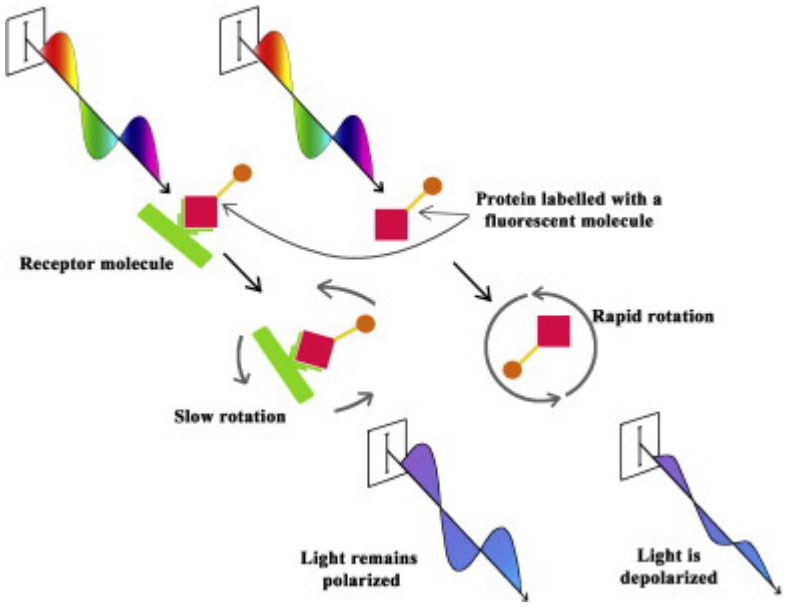


Fig. 1.

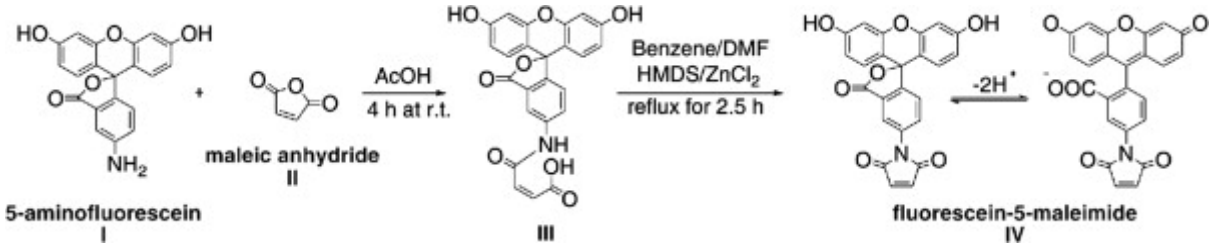
Principle of the assay of binding reaction using fluorescence anisotropy.

A binding isotherm can be easily constructed by titrating a fixed concentration of fluorescent-tagged molecules (GST labelled with fluorescein-5-maleimide in this case) with a binding protein (the antibody in this case). From such data, Kd and Ka can be obtained by non-linear regression analysis. In a typical procedure, the concentration of the fluorescent-labelled ligand is kept constant, and fluorescence anisotropy is measured by changing the concentrations of the receptor solution. The anisotropy value at each concentration is measured and used to generate a binding isotherm.

There are many applications using fluorescence anisotropy in DNA–protein and DNA–DNA interactions [14], protein–protein interactions [15], protease assays [16], immunoassays [17], conformational changes of proteins [18], and cell-biochemical studies [19].

The molecular basis of the specificity of antigen–antibody interactions is still poorly understood and very few data are available in the literature referring to protein–antibody interaction studied by surface plasmon resonance [20], fluorescence correlation spectroscopy (FCS) [21] and [22] and surface-enhanced Raman spectroscopy (SERS) [23]. This is due partly to the difficulty of analyzing in detail the nature of the molecular contacts. In their study, Altschuh et al. [20] worked on the determination of kinetic constants for the interaction between a monoclonal antibody and some peptides. They described a functional analysis of the interaction between a monoclonal antibody raised against tobacco mosaic virus (TMV) protein and a peptide corresponding to residues 134–146 of this protein. Differences in binding affinity resulting from single substitutions in the peptide were measured using the biosensor technology. They provided evidence of association constants in the order of $2.6–3.7 \times 10^7\ M^{-1}$ depending on the different peptide involved. Another paper [24] reported the kinetic analysis of monoclonal antibody–antigen interactions providing a Ka which varies around $3.7 \times 10^7\ M^{-1}$ and $1.5 \times 10^8\ M^{-1}$. Li et al. [25] utilized affinity capillary electrophoresis (ACE), a form of capillary zone electrophoresis (CZE) to determine the binding constant (Ka) of specific antibodies against bovine serum albumin (BSA) and the healthy prion protein (PrPc), in buffer solutions at fixed pHs, approximating to in vivo conditions. They derived Ka values as being $1.8 \times 10^7\ M^{-1}$ for the Rubenstein antibody and $1.9 \times 10^7\ M^{-1}$ for the VMRD antibody.

In the present work, the interaction between GST protein and its antibody was analysed for the first time. GST protein has been bioconjugated with a fluorophore, i.e. fluorescein-5-maleimide (Scheme 1) and its interaction with its antibody α-GST (B-14) was studied by fluorescence anisotropy. The dissociation and association binding constants (Kd and Ka) have been determined by non-linear regression analysis.



Scheme 1.

Synthesis of fluorescein-5-maleimide.

2. Experimental section

2.1. Materials

GST was produced in E. coli BL21 cells using pGEX vector systems. Bacteria were sonicated in buffer A1 (50 mM TrisHCl pH 7.5, 1% TritonX100, 150 mM NaCl, 5 mM MgCl2, 1 mM DTT) containing protease inhibitors. Lysates were incubated with glutathione-coupled Sepharose 4B beads (GE Healthcare Life Sciences) for 1 h at 4 $^{\circ}$ C. After washing, the bounded beads were incubated with 2 ml of buffer B1 (50 mM TrisHCl pH 8.0, 100 mM NaCl, 2 mM DTT, 20 mM glutathione) overnight to perform affinity-chromatography separation. The eluates were dialyzed against PBS using Slide-A-Lyzer 3.5K (PIERCE). Recombinant protein concentrations were determined by Comassie-stained gels.

α-GST (B-14) is a mouse monoclonal antibody raised against the 26 kDa GST specific domain of a fusion protein encoded by a pGEX.3X recombinant vector. It is recommended for detection of glutathione-S-transferase of S. japonicum origin and GST fusion proteins. It was purchased from Santa Cruz Biotechnology Inc. and used as received.

2.2. Experimental techniques

Fluorescence anisotropy measurements were recorded using an LS55 Perkin Elmer spectrofluorimeter equipped with a xenon lamp source, a 5 mm path length quartz cell, Polaroid filters and a thermostatted bath kept at 25 $^{\circ}$ C. Samples were excited at 494 nm and monitored at 515 nm. Slit widths were 2.5/15 nm and integration time was 10 s. G factor was estimated every day before starting measurements.

UV−Vis measurements were recorded using a Shimadzu UV-1700 Pharma Spec Spectrophotometer equipped with 1.0 cm path length quartz cells.

1H NMR spectra were performed on a Jeol EX400 instrument in DMSO-d6 solution using the DMSO signal as a reference. NMR signals are described by use of s for singlet, d for doublet, t for triplet, m for multiplet.

Mass spectra were collected by a Finnigan Mat TSQ700 Spectrometer. ESI−MS spectra were recorded using an LCQ Deca XP plus spectrometer (Thermo, Rodano, Italy), with electrospray interface and ion trap as

mass analyzer. The flow injection effluent was delivered into the ion source using nitrogen as sheath and auxiliary gas.

2.3. Fluorescein-5-maleimide synthesis

Fluorescein-5-maleimide was synthesised as reported by Reddy et al. [26].

2.3.1. Synthesis of N-(5-fluoresceinyl)maleamic acid

To a stirred solution of amine I (2.88 mmol) in AcOH (300 ml) maleic anhydride II (2.88 mmol) was added and the resulting solution was stirred at r.t. for 4 h. Precipitated amic acid III was filtered, washed with EtOAc (600 ml), dried and used as such without further purification. Yield: 74% (yellow solid), Mp: >300 $^{\circ}$ C.

2.3.2. Synthesis of N-(5-fluoresceinyl)maleimide

HMDS (Hexamethyldisilazane) (1.37 g, 8.48 mmol) was added to a stirred suspension of amic acid III (0.95 g, 2.12 mmol) and ZnCl2 (0.58 g, 4.24 mmol) in a mixture of benzene (115 ml) and DMF (13 ml) and the resulting mixture was refluxed for 2.5 h. After cooling to r.t., the mixture was filtered and filtrate was concentrated under vacuum. The residual DMF portion was poured into ice-water (50 ml) and the aqueous phase was acidified to pH 4.0 by adding 0.1 N HCl. On cooling, fluorescein-5-maleimide IV (0.84 g) was obtained in 92.3% yield as an orange-yellow solid, mp>300 $^{\circ}$ C [26].

1H NMR (DMSO-d6): δ = 10.20 (s, 2H), 8.00 (d, J = 1.1 Hz, 1H), 7.80 (dd, J = 1.6, 8.2 Hz, 1H), 7.42 (d, J = 8.1 Hz, 1H), 7.28 (s, 2H), 6.55−6.71 (m, 6H).

MS (ESI): m/z (%) = 428 (M−1), 458 (M − 1, 33).

2.4. Bioconjugation experiments

GST protein has been bioconjugated with fluorescein-5-maleimide following the reported protocol [27]. The sulfhydryl-containing protein GST was dissolved at a concentration of 1−10 mg/ml in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.2. Fluorescein-5-maleimide was then dissolved in DMF at a concentration of 10 mM protecting it from light and a 25-fold molar excess of fluorescein-5-maleimide solution was added to the protein solution. After for 4 hours of reaction at room temperature in the dark the crude derivative was immediately purified using gel filtration on PD10 columns Sephadex® G-25 (Amersham Bioscience) using a phosphate buffer saline solution (10 mM PBS, pH = 7.4) as eluent. The solutions were protected from light during the chromatography.

2.5. GST−antibody interaction

During observation of anisotropy, the temperature was kept constant because it greatly affects the molecular motion of the conjugate in the solution. Fluorescence anisotropy for each titration point was measured 5 times after a 3 min incubation at 20 $^{\circ}$ C. The final concentration includes a dilution factor to correct for the volume of added protein solution.

The interactions were undertaken with a GST concentration of 1.00×10^{-8} M. GST (B-14) antibody solutions at different concentrations were prepared by diluting a 2.50×10^{-6} M stock solution.

After pre-equilibration, the appropriate amount of protein stock solution was added and the fluorescence signal monitored until stable. The sample was then titrated with aliquots (5 μ l) of antibody solution and the anisotropy values were collected.

3. Results and discussions

3.1. Dye/Protein ratio calculation

The dye/protein ratios (D/P) of the conjugates were determined by the absorption spectra of the labelled proteins, registered in 10 mM PBS (pH = 7.4) according to the relationship reported in equation (3):

equation(3)

$$D/P=A_{max}\cdot \varepsilon_{prot}(A_{280}-cA_{max})\cdot \varepsilon_{dye}$$

where A_{280} is the absorption of the conjugate at 280 nm; A_{max} is the absorption of the conjugate at the absorption maximum of the corresponding fluorescein-5-maleimide; c is a correction factor which must be used to adjust for amount of A_{280} contributed by the dye because fluorescent dyes also absorb at 280 nm and equals the A_{280} of the dye divided by the Amax of the dye (c = 0.29); $\varepsilon_{protein}$ (55310 cm^{-1} M^{-1}) and εdye (63096 cm^{-1} M^{-1}) are the molar absorption coefficients for the protein used and fluorescein-5-maleimide, respectively.

The fluorescein-5-maleimide correction factor was calculated by UV−Vis experiments, the molar absorption coefficient of the free dye (ε_{dye}) was calculated from the slope of a Lambert−Beer plot while the molar absorption coefficient of the protein was estimated from knowledge of its amino acid composition [28]. From the molar absorption coefficient of tyrosine, tryptophan and cystine (cysteine does not absorb appreciably at wavelengths >260 nm, while cystine does) at a given wavelength, the molar absorption coefficient of the native protein in water can be computed using equation (4) [29].

equation(4)

$$\varepsilon=(nW\times 5500)+(nY\times 1490)+(nC\times 125)\varepsilon=(nW\times 5500)+(nY\times 1490)+(nC\times 125)$$

where n is the number of each tryptophan (W), tyrosine (Y) and cystine (C) residue respectively and the stated values are the amino acid molar absorption coefficients at 280 nm.

The bioconjugation was repeated several times and the resulting D/P ratio obtained was around 1 with values varying from 1.17 to 1.71. Moreover, we detected that different fractions coming from the same purification have different D/P ratios. This finding, in combination with the consideration that GST presents only 1 cysteine residue at the N-terminal, suggests that the first fraction (which usually has a D/P closer to 1) consists of the labelled protein alone (where the fluorophore is covalently labelled) while in the following fractions the non covalently labelled fluorophore is also present in solution (leading to higher D/P values). To avoid possible signal interferences, we selected the first fraction for the interaction studies.

To validate the reliability of the proposed bioconjugation, we performed a different purification method found in literature and suitable for peptides [30] which consists in removing the non-labelled fluorescein by precipitation with acetone. After fluorophore precipitation, we evaluated the D/P and the obtained value was 1, confirming that fluorescein-5-maleimide is covalently labelled to GST protein. Unfortunately, the use of acetone for the fluorophore precipitation caused protein denaturation as also reported in literature [30],

therefore this bioconjugate was inappropriate for the binding studies, but able to confirm the validity of the gel filtration method.

3.2. Data analysis and results

The interaction between GST and α-GST (B-14) was studied by fluorescence anisotropy. The concentration of the fluorescent-labelled ligand (GST conjugated with fluorescein-5-maleimide) is kept constant, and anisotropy is measured by changing the concentrations of the receptor (B-14) solution. The anisotropy value at each concentration is measured and used to generate a binding isotherm.

Results of a typical experiment are presented in Fig. 2. Data are plotted as the anisotropy of the complex as a function of added GST (B-14). Binding isotherms were fitted to equation (5) by non-linear regression (using the programs Origin 6.1, GraphPad Prism 4 and TableCurve 2D v5.01):

equation(5)

A=Af+(Ab−Af)[Ka[L]1+Ka[L]]

where A is the experimentally measured anisotropy, Af is the anisotropy of free fluorescent molecules, Ab is the anisotropy of bound fluorescent molecules, Ka is the association binding constant and [L] is the ligand concentration (i.e. in this case α-GST concentration) [31]. (A further explanation and the detailed derivation of equation (5) is reported in Supporting information)

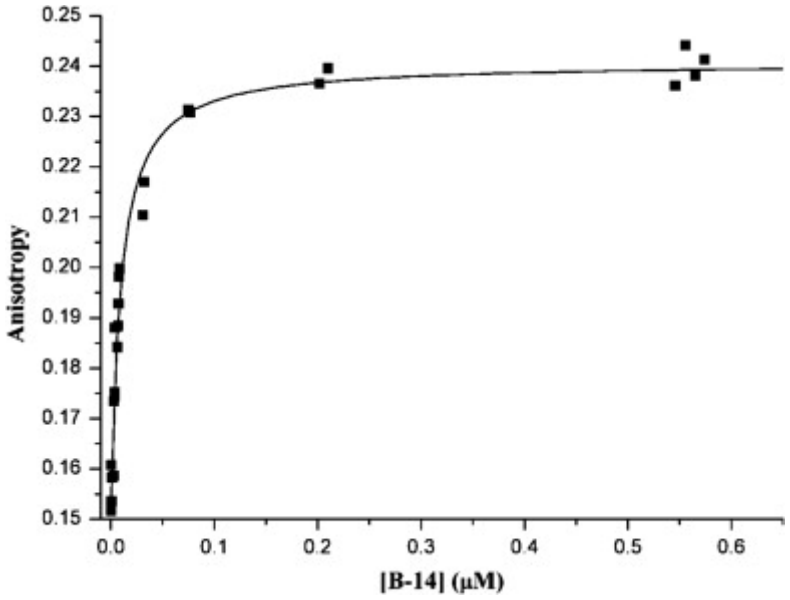


Fig. 2.

Fitting curve of an example of GST − B-14 interactions.

As is evident from the reported graph (Fig. 2), an interaction between the GST protein and its antibody B-14 does occur. Actually, a variation of the anisotropy signal (an increase in particular in this case) by increasing the antibody concentration is visible. The anisotropy value increased because the fluorescent-labelled protein GST, having reacted with the antibody, has decreased its rotational movement leading to a less depolarized emission light.

By fitting the interaction curves for a series of experiments, the following mean results were obtained: Af = 0.140 $\pm$ 0.03, Ab = 0.252 $\pm$ 0.06 and the evaluated association constant Ka was 8.27 $\times$ 10^8 M^{-1} $\pm$ 2.69 $\times$ 10^7 while the dissociation constant Kd was 1.21 $\times$ 10^{-9} M.

Since this interaction has never been analysed before, we are not able to compare the binding constants obtained for this specific protein−antibody interaction. In any case, the binding constants obtained are in agreement with the few data available in literature referring to other protein−antibody interaction. As already reported previously, Altschuh et al. [20] had worked on the determination of kinetic constants for the interaction between a monoclonal antibody and some peptides and provided evidence of association constants in the order of 2.6−3.7 $\times$ 10^7 M^{-1} depending upon the peptide involved. Karlsson et al. [24] reported the kinetic analysis of monoclonal antibody−antigen interactions providing a Ka which varies around 3.7 $\times$ 10^7 M−1 and 1.5 $\times$ 10^8 M^{-1}. Li et al. [25] derived Ka values as being 1.8 $\times$ 10^7 M^{-1}. The binding constants values obtained agree well with the data reported by these authors and are also in good agreement within the same set of interaction providing good repeatability and consistency.

4. Conclusions

The interaction between GST and its antibody α-GST (B-14) was studied by fluorescence anisotropy, after GST bioconjugation with fluorescein-5-maleimide, leading to the determination of the dissociation and association binding constants (Kd and Ka). By comparing the results obtained for GST-B-14 interaction with the data found in literature referring to protein−antibody interactions, we can assert that a good binding specificity is present between GST and the antibody B-14 and that the use of fluorescence anisotropy is a useful and favourable tool to study biochemical problems.

The use of fluorescence anisotropy has provided equilibrium determinations based on the observation of the molecular movement of the fluorescent molecules in solution.

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Corresponding author. Tel.: +390116707598; fax: +390116707591.

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Present address: Department of General Chemistry and Organic Chemistry and NIS, Interdepartmental Centre of Excellence, University of Torino, C.so M. d’ Azeglio 48, I-10125 Torino, Italy.