

# Single-Molecule Studies of HIV-1 Protease Catalysis Enabled by Chemical Protein Synthesis

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**Abstract:** Single-molecule fluorescence studies of the proteolytic activity of the enzyme HIV-1 protease were performed using FRET-pair dye labeled peptide substrates and substrate-derived inhibitors prepared by solid phase peptide synthesis. Chemical protein synthesis was used to prepare homodimeric HIV-1 protease in soluble form and to prepare a covalent dimer 203 amino acid residue HIV-1 protease containing a biotin tether at the mid-point of the synthetic protein molecule. The biotin-tagged HIV-1 protease was immobilized on a neutravidin-coated glass slide. Total internal

reflection excitation multiwavelength fluorescence spectroscopy was used to monitor substrate binding and cleavage by the synthetic enzyme molecules. Single-molecule traces for the dye-labeled peptide substrate showed distinct binding and cleavage events; the corresponding dye-labeled peptide inhibitor showed only the binding event. These results constitute strong proof-of-principle for the utility of chemical peptide and protein synthesis for single-molecule studies of enzyme catalysis.

**Keywords:** chemical protein synthesis · FRET · HIV-1 protease · single-molecule studies · total internal reflectance

## 1. Introduction

Understanding the chemistry of enzyme catalysis has been an important objective of chemical research since the time of Emil Fischer. The goal is a detailed understanding of the molecular basis of the chemistry occurring in the enzyme–substrate complex. The physical organic chemistry of enzyme catalysis is potentially more accessible than the study of the corresponding reaction(s) in solution because, in principle, in the enzyme–substrate complex every aspect of the reaction environment (proximity, angles of attack, electronics of reactants/catalytic functionalities, polarity/dielectric constant of the reaction medium) can be controlled. Realizing such control in experimental practice will require the application of new methods. Total chemical synthesis has an important contribution to make, in that it can provide the researcher with atom-by-atom control of the structure (electronic, covalent, and geometric) of both the substrate and the enzyme protein molecule itself.

For an enzymatic reaction, where multiple turnovers occur, individual members of the population of enzyme–substrate complexes are not synchronized in their dynamic behaviors. At a given point in time, each enzyme–substrate complex will be at a different stage of the reaction mechanism.<sup>[1]</sup> Thus, ensemble measurements represent data averaged over the unsynchronized population of enzyme molecules; this averaging results in the loss of critical detail concerning steps in the reaction mechanism. In contrast to ensemble measurements, when individual molecules are observed synchronization is not an issue:

reaction steps can be monitored for each individual molecule. Spectroscopic studies of enzyme catalysis on a single-molecule level<sup>[2]</sup> provide an additional level of spatio-temporal resolution enabling the observation of transient intermediate states in catalysis, which may be lost in ensemble measurements. In such a way, the dynamic coupling between protein motions and catalysis within a single molecule can be obtained.<sup>[3,4]</sup> For example, in the pioneering work of Xie and co-workers,<sup>[3,5]</sup> it became apparent that enzymes display fluctuations in catalytic rates caused by conformational heterogeneity of enzyme molecules.<sup>[6]</sup> Elegant single-molecule work has been done on protein enzymes acting on DNA,<sup>[7,8]</sup> on the folding and catalysis of ribozymes,<sup>[9–11]</sup> and on the DNA replication machinery.<sup>[12,13]</sup> Synthetic peptide and protein chemistry has been used to enable single-molecule studies of aspects of protein folding and function.<sup>[14–16]</sup>

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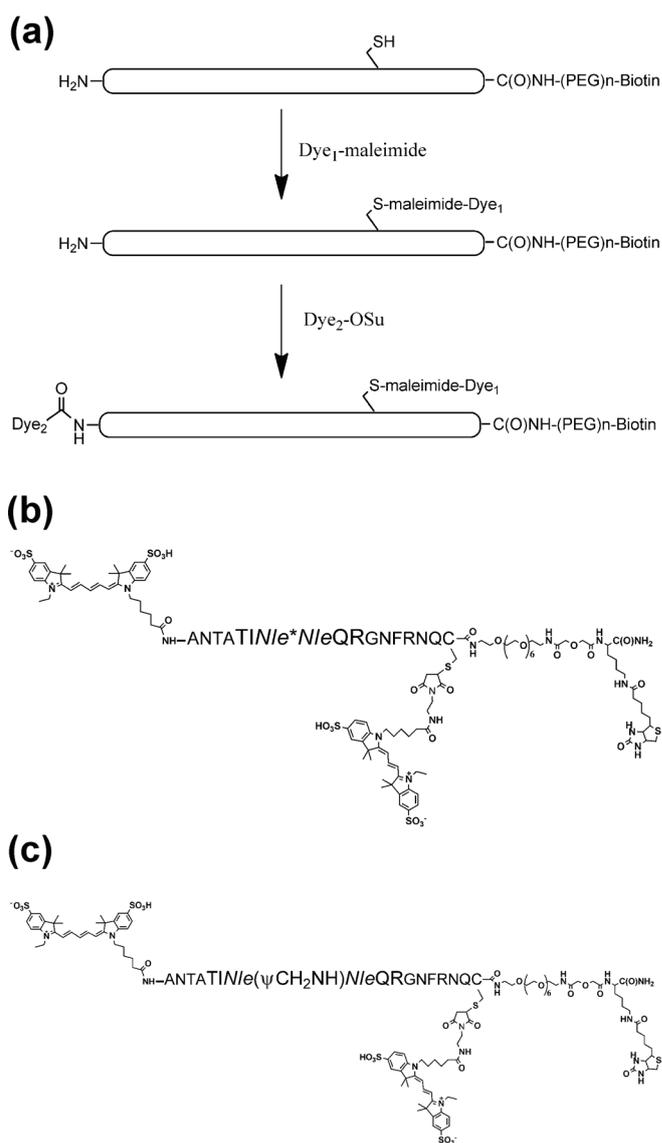
Single-molecule studies of enzyme action most often make use of fluorescence spectroscopy due to the inherent sensitivity of fluorescence detection and to the wide availability of various dye molecules equipped with functionalities for labeling of biomolecules.<sup>[2]</sup> Current approaches to labeling proteins with dye molecules are severely restricted — typically, recombinant protein molecules are engineered to contain a unique Cys residue, so that a single dye moiety can be introduced by chemoselective reaction with the side chain thiol group. Chemical protein synthesis has an important potential contribution to make to single-molecule studies of enzyme action by fluorescence spectroscopy: once access to an enzyme has been established by total chemical synthesis,<sup>[17]</sup> the experimenter can introduce multiple different labels in a site-specific manner at any position in the protein molecule.

In this paper we describe single-molecule studies on the virally encoded HIV-1 protease enabled by chemical synthesis of both peptide substrates and the enzyme molecule itself. Proteases are an important class of enzymes that are, moreover, implicated in many diseases. The HIV-1 protease is essential for the replication of the AIDS virus. It is a homodimeric protein molecule, made up of two identical 99-residue polypeptide chains that together make up a single enzyme active site. The HIV-1 protease functions *in vivo* to cleave the Gag-Pol polyprotein translation product from the HIV genome, and inhibition of the HIV-1 protease by “protease inhibitor” therapeutics is an important component of anti-retroviral treatment for AIDS.

We set out to examine the feasibility of studying HIV-1 protease catalysis using single-molecule fluorescence spectroscopy. Here we report the results of a series of proof-of-principle experiments in which the proteolytic cleavage by the HIV-1 protease of FRET (fluorescence resonance energy transfer)-labeled peptides was monitored by single-molecule fluorescence spectroscopy.

## 2. Experimental Section

Peptides were synthesized by Boc chemistry SPPS using “*in situ* neutralization” protocols.<sup>[18a]</sup> Substrate peptides were fluorophore-labeled as shown in Figure 1 (see also Supporting Schemes 1–3 and Supporting Figures 1–4 for the structures of labeled peptides and their analytical characterization). Reduced isostere peptide inhibitors were synthesized as previously described.<sup>[18b]</sup> Homodimeric HIV-1 protease was prepared by total chemical synthesis as previously described.<sup>[18c]</sup> “Covalent dimer” HIV-1 protease was synthesized as previously described<sup>[18d]</sup> except that the final alkylation step was replaced by reaction with a biotin-containing tether unit through maleimide chemistry. Peptides or “covalent dimer” HIV-1 protease were immobilized at 20–100 pM concentration in their stock solutions in 50 mM NaOAc, 0.4% glucose, 1% mercaptoethanol (v/v), 10% glycerol (v/v), 1% Gloxy (catalase, glucose oxidase). Images were obtained in a wide-field total-internal-reflection microscope with 30–500 ms time resolution using an electron multiplying charge-coupled device (CCD) camera and homemade C++ program



**Figure 1.** Preparation of substrates and inhibitors for single-molecule spectroscopic observation of proteolytic activity of HIV-1 protease. (a) Synthetic scheme. Peptides were synthesized by Boc chemistry stepwise SPPS using a linker that generated a -(PEG)*n*-biotin tag at the C-terminal after cleavage from the resin; each peptide contained one amino-group functionality (either the peptide N-terminus or the amino-group of lysine) and one sulfhydryl group on the side chain of a single cysteine residue. This scheme allowed stepwise site-specific labeling of each peptide with two different dyes (“donor” and “acceptor” fluorophores). (b) Chemical structure of a peptide substrate labeled with Cy3 and Cy5 fluorescent dyes, and with a C-terminal PEG–biotin tag; (c) Chemical structure of a substrate-derived peptide inhibitor labeled with Cy3 and Cy5 fluorescent dyes, and with a C-terminal PEG–biotin tag. The peptide sequence used was based on the p2/NC proteolytic cleavage site in the Gag-Pol polyprotein encoded by the HIV-1 viral genome, with the -Met-Met- residues at the cleavage site replaced by -Nle-Nle- residues.

written by S. A. McKinney (Taekjip Ha laboratory at University of Illinois at Urbana-Champaign). All measurements were per-

formed at 22 °C in 50 mM NaOAc, 0.4 % glucose, 1 % mercaptoethanol (v/v), and 1 % Gloxy (catalase, glucose oxidase) added as an oxygen scavenging system to slow photobleaching. HIV-1 protease was added at 0.5–18  $\mu\text{M}$  concentrations. FRET values were calculated as the ratio between the acceptor intensity and the total intensity. More details of the experimental setup can be found elsewhere.<sup>[12,13,19]</sup>

### 3. Results and Discussion

Two different schemes for performing single-molecule measurements of HIV-1 protease activity were used. In the first, “tethered substrate” approach, a peptide corresponding to p2/NC cleavage site in the Gag/Pol polyprotein was labeled with a FRET pair of interacting dye molecules, and equipped with a biotin tag (Figure 2a). This strategy allowed the immobilization of the substrate on a neutravidin-coated surface and the monitoring, with the help of total internal reflection microscopy, of the proteolytic cleavage reaction upon addition of soluble, homodimeric HIV-1 protease. In the second, “tethered enzyme” approach, a covalent dimer form of the HIV-1 protease with a biotin tether was immobilized on the neutravidin-coated surface and the proteolytic cleavage reaction, upon addition of FRET-pair labeled substrate, was monitored with the help of total internal reflection microscopy.

#### 3.1. Tethered Substrate Experiments

In the first experimental design, we used a 17-residue substrate peptide that contained only one  $\text{NH}_2$ -functionality at its N-terminus, and only one SH group on the side chain of the sole cysteine residue (Figure 1a; see Experimental Section). These amino and thiol functional groups were used for fluorophore-labelling via succinimide active ester and maleimide chemistries, respectively. The labeled peptide substrate (see Figure 1b) was tethered to a polymer-passivated quartz surface coated with neutravidin, and a solution of chemically synthesized homodimeric HIV-1 protease was added at 0.5–18  $\mu\text{M}$  concentrations. The fluorescence behavior of the substrate was monitored by optical imaging with time resolution of 30–500 ms.

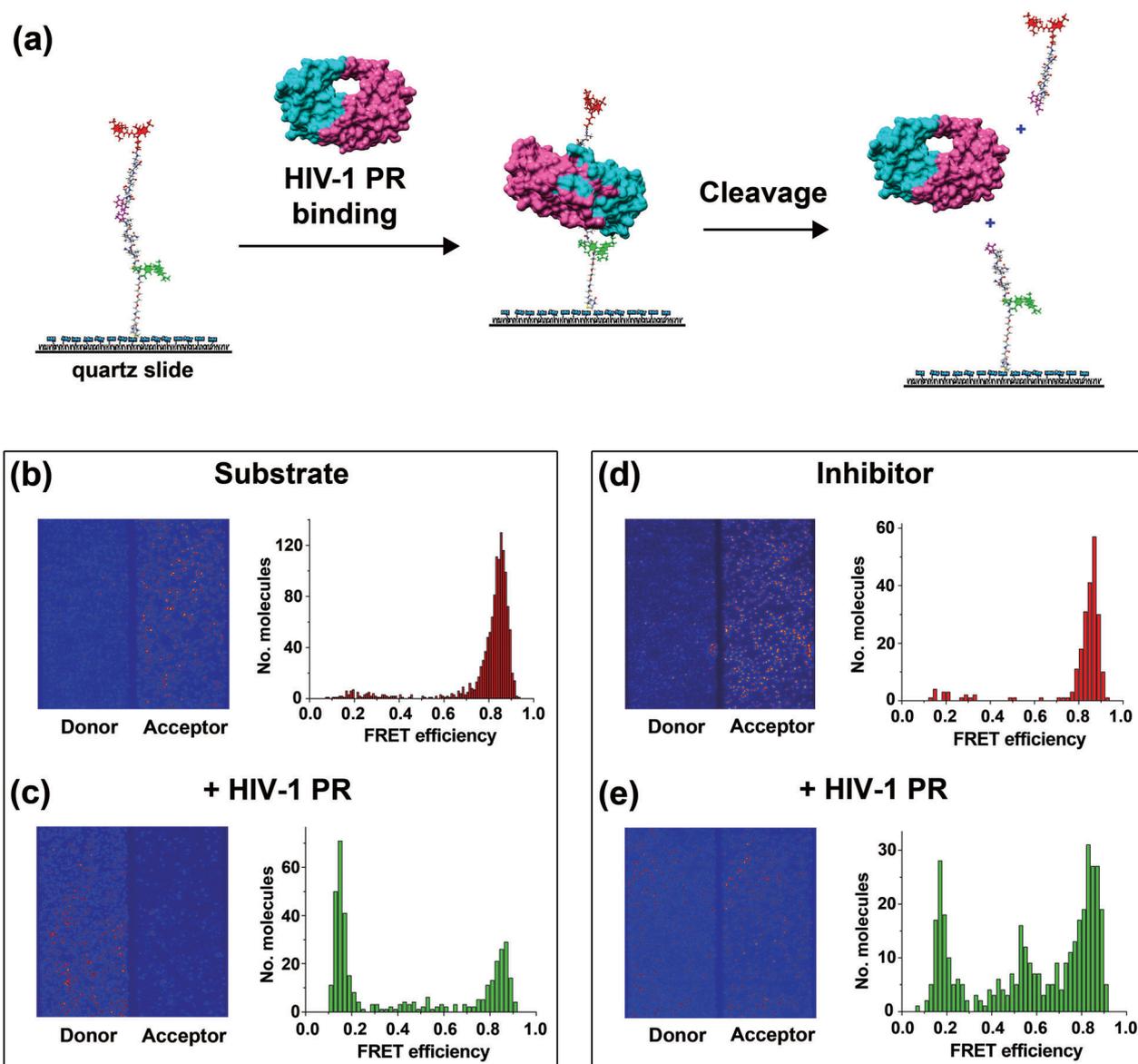
We expected that the peptide substrate would adopt a random coil conformation in the free state (confirmed by circular dichroism measurements at pH 5.5), with a rather close spacing between the dyes and consequently high FRET values, and upon cleavage would have no FRET-transfer due to separation of the donor- and acceptor-containing fragments (see Figure 2a). Meanwhile, while trapped in the binding pocket of the HIV-1 protease, the substrate polypeptide would adopt an extended  $\beta$ -strand peptide conformation with larger separation between donor and acceptor moieties than in the free state, which would correspond to intermediate FRET values. Indeed, high FRET transfer was observed for immobilized sub-

strate molecules alone, resulting in higher fluorescence intensity of the acceptor dye (see Figure 2b). In the case of substrate labeled with Cy-5 dye at its N-terminus, addition of synthetic HIV-1 protease resulted in proteolytic cleavage and the rise of the donor fluorescence signal, combined with the disappearance of the acceptor signal due to dissociation of Cy5-containing product peptide (see Figure 2a–c). Interestingly, observations on individual substrate molecules as a function of time showed that the increase of the donor fluorescence and concomitant decrease of acceptor signal was not abrupt but occurred in a step-wise manner (see Figure 3a). Such behavior could reflect observation of an initial binding step, in which the labeled peptide has an extended  $\beta$ -strand conformation in the enzyme–substrate complex resulting in lowered FRET efficiency, followed by a second step in which the substrate is cleaved and dissociation of one or both fragments lead to an effectively near-zero FRET efficiency.

To confirm that this is indeed a multistep binding/cleavage process and not an artifact such as multi-step photobleaching,<sup>[20]</sup> we performed the same experiments with the identical FRET-pair labeled peptide where the native amide bond [-C(O)NH]- at the p2/NC cleavage site was replaced by a reduced isostere moiety [ $\psi\text{CH}_2\text{NH}$ ],<sup>[18(b)]</sup> effectively transforming the substrate into an inhibitor of the HIV-1 protease (Figure 1c). In experiments with this fluorophore-labelled inhibitor, we again observed increase of the donor fluorescence and corresponding decrease of acceptor fluorescence (Figure 2d,e); however, the intermediate FRET-level state was much longer lived. In many traces, transition to near-zero FRET efficiency was not observed (Figure 3b).

Other experiments supporting that binding followed by proteolysis was indeed observed in these single-molecule traces were: concentration dependence of apparent rate of decrease of Cy5-fluorescence and concomitant increase of Cy3-fluorescence: a 10-fold increase of the concentration of HIV-1 protease from 560 nM to 5.6  $\mu\text{M}$  led to visibly faster FRET-decrease rate (see Supporting Figure 6); and, the observation of a high population of the intermediate-FRET state in experiments with the corresponding inhibitor (compare Figures 2c and 2e).

The Cy3/Cy5 dyes are known to undergo rapid photobleaching, which was indeed observed in the measurements and complicated the interpretation. For example, there were instances of drastic decrease of FRET efficiency, similar to that observed as “cleavage”, even though the experiment was done with inhibitor. An alternative substrate was tested, where the N-terminus was labeled with the Cy3-donor and the Cy-5 acceptor was now attached closer to the surface (see Supporting Scheme 1c,d). In such a configuration, one would expect a decrease of Cy5 (acceptor)-fluorescence and concomitant decrease of Cy3 (donor)-fluorescence due to cleavage of Cy3–peptide product and its dissociation and diffusion

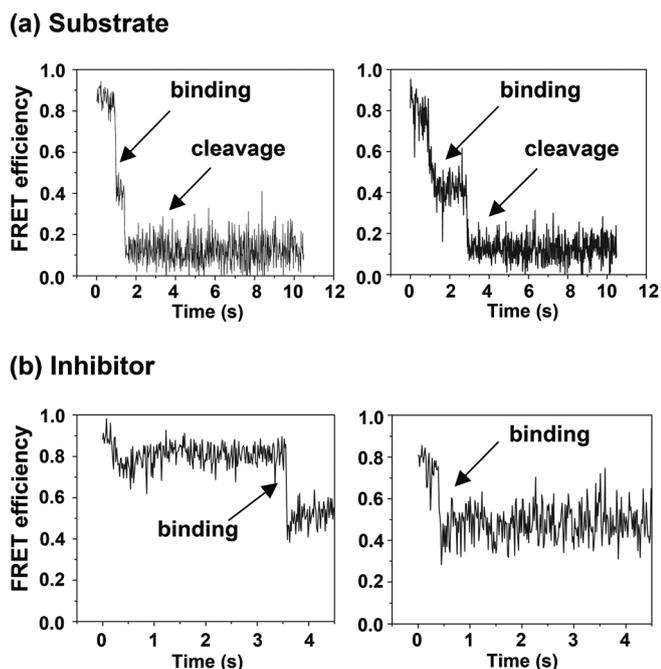


**Figure 2.** Tethered Substrate Experiments. (a) Cartoon of a peptide substrate labeled with a FRET-pair (Cy3 and Cy5 dyes) and equipped with a biotin tag for immobilization on a neutravidin-coated quartz slide. Protease is then added and the progress of the proteolytic reaction is then monitored by fluorescence changes, using a total internal reflection (TIR) fluorescence microscope. (b, c) Monitoring the cleavage reaction of Cy5-substrate-Cy3-biotin with TIR microscopy. (b) "Donor" (left) and "acceptor" (right) channels for surface-immobilized substrate are shown, where FRET efficiency reaches ~85% (shown in the histogram). In (c), HIV-1 protease is added, leading to cleavage of the substrate after which the Cy5 dye-labeled fragment diffuses from the detection volume, leading to reduction in the intensity of the high-FRET peak (see histogram). (d, e) Monitoring the binding of HIV-1 protease to Cy5-inhibitor-Cy3-biotin with TIR microscopy. In (d) FRET efficiency reaches ~85% for inhibitor immobilized to a surface. In (e) HIV-1 protease is added, leading to decrease to near equivalence of the fluorescence intensities in the donor and acceptor channels. The histogram in (e) shows high abundance of high FRET-peak (~85%) as well as a medium FRET-peak (~55%) not observed in the case of substrate (see (c)). [Note: the peak at 20% is leakage of "donor" light due to imperfections in optical filtering.]

into bulk solution. In many single-molecule trajectories we have, however, noticed that fluorescence of the Cy3-fluorophore *did not* diminish upon adding HIV-1 protease, whereas fluorescence of the Cy5-fluorophore decreased. This may indicate that Cy3-dye does not evade volume of detection and remains attached either to the

protein molecule or a surface nearby, perhaps due to Cy3-dye hydrophobicity, or again the photobleaching of Cy5-dye might be a complicating factor in the interpretation of the data.

In order to improve characteristics of the substrate we tried alternative dye configurations, such as Alexa555/



**Figure 3.** Selected single-molecule FRET efficiency traces for reaction of HIV-1 protease with surface-immobilized Cy5-substrate-Cy3-biotin and with Cy5-inhibitor-Cy3-biotin. (a) Examples of FRET efficiency versus time for treatment of Cy5-substrate-Cy3-biotin with HIV-1 protease. (b) Examples of FRET efficiency versus time for treatment of Cy5-inhibitor-Cy3-biotin with HIV-1 protease. For the inhibitor case, many molecules displayed behavior where the FRET-level of medium intensity was sustained.

Alexa 647 (Supporting Scheme 3) and Cy3B/Cy5 (Supporting Scheme 3a). Alexa dyes are known for their hydrophilic properties and high photostability; this could potentially improve the assay. Cy3B is a more photostable analogue of Cy3-dye.<sup>[21]</sup> With the new substrates we observed noticeably higher photostability; however, in many single-molecule traces photobleaching was still taking place, complicating accurate quantitative analysis of the data. We have performed assays in which the initial timing of optical detection was set to be coincidental with the addition of HIV-1 protease. In such cases, we found Alexa647-fluorescence was more stable than without protein, meaning that the protein stabilizes acceptor dye fluorescence against photobleaching. One can speculate, therefore, that in the peptide, which has a random coil structure, close interactions of donor dye and acceptor dye may take place, inducing quenching and photobleaching.

### 3.2. Tethered Enzyme Experiments

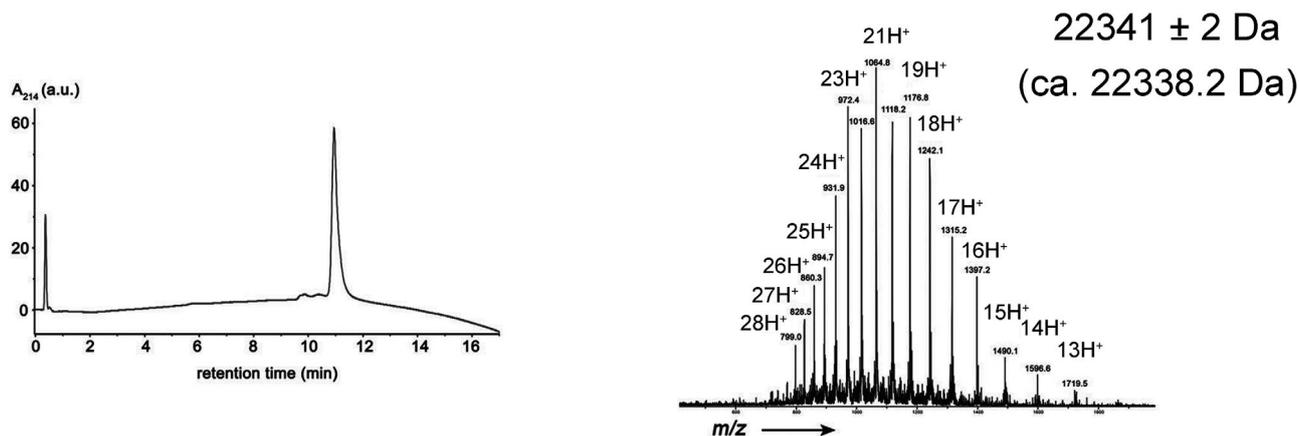
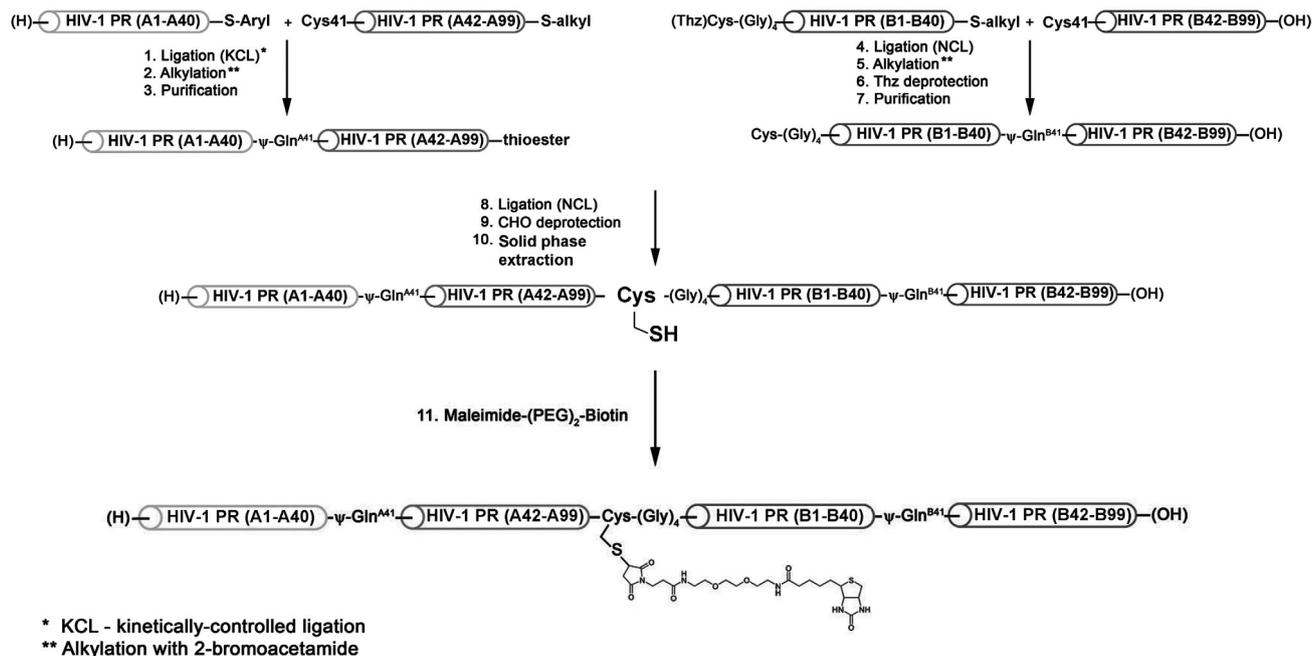
In a second approach used to investigate the feasibility of single-molecule measurements of HIV-1 protease activity, a “covalent dimer” HIV-1 protease equipped with a biotin tag was prepared by total chemical synthesis

(Figure 4) and immobilized on the neutravidin-coated surface (Figure 5a). A short peptide substrate labeled with a FRET dye pair was then added and the reaction was again monitored by TIR microscopy. The major goal for this experiment was to validate a total synthesis approach for the HIV-1 protease construct molecule labeled with a biotin-tag moiety, as well as to test whether such a molecule would possess enzyme activity — detectable by total internal reflectance excitation fluorescence spectroscopy — when tethered to the surface of a glass slide. Synthesis of the required 203-residue protein molecule was performed as previously described, in a fully convergent fashion from four synthetic peptide segments (Figure 4).<sup>[18d]</sup> At the final stage, the two large “halves” were condensed by native chemical ligation to yield the full-length polypeptide, containing just a single free thiol group that was reacted with a maleimide-functionalized PEGylated biotin moiety. The synthetic enzyme molecule was purified to homogeneity by HPLC and then characterized by analytical HPLC and mass-spectrometry (Figure 4).

After surface immobilization of the “covalent dimer” HIV-1 protease and addition of the fluorophore-labeled substrate, we could observe transient binding/cleavage events on the surface probed by TIR spectroscopy. Initially, we could observe a rather high density of fluorescent substrate (Supporting Figure 8, left panel, top), whereas after two hours of incubation of the substrate over the surface and then inspecting the surface with TIR, only a few bright spots (fluorescent substrate molecules) were observed (Supporting Figure 8, right panel, top), indicating that most of the substrate has undergone proteolysis. Individual positions on the glass slide showed fluorescent events that were consistent with binding of the soluble FRET-pair labeled peptide substrate to the tethered enzyme molecule, followed by proteolytic cleavage of the substrate peptide chain; two such traces are shown in Figure 5b. Note the distinct “dwell” times, between binding of the labeled substrate to the tethered enzyme molecule and subsequent cleavage/dissociation of the labeled fragments.

## 4. Conclusions

We have carried out proof-of-principle single-molecule studies of proteolytic cleavage by the HIV-1 protease using chemical synthesis combined with TIR fluorescence microscopy. We attempted to record binding of HIV-1 protease to fluorophore-labeled substrate/inhibitor, and to be able to distinguish cleavage from binding. We expected that this would be a feasible experiment since binding of the labeled peptide would lead to high-to-intermediate FRET change, whereas cleavage would lead to elimination of FRET after dissociation of one of the proteolytic reaction products.

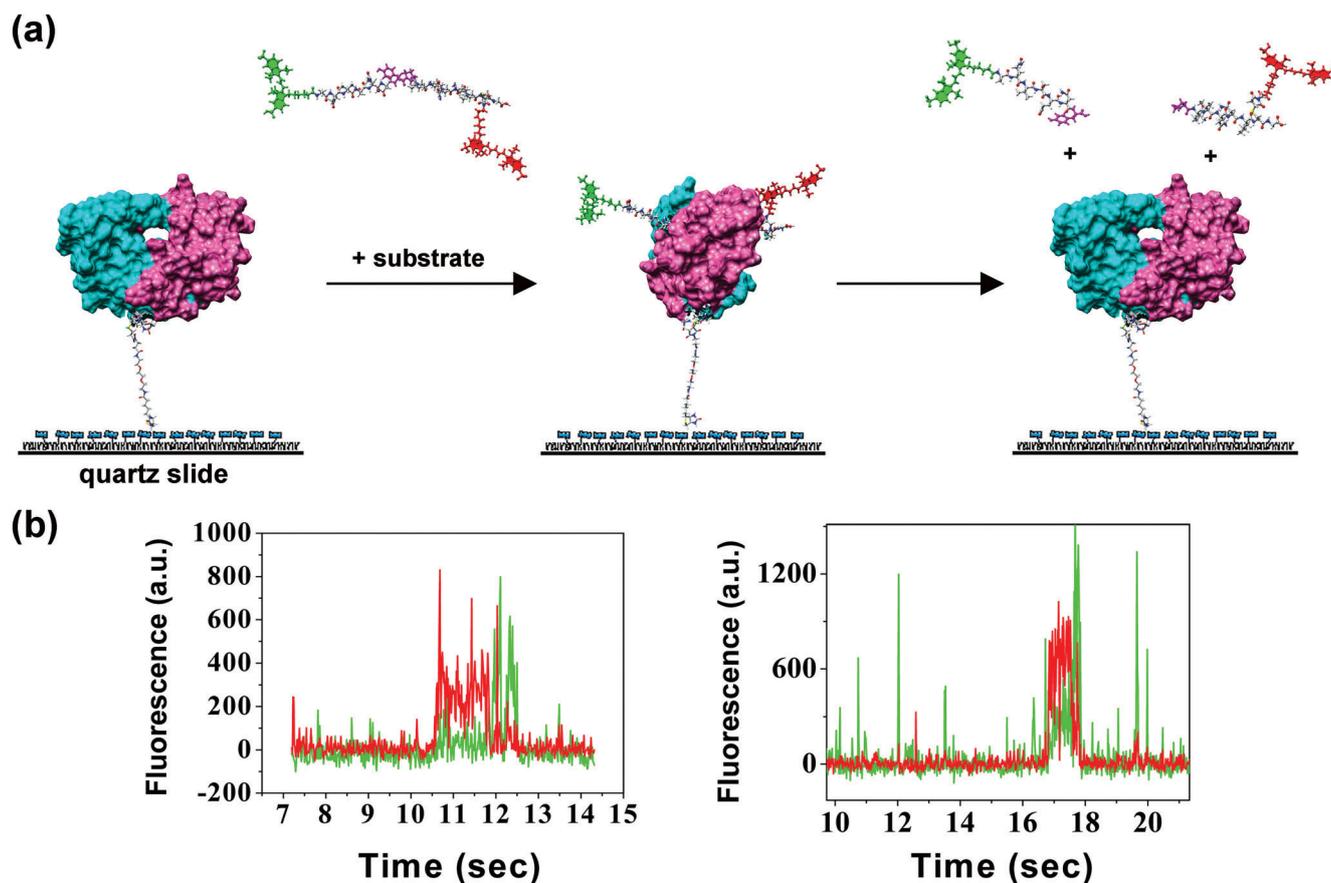


**Figure 4.** Total chemical synthesis of a biotin-tagged covalent dimer HIV-1 protease molecule. The final step is an alkylation reaction to give the full-length protein product containing a PEGylated-biotin tag at the mid-point of the 203-residue polypeptide chain. The biotin is used to tether the synthetic HIV-1 protease protein construct to a neutravidin-coated quartz surface.

The data obtained show that indeed we have observed distinct binding and cleavage steps, where the slow enzyme ( $k_{\text{cat}} = 21 \text{ s}^{-1}$ )<sup>[18c]</sup> HIV-1 protease first “searches” for a cleavage location (which can lead to stepwise changes of the FRET signal), and both substrate and enzyme may undergo multiple conformational isomerizations. Analysis of the data was however hindered by photobleaching of the dye molecules. We have tested various configurations of dyes and, although improvement of the photostability was observed with ALEXA dyes, photobleaching could not be eliminated entirely. This is most probably due to the high flexibility of the substrate/inhibitor peptide structures in comparison with, for example,

DNA strands, where such a fluorescent dye-labeling approach is well-established. The solution to this problem may be implementation of an alternating (or intermittent) laser excitation (ALEX) FRET setup, which greatly extends the lifetime of the fluorescent dyes.<sup>[22]</sup>

In the work reported here, we have also described the total chemical synthesis of a “covalent dimer” HIV-1 protease molecule construct which can be tethered to the surface, and we have verified that such a protein molecule possesses proteolytic activity on FRET-pair-labeled substrates that can be observed by single-molecule spectroscopy. Further study should be performed in which the tethered enzyme protein molecule itself is labeled with a



**Figure 5.** Tethered Enzyme Experiments. (a) Cartoon of HIV-1 protease chemically synthesized as a covalent dimer bearing a biotin tag, immobilized on a neutravidin-coated quartz surface. Substrate peptide labeled with Cy3 and Cy5 dyes is then added, and reaction is monitored using a TIR microscope. (b) TIR-monitoring of the proteolytic activity of HIV-1 protease tethered to the quartz slide. Single-molecule fluorescence traces, where the molecule first binds (high-FRET) then undergoes cleavage, leading to dissociation of one of the dye-labeled peptide fragments.

FRET-pair in order to directly observe conformational isomerizations of the protein molecule during catalysis. In other work, we have developed a series of uniquely chemical analogues of the HIV-1 protease with a range of interesting catalytic properties that are attractive candidates for such single-molecule studies, particularly the asymmetric covalent dimer [D-Ala51, L-Ala51']HIV-1 protease.<sup>[23]</sup>

Finally, using these kinds of chemistries applied to the HIV-1 protease molecule, it should be possible to combine fluorescence monitoring with the use of optical tweezers to simultaneously carry out force measurements on the enzyme molecule in the act of substrate cleavage.<sup>[24]</sup> Such studies could cast further light on the role of the two mobile “flap” structures in catalysis by this important enzyme.

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