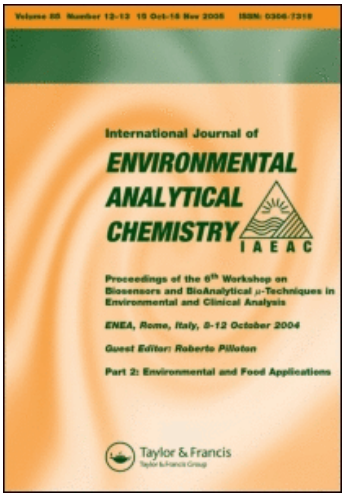
This article was downloaded by:
On: *17 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

New HIV-protease assays applying self-quenching peptide substrates in combination with time-resolved fluorescence single-molecule spectroscopy

Thorsten Martin Staudt[a]; Hans-Georg Kräusslich[b]; Jens-Peter Knemeyer[c]; Nicole Marmé[d]
[a] High Resolution Optical Microscopy, German Cancer Research Center, 69120 Heidelberg, Germany [b] Department of Virology, Hygiene Institute, 69120 Heidelberg, Germany [c] Functional Genome Analysis, German Cancer Research Center, 69120 Heidelberg, Germany [d] Department of Physical Chemistry, University of Heidelberg, 69120 Heidelberg, Germany

Online publication date: 18 November 2010

To cite this Article Staudt, Thorsten Martin , Kräusslich, Hans-Georg , Knemeyer, Jens-Peter and Marmé, Nicole(2007) 'New HIV-protease assays applying self-quenching peptide substrates in combination with time-resolved fluorescence single-molecule spectroscopy', International Journal of Environmental Analytical Chemistry, 87: 10, 731 — 743
To link to this Article: DOI: 10.1080/03067310701417039
URL: http://dx.doi.org/10.1080/03067310701417039

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

Intern. J. Environ. Anal. Chem.
Vol. 87, Nos. 10–11, 20 August–15 September 2007, 731–743

Taylor & Francis
Taylor & Francis Group

New HIV-protease assays applying self-quenching peptide substrates in combination with time-resolved fluorescence single-molecule spectroscopy

THORSTEN MARTIN STAUDT†, HANS-GEORG KRÄUSSLICH‡, JENS-PETER KNEMEYER*§ and NICOLE MARMÉ*¶

†High Resolution Optical Microscopy, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
‡Department of Virology, Hygiene Institute, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany
§Functional Genome Analysis, German Cancer Research Center, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany
¶Department of Physical Chemistry, University of Heidelberg, Im Neuenheimer Feld 229, 69120 Heidelberg, Germany

(*Received 13 November 2006; in final form 24 April 2007*)

Downloaded At: 14:22 17 January 2011

This work describes the optimization and adoption of an assay system for the Human Immunodeficiency Virus (HIV)-protease, whose inhibition plays a central role in HIV therapy. The HIV-protease, which is an essential enzyme during viral maturation, has a specific cleavage site of eight amino acid residues (SQNY*PIV). Adding two amino acid residues at the N-terminus and enclosing the resulting sequence by a dye-labelled lysine residue and a tryptophan residue leads to the substrate (K(dye)CGSQNY*PIVW) in which the fluorescence of the fluorophore is efficiently quenched by the intrinsic tryptophan due to a photoinduced electron transfer reaction. After cleavage of the substrate by the target enzyme, the dye and the tryptophan residue are separated, effecting a significant increase in fluorescence intensity. Measuring the fluorescence *versus* time enables an online-monitoring of the enzyme activity. With this method, a HIV-PR concentration of 10^{-9} M is detectable within minutes, which is comparable with commercially available assays using doubly labelled substrates based on a fluorescence resonance energy transfer. We were able to further increase the sensitivity to the subnanomolar range by using confocal single-molecule spectroscopy.

Keywords: Proteolytic enzymes; HIV-protease; Photoinduced electron transfer; Single-molecule spectroscopy

1. Introduction

Acquired Immunodeficiency Syndrome (AIDS) is one of the most destructive epidemics. More than 25 million people have fallen victim to AIDS since it was first

*Corresponding authors. Fax: +49-12120-175486. Email: knemeyer@single-molecule-spectroscopy.de; Fax: +49-6221-545050. Email: marme@single-molecule-spectroscopy.de

International Journal of Environmental and Analytical Chemistry
ISSN 0306-7319 print/ISSN 1029-0397 online © 2007 Taylor & Francis
DOI: 10.1080/03067310701417039

recognized in 1981. Almost 5 million people (700 000 children) were newly infected by the Human Immunodeficiency Virus (HIV), the causative agent of AIDS, in the year 2005 [1].

The HIV-1 protease (HIV-PR) plays a central role during the maturation of HIV-1. It acts as a dimer and belongs to the aspartic proteases. The enzyme catalyses a number of cleavages within viral precursor polyproteins yielding individual functional proteins [2]. Inhibition of the HIV-PR leads to immature, non-infectious viruses [3] and thus has become an important element in the antiretroviral therapy [4, 5].

Different fluorescence-based assays to prove the presence of a specific protease have been developed so far. The fluorometric assay described by Wang and co-workers is based on intramolecular fluorescence resonance energy transfer (FRET) [6, 7] and uses a quenched fluorogenic substrate, labelled with a donor dye (5-(aminoethyl)-aminonaphthalene sulfonate, EDANS) at one end of the eight amino acids containing the cleavage site and an acceptor dye (4′-dimethylaminoazobenzene-4-carboxylate, DABCYL) at the other end. After the cleavage of the peptide substrate by a protease, both chromophores are separated. This disruption of the FRET system enables the direct monitoring of protease activity by measuring the 40-fold increase in donor fluorescence intensity within a few minutes. Matayoshi *et al*. detected the HIV-Protease at a concentration of about 10^{-8} M [7].

Downloaded At: 14:22 17 January 2011

Alternatively, the substrate can be labelled with the same dye at each end of the specific cleavage sequence [8–11]. Under aqueous conditions, the fluorophores form non- or only weakly fluorescent dimers [12–14]. The formation of non-fluorescent dye dimers has also been successfully applied in DNA assay systems [15, 16].

It is apparent that every chemical modification of the substrate leads to an altered affinity of the substrate to the enzyme, and hence, the detection sensitivity of the assay is changed. Therefore, the number of modifications should be kept as low as possible in order to design high-affinity molecular probes for proteases. Furthermore, peptide probes with two labels require site-specific labelling with two extrinsic fluorophores. The synthesis of peptide substrates with two modifications in a distinct manner is still time-consuming and expensive. The purification of such substrates needs to be carried out accurately. Incomplete labelled probes, i.e. probes with only one extrinsic fluorophore, lead to a higher background level and to a lower signal response, and this hinders a highly sensitive test.

Here, we present a new assay format taking advantage of fluorescence quenching by the amino acid tryptophan. In the past, it has been shown that properties of some fluorescent dyes are affected by several amino acids. For instance, the fluorescence quantum yield of Fluorescein [17] and Bodipy dyes [18, 19] is decreased by tryptophan and partly also by tyrosine and methionine. Lately, the influence of amino acids on fluorescent dyes absorbing in the visible red region was examined [20]. It is advantageous to use these dyes instead of blue or green absorbing dyes because of the reduced background of biological samples in the red region. The major sources of background are fluorescence of impurities and elastic (Rayleigh) and inelastic (Raman) scattering. Both Raman and Raleigh scattering are dramatically reduced by shifting the excitation to longer wavelengths as they are proportional to $1/\lambda^4$. Furthermore, low-cost, energy-efficient, robust, and pulsed (collecting lifetime information, see below) diode lasers can be used for excitation. These advantages led to the development of new fluorescent dyes which are absorbing and emitting above 620 nm but still having a sufficient fluorescence quantum yield [21]. Especially oxazine

derivatives like MR121 have been used in many applications, e.g. ultrasensitive detection of DNA [22–25]. Recently, the possibility of quenching the fluorescence of MR121 with tryptophan was applied to detect single p53 antibodies in human blood serum [26].

In this article, we report on the development of a new method for the detection of HIV-PR activity based on single molecule detection applying self-quenching substrates. These oligopeptide substrates containing the cleavage site of HIV-PR are labelled with only one chromophore at the N-terminus, and thus, the synthesis and purification of the dye-peptide conjugates are much easier. As a fluorophore, we chose the red-adsorbing oxazine derivative MR121 and the boradiazaindacene derivative Bodipy 630/650, which are both efficiently quenched by a tryptophan residue located on the other end of the cleavage site, via photoinduced electron transfer (PET). Due to digestion by the HIV-PR, the spatial contact between fluorophor and quencher gets lost, and a fluorescence increase can be monitored. To increase the sensitivity even more, the fluorescence signal was not measured via a standard fluorescence spectrometer but using a confocal microscope. Small detection and excitation volumes reduce the background considerably. Detection volumes in the range of 1fL allow the observation and the characterization of single molecules in concentrations of about 10^{-9}–10^{-12} M. These molecules generate a fluorescent burst with a high signal-to-noise ratio whenever diffusing through the focus. Since that method is also capable of detecting small subpopulations of molecules with slightly shifted spectroscopic properties compared with the majority, it is possible to differentiate between quenched uncleaved probes and cleaved fluorescent probes via different burst intensities. The great advantage of single-molecule detection is the derivation of the spectroscopic properties of single molecules and events, and not the properties of ensembles. It is additionally possible to collect the lifetime information of the different molecule fractions in solution obtaining an accessory discrimination criterion. This is achieved by Time Correlated Single Photon Counting (TCSPC), where the time between a fluorescence photon (start signal) and an external trigger signal (stop signal from the pulsed laser source) is measured. Details of the setup and the data processing are described elsewhere [27].

Downloaded At: 14:22 17 January 2011

2. Experimental

2.1 *Fluorescent dyes and conjugates*

The oxazine derivative MR121 was kindly provided by the group of K.H. Drexhage (Universität-Gesamthochschule Siegen, Germany) as free carboxic acid. The dye was converted into a *N*-hydroxysuccinimidylester by an equimolar amount of *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) in acetonitrile. Bodipy 630/650 was purchased as NHS-ester from Molecular Probes.

The peptide KCGSQNYPIVW was synthesized by the peptide synthesis unit of the German Cancer Research Center. The cysteine residue provides an alternative possibility for labelling the peptide via a maleimide derivative of the fluorescent dye. However, these data are not presented in this article.

For coupling the fluorophore to the lysine residue of the peptides, 20 μL of the activated dye (0.1 mM in acetonitrile) was added to an excess of peptide, dissolved in 50 μL of carbonate buffer (0.1 M, pH 8.3). The solution was incubated for 6 h at room temperature and then purified by reverse-phase (Knauer, Berlin) HPLC (Hewlett-Packard, Böblingen, Germany) with octadecylsilane-hypersil C18. Separation was performed in 0.1 M of triethylammonium acetate, using a linear gradient from 0 to 75% acetonitrile in 20 min. Molecular weights were determined by mass spectrometry. Yields of approximately 70% were obtained. The dye–peptide conjugates are stable for several months if stored as aliquots frozen at −20°C. The inhibitor Idinavir was purchased from Merck (Darmstadt, Germany), and the expression and purification of the HIV protease are described in the literature.

2.2 *Spectroscopy*

All absorption spectra were recorded with a Cary 500 UV-Vis-NIR spectrometer (Varian, Darmstadt, Germany) in standard cuvettes at room temperature. Steady-state emission spectra were measured with a Cary Eclipse fluorescence spectrometer. To avoid unspecific adsorption of the molecules to the glass walls, the cuvettes were coated with polyethylene glycol before. In all measurements, the concentrations were kept strictly below 1 μM to prevent re-absorption and re-emission effects. Relative fluorescence quantum yields, $\Phi_{f.rel}$, of the dye-labelled peptides were measured with respect to the fluorescence intensity of the free dye under otherwise similar conditions.

Downloaded At: 14:22 17 January 2011

2.3 *Single-molecule experiments*

In the following, the confocal detection scheme, which can be applied for Fluorescence Correlation Spectroscopy (FCS) and for detection of individual molecules in solution, will be explained. The laser beam of the pulsed semi-conductor laser diode (10–80 MHz at 635 nm; Picoquant GmbH, Berlin) is circularly polarized for isotropic excitation in solution using a 1/4 wave retarder and cleaned by an excitation filter (640 DF 10; Omega Optical, Brattleboro, VT). Passing a telescope leads to a wider beam diameter. After reflection by a dichroic mirror (640 DLRP; Omega Optical), the sample was illuminated through an oil-immersion microscope objective (100×, Olympus, 1.4 NA; Tokyo) yielding a diffraction limited excitation volume. Fluorescence emission is collected by the same objective transmitted through the dichroic mirror for separation from Raleigh scattered light. The beam is focused onto a 100 μm pinhole, and after passing two emission filters, focused on an APD (AQ 141; Optoelectronics, Canada) for detection. For acquisition of the fluorescence lifetime, each photon detected is correlated with the subsequent laser pulse which is fed into a single photon counting PC-card (SPC-630; Becker & Hickl, Berlin) as start and stop trigger, respectively, to determine the time between the laser pulse and photon detection (TCSPC).

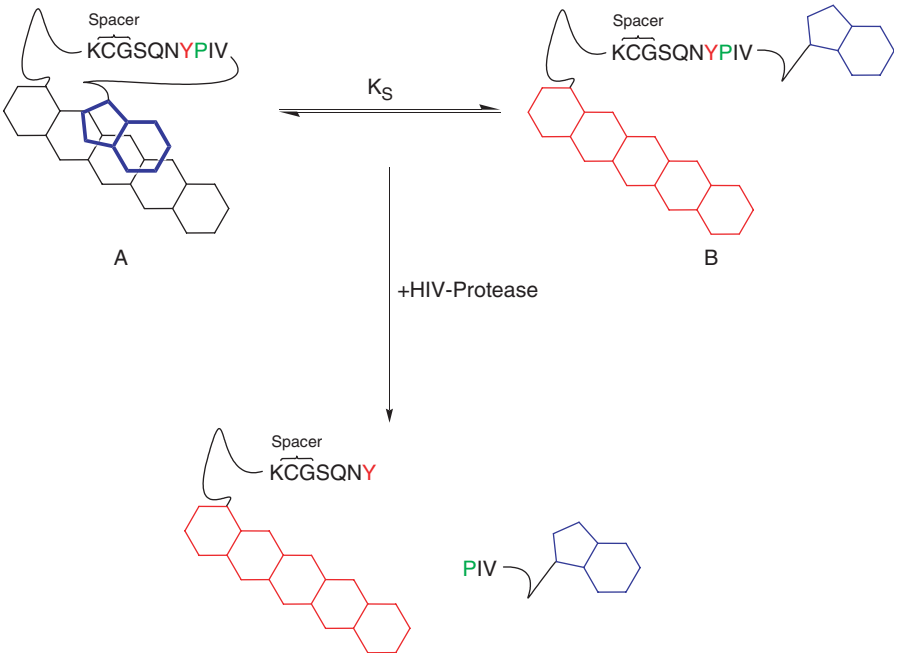


Figure 1. Working mechanism of the self-quenching peptide substrate. The peptide containing the specific cleavage site SQNY*PIV is labelled at the N-terminal lysine with a chromophore. Its fluorescence is quenched by the C-terminal tryptophan residue due to contact formations. Upon cleavage by the HIV-PR, dye and tryptophan are separated, and the fluorescence intensity increases.

Downloaded At: 14:22 17 January 2011

3. Results

3.1 *Mechanism*

A highly sensitive protease assay via tryptophan-quenched peptide probes based on photoinduced electron transfer (PET) was recently applied to test for exopeptidases below 10^{-13} M concentrations [28, 29]. In this manner, it was also possible to detect the relatively unspecific endopeptidase trypsin, which cleaves peptide bonds in the middle of a substrate in the picomolar range. In the following, the design of a substrate for a protease with different recognition sequences, and the applications concerning the detection and the search for resistances will be described by means of the HIV1-PR. The basic idea of the experiment is that the fluorescence of suitable fluorophores is efficiently quenched by tryptophan residues via PET [20]. Except for tryptophan itself, all other amino acids do not quench, or do so only at rates that are substantially smaller. As the recognition sequence for the HIV1-PR, the sequence around the cleavage site of the matrix and the capsid protein within the Gag or Gag/Pol poly protein was chosen [30]. The basic sequence motif SQNYPIV, necessary for the recognition process, was labelled on one side with the red fluorescent dye MR121 and Bodipy 630/650, respectively. The other side of the peptide was elongated by a tryptophan residue acting as the quencher molecule (figure 1).

A spacer (cystein-glycin; CG) was incorporated to ensure the required space for the protease. The dye is coupled to the side chain of an N-terminal lysine residue by means

of an NHS ester method, whereas the N-terminus itself was acetylated to avoid double-labelled products. Equilibrium fluctuations of the backbone lead to conformations where the fluorophore and the tryptophan come into contact. The incubation of this substrates with recombinant HIV-PR leads to hydrolysis of the peptide bond between the tyrosine and proline (SQNY*PIV) residue and therefore to a spatial separation between the dye and the quencher molecule. The formation of static charge transfer ground state complexes (static quenching) due to hydrophobic interactions [31] and collisions between tryptophan residue and chromophore is therefore less probable after the cleavage reaction. This in turn leads to prevention of the PET between the fluorophore and the quencher and to a measurable increase in fluorescence intensity. Several different cleavage sites have been reported for the HIV protease. We choose the sequence SQNY*PIV because this sequence is relatively inert to other endopetidases due to the proline residue. For example, this substrate is not digested by blood serum.

To quantify the quenching efficiencies of the substrates for the HIV-PR, the quantum yields of the probes relative to the quantum yields of the free dyes were determined. The quantum yield is the ratio of the number of photons emitted to the number absorbed. The fluorescence intensities of the free dye and the probes were measured and corrected with the absorbences to take different concentrations into account.

$$Q_{\text{rel}} = \frac{E_{\text{Probe}}}{E_{\text{Dye}}} \cdot \frac{A_{\text{Dye}}}{A_{\text{Probe}}}. \tag{1}$$

The MR121 labelled substrate exhibits a quantum yield of 0.19 in HIV-PR buffer. This means that the fluorescence intensity is reduced due to quenching to 20%

Downloaded At: 14:22 17 January 2011

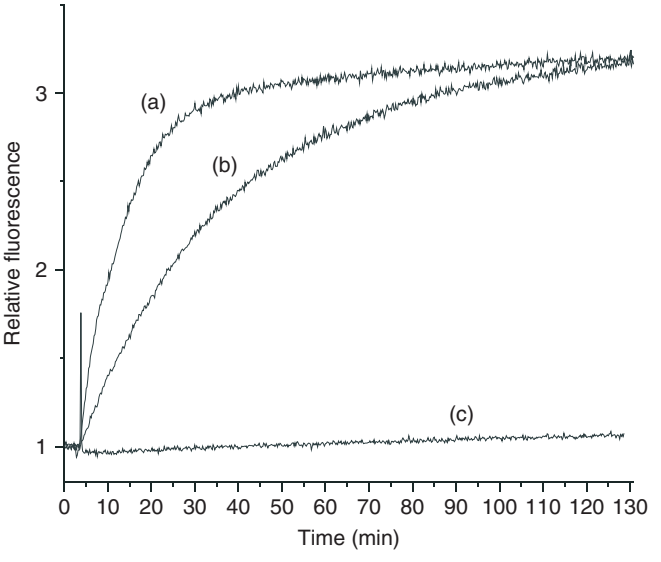


Figure 2. Relative fluorescence signal of the substrate Ac-K(MR121)CGSQNY*PIVW (8×10^{-7} M) at 680 nm *vs.* time ($\lambda_{Ex} = 650$ nm). At 2 min, 2×10^{-8} M (a) and 2×10^{-9} M (b) HIV protease is added. As a control experiment, the inhibitor Indinavir (10^{-6} M) is added, before addition of 2×10^{-8} M protease (c). Measurements were carried out in 0.1 NaOAc buffer (pH = 4.7) containing 4 mM EDTA and 100 mM NaCl (HIV-PR) at 25°C.

compared with the free dye. Therefore, a cleavage reaction could induce a maximal fivefold increase in fluorescence intensity.

3.2 *Digestion of the substrate recorded with standard fluorescence spectrometry*

Incubation of the MR121-labelled substrate (8×10^{-7} M) with the HIV-PR – 2×10^{-8} M and 2×10^{-9} M in HIV-PR buffer (0.1 NaOAc buffer, pH = 4.7 containing 4 mM EDTA, 300 mM NaCl and 1 mg mL^{-1} bovine serum albumin, BSA) at 25°C—led to a threefold increase in fluorescence intensity due to the separation of quencher and dye and the disruption of the PET system (figure 2). The fluorescence intensity was monitored over the complete time period.

The substrate was also digested with the exopeptidase carboxypeptidase A (CPA, 10^{-6} M) in Tris/HCl buffer at 37°C. CPA cleaves every peptide bond step by step, beginning at the C-terminus to the N-terminus and therefore separates quencher and dye. Indeed, proline residues cannot be digested by CPA, and thus the substrate is not completely digested. However, the tryptophan residue is removed, and the increase in fluorescence intensity is also threefold but occurred within seconds (data not shown). The increase in fluorescence intensity is not as high as predicted by the quantum yield, which can probably be explained by the tyrosin residue (providing also a measurable quenching property) that is part of the dye-containing fragment of the cleaved probe in both cases. In order to prove that the cleavage is due to the activity of HIV-PR, a control experiment was accomplished. Here, the HIV protease was inhibited by Indinavir, which is a drug for the anti-retroviral therapy, and incubated with the MR121-labelled substrate. An increase in fluorescence intensity was not observed (figure 2c). This control experiment also shows the potential of the new probes to test for resistances of the HIV protease from special HI-viruses to distinct

Downloaded At: 14:22 17 January 2011

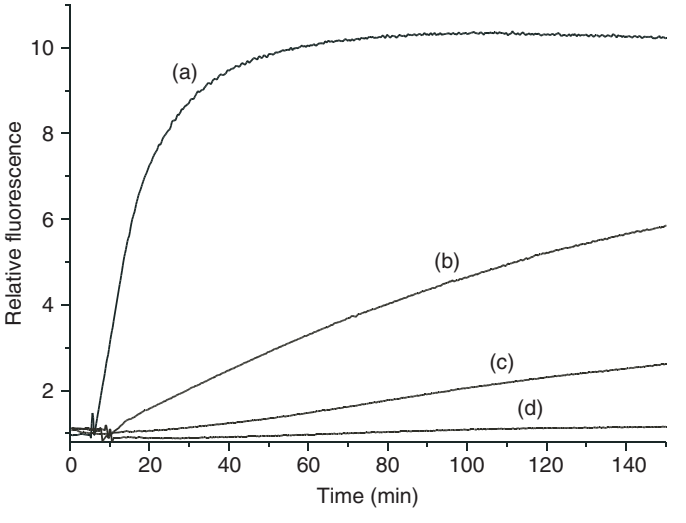


Figure 3. Relative fluorescence intensity of the SubB630 (4×10^{-7} M) in the presence of (a) 2.5×10^{-8} M, (b) 2.5×10^{-9} M, (c) 2.5×10^{-10} M, and (d) 2.5×10^{-11} M HIV-protease. Measurements were carried out in HIV buffer (without BSA) at 37°C.

HIV-PR inhibitors. This in turn provides important information for the anti-retroviral therapy (ART).

Whereas the oxazine derivative MR121 is establishing a ground-state complex with the quencher molecule tryptophan, and the fluorescence is mostly static quenched, the bora-diaza-indazene derivative Bodipy 630/650 ($\lambda_{Abs} = 625\,nm$ and $\lambda_{Em} = 640\,nm$) is partly dynamically quenched [20]. The two different quenching mechanisms could be distinguished from each other by investigating the influence of the quencher concerning the fluorescence lifetime of the dye. In the case of dynamical quenching, the fluorescence lifetime is reduced due to an additional relaxation rate of the excited state which can be used as a second detection criterion besides the fluorescence intensity. Therefore, we synthesized and investigated a Bodipy 630/650 labelled substrate (SubB630). According to the astonishingly low quantum yield of 0.13 in HIV-PR buffer (without BSA), a 10-fold increase in fluorescence intensity was obtained by incubating the substrate ($4 \times 10^{-7}\,M$) with the HIV-PR ($2.5 \times 10^{-8}\,M$) in HIV-PR buffer at 37°C (figure 3). We removed the BSA from the buffer because the relative hydrophobic dye could interact with proteins leading to an increase in fluorescence. With this substrate, it was possible to detect the HIV-PR in a concentration of $10^{-10}\,M$ with a twofold increase in intensity within 2 h (figure 3c). The unequal intensity end levels in the data are likely due to insufficient stability of the HIV-PR at 37°C.

To check whether the enzymes active site is responsible for the increase in fluorescence intensity and to exclude unspecific adsorption of the enzyme to the substrate, which could also lead to an inhibition of the quenching process, we inhibited the HIV-PR with Indinavir and repeated the experiment (data not shown). No increase in fluorescence intensity was detected.

Downloaded At: 14:22 17 January 2011

3.3 *Assaying HIV-PR applying single-molecule spectroscopy*

Generally, both dyes used here are appropriate for confocal single-molecule fluorescence spectroscopy offering a higher detection sensitivity. For this, the dyes were excited with a pulsed (80 MHz) laser diode (635 nm), and the fluorescent bursts were detected with an avalanche photodiode (APD). The time information between the laser pulse and detection of the photon for extracting the fluorescence lifetime was determined via TCSPC. However, for the single-molecule experiments, we applied only the Bodipy 630/650 labelled substrate, because the MR121-labelled substrate is worse quenched and shows no change in fluorescence lifetime.

Because of the fact that the reaction velocities decrease with decreasing enzyme concentrations, long-lasting reaction times had to be realized. To detect single molecules in solution, the substrate concentrations have to be in the range of $10^{-9}\,M$; otherwise averaged values of the spectral characteristics of the molecules in the focus are obtained. To realize a high reaction velocity, the cleavage reactions were carried out with relatively high substrate concentrations at 20°C. Thus, a comparable high number of substrates are cleaved, and, compared with the reference, a good discrimination is possible. Directly before the measurement the reaction mixture was diluted to yield an adequate concentrated solution in order to detect single molecules. To avoid adsorptions of the enzyme or the substrate on the surfaces, the reactions were carried out in small glass vessels coated with polyethylene glycol (PEG). The coverslips used for the single molecule experiments were also PEG-coated. This is essential for the

experiments when applying the Bodipy 630/650-labelled substrate, otherwise the probe will adsorb to the surface due to its hydrophobicity. For this experiment, the substrate (2×10^{-7} M) was incubated with variably concentrated HIV-PR (8×10^{-10} M to 8×10^{-12} M) over 24 h at 20°C. Before measurement, the reaction mixture was diluted 1 : 100, to separate single bursts within the multifluorescence intensity trace (figure 4). In a control experiment, the HIV-PR (8×10^{-10} M) was inhibited by Indinavir (5×10^{-7} M).

Plotting the number of the detected photons within a certain time interval (binning 1 ms) *versus* the time leads to fluorescence intensity traces. After the reaction of the enzyme with the Bodipy 630/650-labelled substrate, the fluorescence intensity increases, and thus the intensity or the total number of the fluorescence bursts. A burst is the sum of photons within a peak. To quantify the results, a burst analysis was carried out. For this, a threshold was defined to distinguish the signal from the background. The sample with the inhibited HIV-PR shows approximately 35 fluorescence signals per minute over a threshold of 50 kHz, whereas this number increases significantly when the inhibitor is missing (approx. 600 signal min^{-1}). Even at a protease concentration of 8×10^{-11} M, around 100 signals with maximum count rates over 50 kHz were detected. Although there is no significant increase in the overall intensities (sum of all detected photons) of the three samples, the numbers of high fluorescence bursts differ up to a factor of 18, which clearly demonstrates the advantage of applying single-molecule spectroscopy. Furthermore, the fluorescence lifetime of each fluorescence burst can be determined separately. Plotting these data on a histogram gives a fluorescence lifetime derivation of the respective sample (figure 5). This method also allows the detection of the enzyme because of the shift in the lifetime in the case of a cleavage reaction.

The free dye shows a lifetime of about 3.8 ns. The Bodipy 630/650-labelled substrate (K(Bodipy 630/650)CGSQNY*PIVW) incubated with inhibited HIV-PR (reference) shows a reduced lifetime of about 2 ns, corresponding to the model of dynamic quenching. The reaction mixture of SubB630 with HIV-protease in a concentration of 8×10^{-11} M contains two species: one with a lower (2.8 ns, quenched probe), and one with a higher (4.5 ns, cleaved probe) lifetime. Therefore, the lifetime determination is also an appropriate tool for discrimination between cleaved and uncleaved substrates.

Downloaded At: 14:22 17 January 2011

4. Discussion

Here, we present new self-quenched enzyme substrates for the specific detection of HIV-PR. These substrates are also appropriate for using highly sensitive single-molecule detection methods. The fluorescence of the applied substrates is quenched by a photon-induced electron-transfer reaction (PET). If the target enzyme is present, a peptide bond within the recognition sequence is cleaved, quencher and fluorophore are separated from each other, the fluorescence intensity increases, and the fluorescence lifetime is altered in the case of the Bodipy630-labelled substrate. The increased fluorescence intensity is detectable in single-molecule experiments via an increase in the number of signals above a certain threshold. When single-molecule spectroscopy was used, the HIV-PR was detected at a concentration of 8×10^{-11} M. The advantage of the presented substrates is the usage of the naturally occurring amino acide tryptophan as a quencher molecule. This fact avoids the time-consuming synthesis of dual

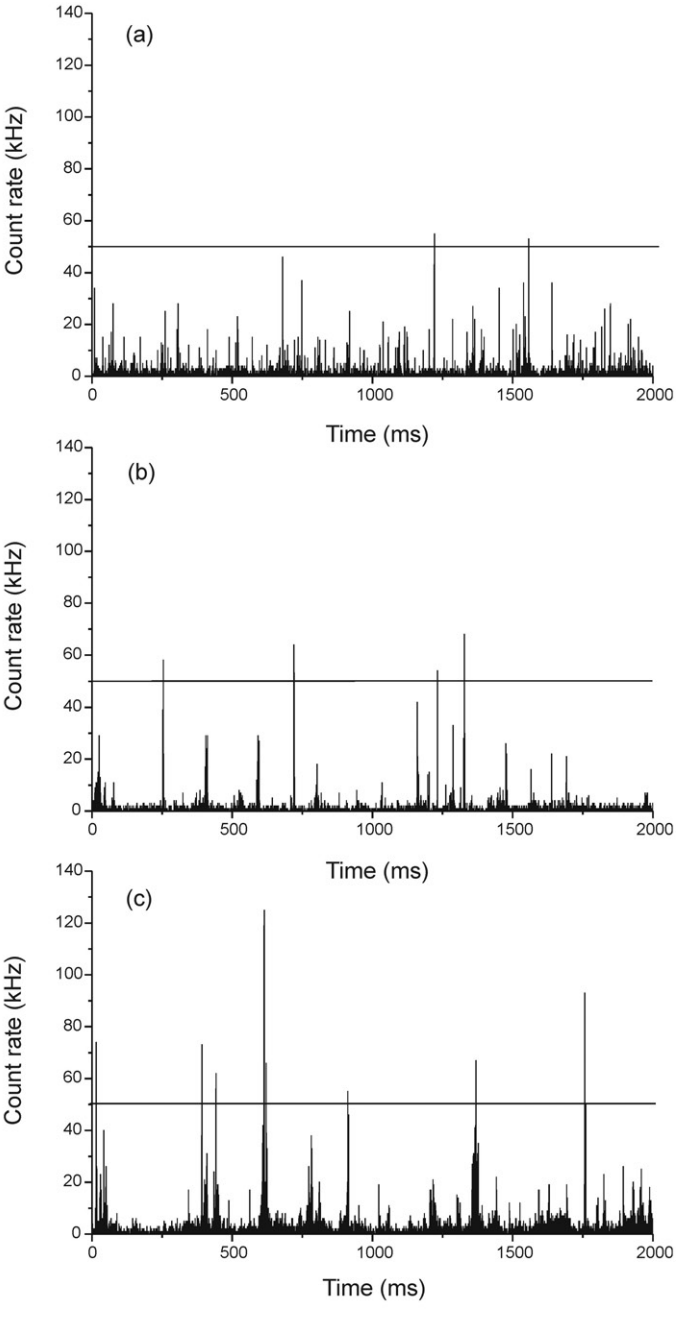


Figure 4. Fluorescence intensity traces after 24 h incubation and dilution (1:100) of the samples containing the substrate SubB630 (2×10^{-7} M) and (a) 8×10^{-10} M HIV-PR inhibited by Indinavir (reference), (b) 8×10^{-11} M HIV-PR, and (c) 8×10^{-10} M HIV-PR. Two seconds of a 1-min measurement is shown, and the time binning is 1 ms.

Downloaded At: 14:22 17 January 2011

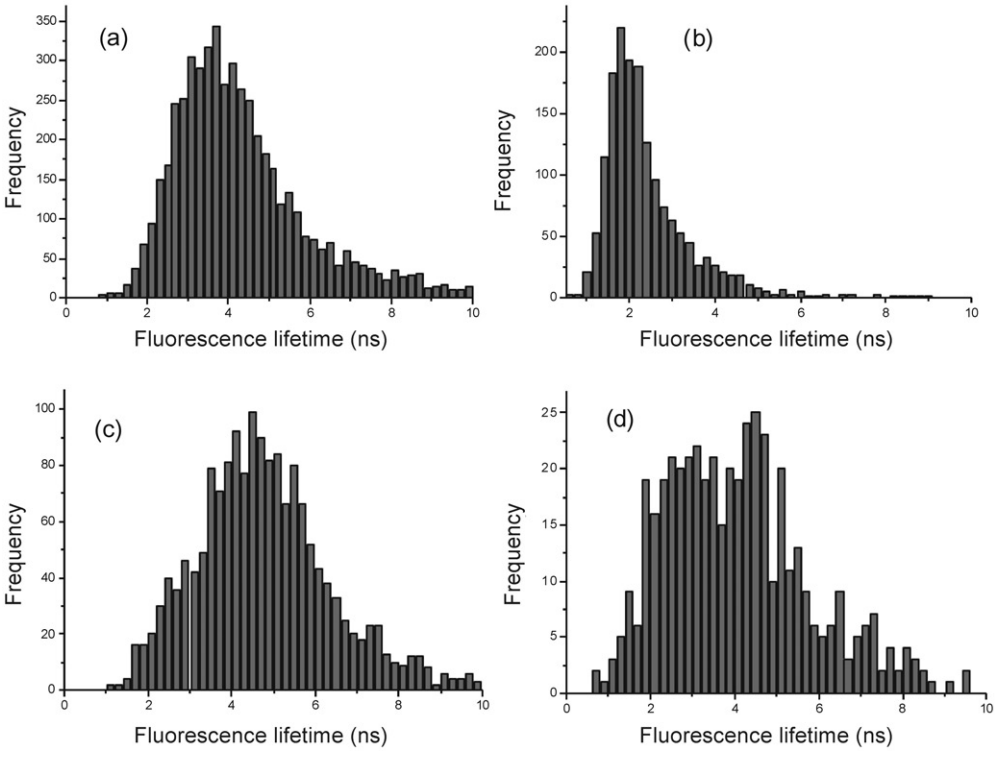


Figure 5. Fluorescence lifetimes of the samples plotted *vs.* the frequency for (a) pure Bodipy 630/650, (b) K(Bodipy 630/650)CGSQNY*PIVW + HIV-PR inhibited by Indinavir (reference), (c) K(Bodipy 630/650)CGSQNY*PIVW + 8×10^{-10} M HIV-PR, and (d) 8×10^{-11} M HIV-PR. C = cysteine, G = glycine, S = serine, Q = glutamine, N = asparagine, Y = tyrosine, P = proline, I = isoleucine, V = valine, W = tryptophan.

Downloaded At: 14:22 17 January 2011

labelled probes. Due to the minimized number of modifications within the probe, the affinity of the enzyme to the substrate should often be less altered. Compared with the commercially available probe which is based on FRET, the new substrates exhibit easier synthesis and fewer modifications.

The Bodipy 630/650-labelled substrate shows the general application of dynamically quenched substrates in the detection of proteases taking the fluorescence lifetime information into account. The detection sensitivity is considered to be comparable with the sensitivity delivered by the intensity criterion. The combination of both information could lead to lowered detection limits as already demonstrated for DNA assays [22, 25].

The inhibition of the HIV-PR with a 50-fold excess of the inhibitor Indinavir proved that the new substrates are generally appropriate for testing new inhibitors. The mutation rate of the virus is very high due to the high failure rate of the reverse transcriptase, and the absence of a proof-reading function. Therefore, a fast and sensitive test for the resistance of the HIV-PR is very important to optimize the treatment. Before screening inhibitors for already resistant proteases, it has to be verified whether the substrate is adequate also for the respective resistant proteases, and eventually the substrate has to be modified.

The next step to establish new peptide substrates is the further increase in sensitivity, for example due to binding of the substrates to surfaces. A major goal would be the

direct detection of the HIV-PR activity in the blood serum of HIV-infected people to obtain an alternative to the diagnosis by RT-PCR and to detect viral parts in an early stage, when no antibodies are present. Therefore, the sensitivity must be increased by a factor of 1000 and the interference between the substrates amd other proteases, which are present in blood sera, and their inhibition must be tested. To summarize, we have demonstrated the potential of the new class of self-quenching peptide substrates for assaying HIV-protease and testing inhibitors with standard fluorescence spectrometers and also single-molecule spectroscopy.

Acknowledgements

The authors are indebted to J. Wolfrum for discussions and his support. This work was financially supported by the Landesstiftung Baden-Württemberg, Alexander von Humboldt foundation, and the Boehringer Ingelheim Fonds.

Downloaded At: 14:22 17 January 2011

References

[1] AIDS epidemic update: December 2005. Available online at: http:\\www.unaids.org/epi/2005/doc/report_pdf.asp (accessed April 2007).
[2] B.N. Fields, D.M. Knipe, P.M. Howley. *Fundamental Virology*, 3rd Edn, Lippincott-Raven, Philadelphia, PA (1996).
[3] N.E. Kohl, E.A. Emini, W.A. Schleif, L.J. Davis, J.C. Heimbach, R.A. Dixon, E.M. Scolnick, I.S. Sigal. *Proc. Natl. Acad. Sci. USA*, **85**, 4686 (1988).
[4] T.D. Meek, D.M. Lambert, G.B. Dreyer, T.J. Carr, T.A. Tomaszek, M.L. Moore, J.E. Strickler, C. Debouck, L.J. Hyland, T.J. Matthews, B.W. Metcalf, S.R. Petteway. *Nature*, **343**, 90 (1990).
[5] T.J. McQuade, A.G. Tomasselli, L. Liu, B. Karacostas, B. Moss, T.K. Sawyer, R.L. Heinrikson, W.G. Tarnley. *Science*, **247**, 454 (1990).
[6] G.T. Wang, E.D. Matayoshi, J. Huffaker, G.A. Krafft. *Tetrahedron Lett*., **31**, 6493 (1990).
[7] E.D. Matayoshi, G.T. Wang, G.A. Krafft, J. Erickson. *Science*, **247**, 954 (1990).
[8] M.J. Blackman, J.E. Corrie, J.C. Croney, G. Kelly, J.F. Eccleston, D.M. Jameson. *Biochemistry*, **41**, 12244 (2002).
[9] B.Z. Packard, A. Komoriya, V. Nanda, L. Brand. *J. Phys. Chem*., **102**, 1820 (1998).
[10] B.Z. Packard, D.D. Toptygin, A. Komoriya, L. Brand. *Proc. Natl. Acad. Sci*., **93**, 11640 (1996).
[11] B.Z. Packard, D.D. Toptygin, A. Komoriya, L. Brand. *Methods Enzymol*., **278**, 15 (1997).
[12] M. Kasha, H.R. Rawls, M. Ashraf El-Bayoumi. *Pure Appl. Chem*., **II**, 371 (1965).
[13] M. Kasha.. *Radiad. Res*., **20**, 55 (1963).
[14] N. Marmé, G. Habl, J.P. Knemeyer. *Chem. Phys. Lett*., **408**, 221 (2005).
[15] J.P. Knemeyer, N. Marmé, B. Häfner, G. Habl, G. Schäfer, M. Müller, O. Nolte, M. Sauer, J. Wolfrum. *Int. J. Environ. Anal. Chem*., **85**, 625 (2005).
[16] N. Marmé, A. Friedrich, D. Denapaite, R. Hakenbeck, J.P. Kenmeyer. *Chem. Phys. Lett*., **428**, 440 (2006).
[17] R.M. Watt, E.W. Voss. *Immunochemistry*, **14**, 533 (1977).
[18] N. Emans, J. Biwersi, A.S. Verkman. *Biophys. J*, **69**, 716 (1995).
[19] J. Karolin, L.B.-A. Johansson, L. Strandberg, T. Ny. *J. Am. Chem. Soc*., **116**, 7801 (1994).
[20] N. Marmé, J.P. Knemeyer, M. Sauer, J. Wolfrum. *Bioconjugate Chem*., **14**, 1133 (2003).
[21] M. Sauer, K.T. Han, V. Ebert, R. Müller, A. Schulz, S. Seeger, J. Wolfrum, J. Arden-Jacob, G. Deltau, N.J. Marx, C. Zander, K.H. Drexhage. *J. Fluoresc*., **5**, 247 (1995).
[22] J.P. Knemeyer, N. Marmé, M. Sauer. *Anal. Chem*., **72**, 3717 (2000).
[23] O. Piestert, H. Barsch, V. Buschmann, T. Heinlein, J.P. Knemeyer, K.D. Weston, M. Sauer. *Nano Lett*., **3**, 979 (2003).
[24] T. Heinlein, J.P. Knemeyer, O. Piestert, M. Sauer. *J. Phys. Chem. B*, **107**, 7957 (2003).

[25] N. Marmé, A. Friedrich, M. Müller, O. Nolte, J. Wolfrum, J.D. Hoheisel, M. Sauer, J.P. Knemeyer. *Nucleic Acids Res*., **34**, e90 (2006).
[26] H. Neuweiler, A. Schulz, A.C. Vaiana, J.C. Smith, S. Kaul, J. Wolfrum, M. Sauer. *Angew. Chem. Int. Ed*., **114**, 4964 (2002).
[27] M. Sauer, B. Angerer, W. Ankenbauer, Z. Foldes-Papp, F. Goebel, K.T. Han, R. Rigler, A. Schulz, J. Wolfrum, C. Zander. *J. Biotechnol*., **86**, 181 (2001).
[28] N. Marmé, J.P. Knemeyer, J. Wolfrum, M. Sauer. *Angew. Chem*., **116**, 3886 (2004).
[29] N. Marmé, T.M. Staudt, J.P. Spatz, J.P. Knemeyer. *Int. J. Environ. An. Ch*., **85**, 741 (2005).
[30] H.G. Kräusslich, H.G. Ingraham, M.T. Skoog, E. Wimmer, P.V. Pallai, C.A. Carter. *Proc. Natl. Acad. Sci*., **86**, 807 (1989).
[31] J.R. Lakowicz. *Principles of Fluorescence Spectroscopy*, 2nd Edn, Kluwer Academic Plenum, Amsterdam (1999).

Downloaded At: 14:22 17 January 2011