Site-Specific Interaction of Thrombin and Inhibitors Observed by Fluorescence Correlation Spectroscopy

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ABSTRACT We report on the application of fluorescence correlation spectroscopy (FCS) to observe the interaction between thrombin and thrombin inhibitors. Two site-specific fluorescent labels were used to distinguish between inhibitors directed to the active site, the exosite, or both binding sites of thrombin. For several well-known inhibitors of thrombin, the binding sites observed by FCS correspond to previous studies. The interaction of the recently discovered thrombin inhibitor ornithodorin from the tick *Ornithodorus moubata* with thrombin was investigated. It was found that this inhibitor, like hirudin and rhodniin, binds to both the active site and exosite of thrombin simultaneously. This study shows the feasibility of FCS as a sensitive and selective method for observing protein-ligand interactions. As an additional technique, simultaneous labeling with both fluorescent labels was successfully demonstrated.

INTRODUCTION

Fluorescence correlation spectroscopy (FCS) is an analytical tool that uses number fluctuations of fluorescent molecules (or particles) in a very small observation volume (the confocal volume of a set-up analogous to confocal laser microscopy) to obtain information on the diffusive properties of these molecules. Molecular interactions that lead to the formation of a complex between the fluorescent molecules and other molecules show up in a reduced diffusion constant of the fluorescent molecules, as the thermal diffusion of the complex will be slower than that of each component. Together with the high sensitivity and the spectral selectivity, the ability to derive diffusion coefficients of the observed fluorescent molecules, and therefore to distinguish between different states of these molecules without physical separation, makes FCS an interesting technique for many problems in biochemistry (Rigler, 1995). FCS, conceived more than 20 years ago (Magde et al., 1972), has recently seen a surge in interest. There are two main reasons for this surge in interest.

First, technical advances (Thompson, 1991), like progress in confocal laser microscopy and high-quantum-efficiency solid-state light detectors, have made it possible to carry out FCS measurements with low background and short measurement durations at very low concentrations of the analyte, even down to single molecule detection (Rigler, 1995).

Second, fluorescent techniques are becoming more important in the discovery of new lead structures for drugs. This discovery still involves brute-force testing of large compound libraries for every new pharmaceutical target, despite the considerable progress of more knowledge-based approaches, like molecular modeling (Campbell, 1996; Böhm et al., 1996). With the advent of new synthesis strategies like combinatorial chemistry (Campbell, 1996), the size of compound libraries is seeing a vast expansion. To screen these large compound libraries, it is necessary to develop fast screening methods. Many screening methods for new drugs currently employed are heterogeneous; that is, they rely on a physical separation of bound and unbound ligands. This separation is time consuming because of repetitive pipetting and washing steps. An approach to avoiding this would be a homogeneous assay principle like FCS, concentrating on a change of physical parameters of fluorescent reference compounds, like rotational or translational diffusion constants. Such homogeneous assay principles can detect the release of the fluorescent reference compound from the target through competition from a compound to be tested without separating bound from unbound reference compounds, thus offering the promise of fast assays. The goal of the present work is to investigate in detail the feasibility of homogeneous FCS assays for a well-known target.

THROMBIN AND INHIBITORS

The biochemical system investigated is shown in Fig. 1. We employed thrombin as a model system to evaluate FCS as an analytical technique for drug screening. Thrombin (m = 39 kDa) is the central protease in the blood coagulation cascade (Jackson, 1980; Davie et al., 1991). Thrombin cuts two small peptides from the protein fibrinogen, forming fibrin, which, in turn, polymerizes. To achieve this, thrombin is equipped with two binding sites. The exosite is a positively charged surface area that binds fibrinogen and orients it according to distance and location toward the active site. The active site contains a catalytic pocket of the trypsin type. Two peptide loops restrict the access of proteins to this binding site and therefore are responsible for the high specificity of thrombin.

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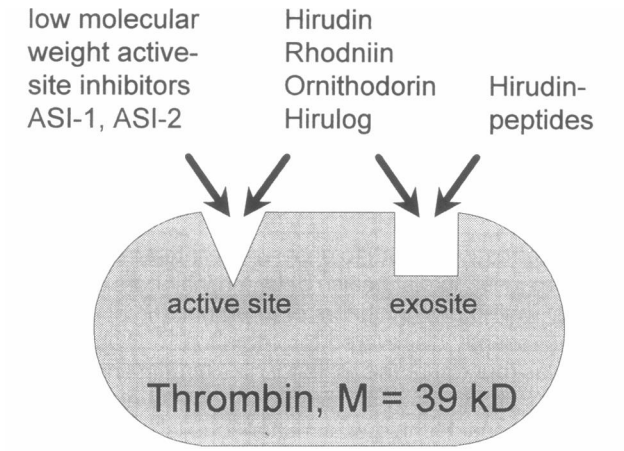
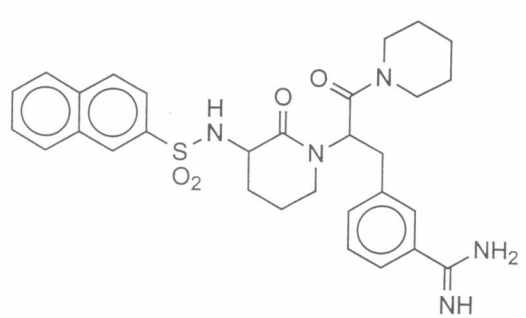
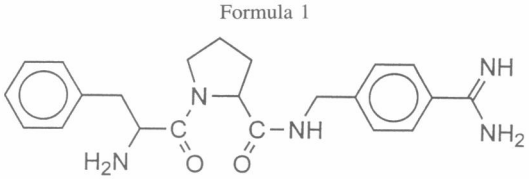


Inhibitors	M	
Hirudin	6.5 kD	natural inhibitor (*Hirudo medicinalis*)
Rhodniin	11 kD	natural inhibitor (*Rhodnius prolixus*)
Ornithodorin	12 kD	natural inhibitor (*Ornithodorus moubata*)
Hirulog	3 kD	C-terminal sequence of Hirudin, linked to active-site inhibitor
Hirudin peptides	1471 D 1644 D 1835 D	3 C-terminal sequences of Hirudin: 1: N-acetyl-FEEIPEEYLQ-COOH 2: N-acetyl-DFEEIPEEYLQ-COOH 3: N-acetyl-HNDGFEEIPEEYLQ-COOH
active site inh. ASI-1	394 D	*(see formula 1)*
active site inh. ASI-2	394 D	*(see formula 2)*
Fluorescent reference compounds		
ASI-2* (active site)	872 D	FITC-labeled active site inh. ASI-2
JR-Sym* (exosite)	5600 D	Rhodamine labeled DNA oligomer



Formula 1



Formula 2

FIGURE 1 Thrombin inhibitors and reference compounds used in this study.

As thrombin is responsible for the fibrin polymerization and in part for the formation of blood clots in different coagulation-related diseases, it is a well-studied target, and several thrombin inhibitors are known. The inhibitors used in this study are also shown in Fig. 1. Hirudin, rhodniin, and ornithodorin are proteins isolated from blood-sucking animals. Hirulog and the hirudin peptides are shorter parts of hirudin. The natural proteins and hirulog are known to bind to both binding sites, the small-molecular-weight compounds to the active site only, and the hirudin peptides only to the exosite (Dodt, 1995). As shown by recent crystal structure studies, ornithodorin should bind to both active site and exosite (van de Locht et al., 1996).

MATERIALS AND METHODS

FCS

All measurements were made on a homemade FCS apparatus, based on a modified commercial confocal laser scan microscope (type Leica DM-RB; Leica GmbH, Heidelberg, Germany). The set-up is similar to one recently described in the literature (Rigler et al., 1993), so only a short description is given here. The light of a 20-mW argon laser (488 or 514 nm) is coupled to a single-mode optical fiber and passes through the illumination pinhole (diameter 30 μm) onto a partially reflective (20%) mirror. The reflected light is focused by a water immersion microscopy objective (magnification 50×, NA 0.75; Leica) into the sample. Absolute light intensities at the point of focus are between 0.05 and 0.5 mW. The samples are presented in rectangular glass capillaries (Microslide; Camlab, Cambridge, England; inner dimensions 1.000 μm × 200 μm, wall thickness 185 μm, length 5 cm, sample volume 10–20 μl). The excited fluorescence is collected by the objective and focused through the partially reflective mirror (80% transmission) and the detection pinhole (variable diameter, set to 130 μm in this study) onto the sensitive area of a silicon avalanche diode module (SPCM 200; EG&G Instruments, Princeton, NJ). The electric signal of this detector is fed into a hardware correlator (ALV-5000; ALV, Langen, Germany). The standard measurement duration for this study was 60 s, at signal count rates of 10–100 kHz and a background of 1–2 kHz.

Data evaluation procedure

The data evaluation procedure in FCS is similar to the one used for photon correlation spectroscopy (or dynamic light scattering) (Berne and Pecora, 1976) and has been described in the literature (Rigler et al., 1993).

In short, the direct result of a FCS measurement, the autocorrelation function (ACF) $G(t)$ of the observed fluorescence intensity, is fitted to the equation

$$G(t) = 1 + \frac{1}{N}\left(\frac{1}{1 + (t/\tau)}\right) \quad (1)$$

where N is the average number of fluorescent molecules and τ is the average duration of their stay in the observation volume. For molecules moving under thermal diffusion, τ is given by

$$\tau = \frac{r^2}{4D} \quad (2)$$

where D is the thermal diffusion coefficient of the molecules and r is the radial width of the laser profile (1/e point). The value of r must be obtained by calibrating the instrument once with a sample with a known diffusion coefficient. To give more intuitively accessible results, the diffusion coefficients in this study are converted to apparent hydrodynamic diameters according to the Einstein equation

$$d = \frac{kT}{3\pi\eta D} \quad (3)$$

where d is the hydrodynamic diameter of the molecule, k is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the medium.

In the literature, there are several expansions of Eq. 1 that account for deviations of the real experiment from the idealized one, for which Eq. 1 is derived. In our study, however, these expansions are neglected, and the data are evaluated for the simple case of Eq. 1, with its two free parameters N and τ. We chose to do so after evaluation with more complicated equations that include the above expansions gave unstable results with our experimental data. (For the expanded data evaluation, we followed the formula in Widengren et al. (1994). The expansions take into account 1) the finite extent of the observation volume along the optical axis, 2) the pumping of the fluorophores into the triplet state, and 3) the presence of bound and unbound fractions of the reference compound. The programs used to perform the nonlinear curve fitting were 1) a home-written program for nonlinear least-square fit of FCS data, 2) the nonlinear curve-fitting routine of the program package SigmaPlot by Jandel Corporation, and 3) the program FCS ACCESS by EVOTEC BioSystems GmbH (Hamburg, Germany), recently made available to us by Carl Zeiss Jena GmbH.)

So for each ACF, the fit to Eq. 1 yields two parameters: the average number N of fluorescent molecules observed, and their average time τ of stay in the observation volume. τ is converted into a hydrodynamic size by using Eqs. 2 and 3. To give absolute numbers, the instrumental value of r is calibrated with Rhodamin 6G as a molecule of known diffusion coefficient. (The hydrodynamic sizes given in this study are obtained by calibration of the instrument with an aqueous solution of the fluorescent dye Rhodamin 6G. The diffusion coefficient of R6G at 22°C is assumed to be $D = 2.8 \cdot 10^{-10}$ m^2/s, corresponding to a hydrodynamic radius of 1.60 nm. The calibration of our instrument then gives values of $r = 250$ nm and $r = 270$ nm for laser wavelengths of 488 nm and 514 nm, respectively. When a noticeable triplet fraction is present, we ignore the first few channels of the ACF in the data evaluation. Data evaluation for the more photostable reference compound JR-Sym* with the rhodamine label starts at a ACF time lag of 6 μs, for the less photostable reference compound ASI-2* with the fluorescein label at a 6- or 12-μs time lag. At greater time lags than these, the triplet influence in the ACF is negligible.)

For a mixture of two components A and B with the same fluorescent properties but different sizes (bound/free reference compound), the measured average hydrodynamic size $d_{average}$ from the simple fit of Eq. 1 can be calculated from the sizes of the individual components:

$$\log(d_{average}) \cong x_A \log(d_A) + x_B \log(d_B) \qquad (4)$$

where x_A and x_B are the molar fractions of components A and B, respectively. Equation 4 is strictly valid only for d_A and d_B that are very close together. Computer simulations of noise-free two-component ACFs have shown, however, that the error obtained with Eq. 4 when compared to the true two-component evaluation is less than 2%, if the size difference of the two compounds is less than a factor of 3, as is the case in our experiment. In the case of an ideal, noise-free ACF, the molar fractions x and individual times τ of a mixture of two components A and B with the same fluorescent properties can be obtained by fitting the ACF to the equation (Rigler et al., 1993b)

$$G(t) = 1 + \frac{1}{N}\left(\frac{x_A}{1 + t/\tau_A} + \frac{x_B}{1 + t/\tau_B}\right)$$

Thrombin and inhibitors

This study was carried out using commercial bovine thrombin (Sigma no. T 6634). Solutions of 10–50 μM in isotonic NaCl solution were divided into aliquots, stored at −18°C, and thawed before each experiment. Hirudin (Markwardt, 1955) was supplied by Dr. Schweden, BASF-AG. Rhodniin was prepared according to the methods of Friedrich et al. (1993) and van de Locht et al. (1995). Ornithodorin was prepared directly from the tick *Ornithodorus moubata* according to the method of van de Locht et al. (1996) and Otte et al., (manuscript in preparation). Hirulog and the hirudin peptides 1–3 were synthesized on a standard peptide synthesizer (Dodt, 1995).

Active site inhibitors ASI-1 and ASI-2 were synthesized by standard organic synthesis protocols and characterized by mass spectrometry and NMR.

All experiments were carried out in isotonic NaCl solution (145 mM NaCl) or phosphate buffer at pH 7.4 at room temperature (22°C) without further temperature control.

Fluorescent reference compounds

We synthesized two fluorescent reference compounds (ASI-1* and ASI-2*), directed to the active site and the exosite of thrombin, respectively. The fluorophores of these compounds were chosen to be well separated in excitation and emission wavelengths, so that each of them can be addressed separately by using suitable laser wavelengths and filter combinations.

The fluorescent reference compound directed to the active site (ASI-2*) was derived from the active site inhibitor ASI-2. Forty milligrams of ASI-2 (466.41 D, 85.8 μmol) was incubated with 60.8 mg fluorescein(5,6)succinimide ester (128.7 μmol) in 2 ml *N,N*-dimethylforamide and 9 μl triethylamine (86 μmol) for ~16 h at room temperature. The reaction mixture was then diluted with 2 *N,N*-dimethylforamide and applied to a C18-reversed phase HPLC separation (Macherey&Nagel RP 100/5 C, 18 ml, 4 mm diameter, 250 mm main column length, and a precolumn of 4 mm diameter and 11 mm length) on a Merck/Hitatchi HPLC system. For separation a linear gradient was used. (solvent A H_2O with 0.1% formic acid, solvent B CH_3CN with 0.1% formic acid). The labeled active site inhibitor ASI-2* was detected as a new thrombin-inhibiting fraction. Mass spectrometry revealed the expected molecular weight.

The fluorescent reference compound directed to the exosite (JR-Sym*) is a rhodamine-labeled DNA oligomer known from the literature (Wu et al., 1992). The DNA aptamer is synthesized on an Applied Biosystems 394-Synthesizer according to the manufacturers' guidelines with the sequence JR-Sym: GGTTGGTGTGGTTGG with a 5′ aminolink. As a control we synthesized the sequence JR-Neg: GGTGGTGGTTGTGGT (Wu et al., 1992). The rhodamine (ROX, ABI no. 400980) was coupled to the 5′ end in a carbonate buffer (0.5 M $NaHCO_3/Na_2CO_3$, pH 9) overnight. The labeled oligomers were purified by gel electrophoresis. The coupling of the dye was checked by spectral analysis of the end product, comparing absorption at 260 nm (DNA band) and 586 nm (ROX band).

RESULTS

Reference compounds

In a first step of the experiments, the binding of the reference compounds to thrombin was observed. Fig. 2 shows the measured hydrodynamic sizes of the reference compounds versus the concentration of thrombin. At the starting point and at the end point of the titration curves, the measured hydrodynamic sizes correspond to the size of the free and the bound reference compounds, respectively. The solid lines in Fig. 2 are a fit of the data to the model of a two-component complexation in chemical equilibrium, with the resulting observed hydrodynamic size calculated according to Eq. 4. The values for the dissociation constant of the reference compounds obtained from the fit are 200 nM for JR-Sym* and 500 nM for ASI-2*. The scrambled DNA aptamer JR-Neg* shows no complexation with thrombin.

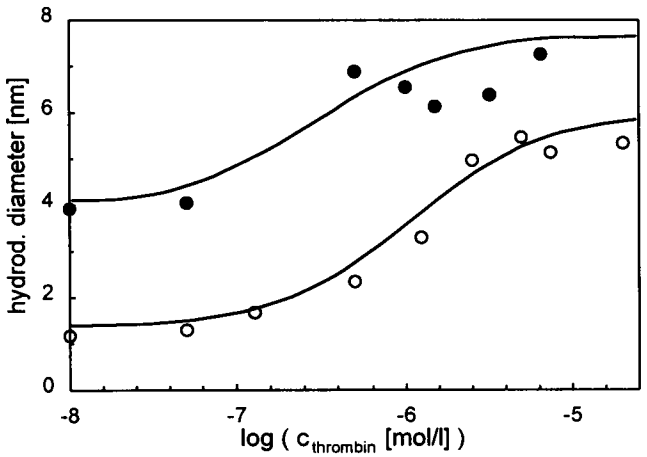


FIGURE 2 Measured hydrodynamic size of the reference compounds JR-Sym* (●) and ASI-2* (○) versus thrombin concentration. (To give more intuitively accessible results, all experimental data in this study are given as apparent hydrodynamic diameters, converting the direct result of the fit to the ACF, τ, according to Eqs. 2 and 3.) Concentration of the reference compounds was 5 nM for JR-Sym* and 13 nM for ASI-2*. The solid lines are a fit to a two-component complexation model in chemical equilibrium with dissociation constants of 200 nM (●) and 500 nM (○), respectively.

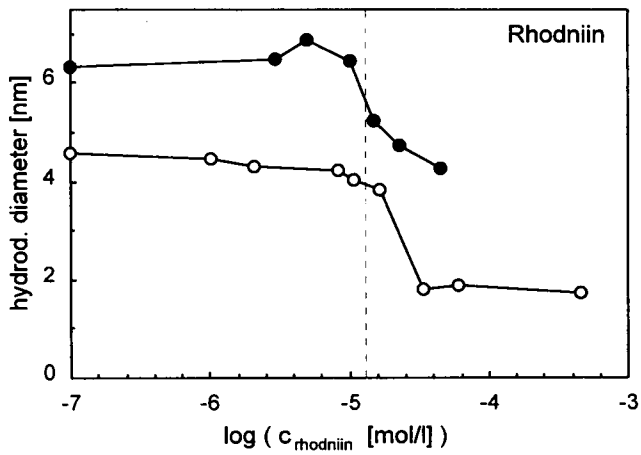


FIGURE 3 Measured hydrodynamic size of the reference compounds JR-Sym* (●) and ASI-2* (○) versus concentration of the thrombin inhibitor rhodniin added to a previously formed complex of the reference compounds with thrombin. Concentrations: 3.3 μM thrombin (both experiments), 33 nM JR-Sym*, 13 nM ASI-2*.

Competition experiments

We form a complex by incubating a small amount of one of the reference compounds with an excess of thrombin. To this complex, various amounts of thrombin inhibitors are added and the apparent hydrodynamic size of the fluorescent species is monitored with FCS. If the reference compound in the complex with thrombin is replaced by the inhibitor, the hydrodynamic size of the reference compound measured by FCS is reduced.

The standard protocol of the experiments is as follows: Solutions (0.5–5 ml of 5–50 nM) of the reference compounds were incubated with 1–50 μM thrombin for 30 min. The sample is then divided into several aliquots. A certain concentration of the thrombin inhibitor under investigation is added to one aliquot. After an additional incubation time of 30 min, part of the aliquot is transferred to a microslide and measured in the FCS setup. Typically, five measurements of 60 s duration each are made for each sample. This procedure is repeated for several inhibitor concentrations.

Fig. 3 shows the result of two such measurement series for the thrombin inhibitor rhodniin. The apparent hydrodynamic sizes of the reference compounds drop from their maximum value of 6.2 nm (JR-Sym*) and 4.7 nm (ASI-2*) for the thrombin/reference compound complex without rhodniin to 4.1 nm (JR-Sym*) and 1.8 nm (ASI-2*) at high rhodniin concentrations. These end sizes correspond to the sizes of the free reference compounds. Similar experiments were carried out for all of the thrombin inhibitors of Fig. 1. Fig. 4 shows the result for the active site inhibitors ASI-1 and ASI-2. The complex between JR-Sym* and thrombin is not broken up by the addition of ASI-1 or ASI-2. Fig. 5 shows the respective results for the recently discovered thrombin inhibitor ornithodorin.

Table 1 gives an overview of the results of all competition experiments. Listed are the measured hydrodynamic sizes of the reference labels at high concentrations of the respective inhibitors, compared to the sizes for the reference compounds themselves and their complexes with thrombin. The errors indicated are standard deviations of at least five repetitions of each measurement.

Double labeling

In one series of experiments, thrombin was incubated with both reference labels at the same time. Measurements of each sample were taken at the two wavelengths 488 nm and

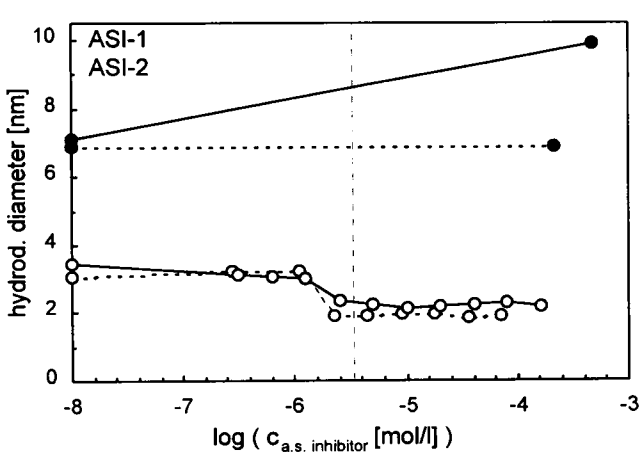


FIGURE 4 Measured hydrodynamic size of the reference compounds JR-Sym* (●) and ASI-2* (○) versus concentration of the thrombin active site inhibitors ASI-1 (——) and ASI-2 (– – –) added to a previously formed complex of the reference compounds with thrombin. Concentrations: 3.3 μM thrombin (both experiments), 33 nM JR-Sym*, 13 nM ASI-2*.

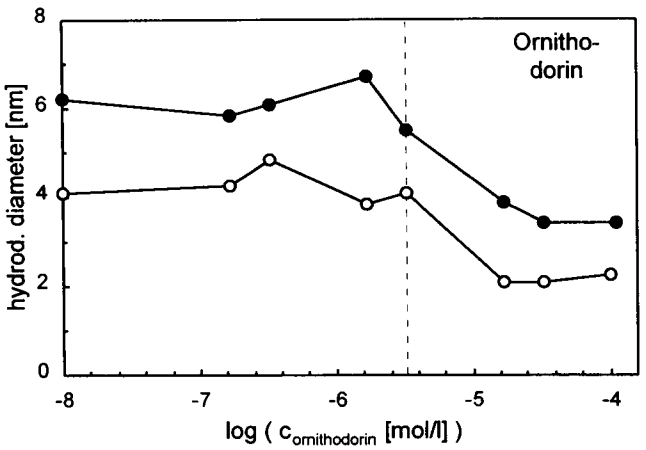


FIGURE 5 Measured hydrodynamic size of the reference compounds JR-Sym* (●) and ASI-2* (○) versus concentration of the thrombin inhibitor ornithodorin added to a previously formed complex of the reference compounds with thrombin. Concentrations: 3.3 μM thrombin (both experiments), 33 nM JR-Sym*, 13 nM ASI-2*.

514 nm, selective for ASI-2* and JR-Sym*, respectively. Otherwise, the experiments were performed in the same way as described above for single labeling. Table 2 shows the results of this double-labeling experiment.

DISCUSSION

The data of Fig. 2 show that FCS is a suitable method for monitoring the formation of molecular complexes in a homogeneous assay without separation steps. The measured hydrodynamic sizes of 7 nm for the bound state and 4 nm

TABLE 1 Summary of competition experiments (single labeling)

Binding site: / Reference compound: / Inhibitor	Active site ASI-2* measured size (nm)	Exosite JR-Sym* measured size (nm)
Hirudin	**1.8 ± 0.2**	**4.1 ± 0.4**
Rhodniin	**1.7 ± 0.3**	**4.1 ± 0.3**
Ornithodorin	**2.1 ± 0.3**	**3.7 ± 0.4**
Hirulog	**1.8 ± 0.2**	**4.8 ± 0.3**
Hirudin peptide 1	4.3 ± 0.5	**4.5 ± 0.5**
Hirudin peptide 2	4.1 ± 0.6	**5.0 ± 0.7**
Hirudin peptide 3	4.1 ± 0.4	**4.5 ± 0.7**
ASI-1	**2.2 ± 0.2**	9.9 ± 0.3
ASI-2	**1.9 ± 0.2**	6.9 ± 0.3
Sizes without inhibitors		
Reference compounds alone	1.3 ± 0.2	3.9 ± 0.3
Complex with 3.3 μM thrombin	4.9 ± 0.4	6.9 ± 0.5

Listed are measured hydrodynamic sizes of the reference compounds JR-Sym* and ASI-2* at high inhibitor concentrations (10–50 μM). The concentration of thrombin was 5–15 μM. For comparison, free reference compounds and complexes between reference compounds and thrombin without inhibitors are also listed. Experiments that show competition of an inhibitor with the respective reference compound (i.e., the inhibitor binds to the corresponding site) are in bold.

TABLE 2 Summary of double-labeling experiment

Binding site: / Reference compound: / Inhibitor	Active site ASI-2* measured size (nm)	Exosite Jr-Sym* measured size (nm)
Rhodniin (27 μM)	**2.1 ± 0.3**	**3.7 ± 0.4**
Sizes without inhibitors		
Reference compounds alone	1.2 ± 0.1	3.9 ± 0.2
Complex with 2.5 μM thrombin	4.6 ± 0.4	6.6 ± 0.5

Measured hydrodynamic sizes of the reference compounds at zero and at high inhibitor concentration (27 μM rhodniin). The concentration of thrombin was 2.5 μM.

for the free state agree reasonably well with expectations from molecular geometry. (From crystallographic data (Stubbs and Bode, 1993; van de Locht et al., 1995), thrombin is known to have an ellipsoidal shape of the approximate dimensions 4.5 × 4.5 × 5.5 nm^3. A DNA 15-mer has a nominal linear extension of 5.4 nm (0.36 nm per base, the ROX label, M = 515 D, has to be considered in addition).) According to Fig. 2, the binding of the two fluorescent reference compounds JR-Sym* and ASI-2* can be described quantitatively by a simple two-component model. The value of the dissociation constant of 200 nM for JR-Sym* agrees well with a published value of 115 nM (IC_{50}) (Wu et al., 1992), given the uncertainties of the fit in Fig. 2. The inability of the randomly scrambled DNA aptamer JR-Neg* to bind to thrombin shows that the binding of JR-Sym* is indeed a property of the sequence and not due to unspecific binding.

Figs. 3 and 4 show that the interaction of the inhibitors with thrombin can be monitored with FCS and that this monitoring is site-specific. The binding of a thrombin inhibitor to one or both binding sites shows up as a release of the corresponding site-specific reference labels. The binding constants of the thrombin inhibitors used in this study are known to be in excess of 10^{10} M^{-1}—much greater than the binding constants of our reference compounds. This leads to an almost complete replacement of the reference compounds from thrombin at inhibitor concentrations greater than the thrombin concentration, reflected in the fact that the observed sizes of the fluorescent reference compounds JR-Sym* and ASI-2* at these inhibitor concentrations are close to the sizes of the free reference compounds. Thus quantitative information about the binding constants of the inhibitors used cannot be obtained from our experiments. This can only be done by a competition method like the one in this study, if the binding constants of the reference compounds and the inhibitors do not differ by more than one order of magnitude. From our experiments we can only deduce that the inhibitors bind more strongly to thrombin than do our reference compounds.

The results of the double-labeling experiments show that the two labels behave independently, i.e., when one reference label is observed with the appropriate laser wavelength, the presence of the other reference label makes no

difference in the results. This behavior can be expected, because the target protein thrombin is present in 100-fold excess over the reference labels. At this excess of target protein there should be no competition between the reference labels. (This excess of target protein is necessary because of the low binding constants of the reference labels. See Fig. 2.)

CONCLUSIONS

The results of this study show that FCS can be successfully employed to yield quantitative information about biochemical complexation reactions without separation steps. In the system thrombin of and thrombin inhibitors, FCS together with site-specific fluorescent reference compounds can distinguish between inhibitors directed to the active site, the exosite, or both sites. For proteins with two binding sites, both sites can be probed simultaneously by using reference compounds with different fluorescence labels and distinguishing them spectroscopically.

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