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Mechanism of Dissociative Inhibition of HIV Protease and its Autoprocessing from a Precursor

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Abstract

Dimerization is indispensable for release of the HIV protease (PR) from its precursor (Gag-Pol) and ensuing mature-like catalytic activity that is crucial for virus maturation. We show that a single-chain Fv fragment (scFv) of a previously reported monoclonal antibody (mAb1696), which recognizes the N-terminus of PR, dissociates a dimeric mature D25N PR mutant with an enhanced dimer dissociation constant (K_d) in the sub-micromolar range to form predominantly a monomer-scFv complex at a 1:1 ratio, along with small (5–10%) amounts of a dimer-scFv complex. Enzyme kinetics indicate a mixed mechanism of inhibition of the wild-type PR, which exhibits a $K_d < 10$ nM, with effects both on K_m and k_{cat} at scFv to PR ratio of 10:1. ScFv binds to the N-terminal peptide P¹QITLW⁶ of PR and to PR monomers with dissociation constants of 30 nM and ~100 nM, respectively. Consistent with a ~400-fold increase in the dissociation of the antibody (K_{Ab}) on even addition of an acetyl group to P¹ of the peptide, the antibody fails to inhibit N-terminal autoprocessing of the PR from a model precursor (at ~5 μ M). However, subsequent to this cleavage, it sequesters the PR thus blocking autoprocessing at its C-terminus. A second monoclonal antibody (PRM1), which recognizes part of the flap region (residues 41–47) of the mature PR and its precursor, does not inhibit autoprocessing and ensuing catalytic activity. However, its failure to recognize drug resistant clinical mutants of PR may be beneficial to monitor the selection of mutations in this region under drug pressure.

Keywords

Retrovirus; HIV protease; dimerization inhibitor; Antibody binding; Polyprotein processing

INTRODUCTION

The mature protease of HIV-1 (PR) is a homodimer of two 99-amino acid subunits, each of which contributes one aspartyl residue to the catalytic site.¹ The dimer interface comprises the conserved active site triad Asp-Thr-Gly and a four-stranded antiparallel β -sheet, formed by the N- (1–4) and C-terminal (96–99) residues of each monomer. In contrast to the mature PR, which is a very stable dimer (K_d in the low nM range), PR within the Gag-Pol prior to its maturation (Fig. 1) exhibits orders of magnitude higher K_d . Polyprotein maturation is a spatio-temporally regulated process in which PR catalyzes its own release at its termini

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(sites 3 and 4 indicated by arrows in Figure 1) from the Gag-Pol. The intramolecular N-terminal cleavage which precedes the C-terminal cleavage and likely occurs via transient dimer formation is concomitant with the appearance of mature-like catalytic activity and stable dimer formation (decrease in K_d).^{2,3} This fully active PR subsequently mediates cleavage at the C-terminus by an intermolecular mechanism to form the mature PR, as well as the release of the mature structural and other functional proteins essential for viral assembly.^{2,4}

The pivotal role of PR autoprocessing in initiating this cascade of cleavages leading to polyprotein and virus maturation, as well as the conserved nature of residues comprising the dimer interface, suggests that effective inhibition of autoprocessing through non-competitive inhibitors to disrupt this interface may provide a novel approach to circumvent the selection of drug-resistant mutants that can occur during therapy with active site directed clinical inhibitors of the mature PR. Consequently, a better understanding of the inhibition of autoprocessing will be beneficial.

We recently developed methods for the expression and purification of PR precursor mimetics containing the N-terminal full-length and truncated TFR (optimized analogues of the PR precursor, Fig. 1), in quantities sufficient for biophysical and structural studies.⁵ Crystal structures of a PR precursor mimetic reveal several unique conformations of the N-terminus including complete disengagement of the P¹QIT⁴ residues of one monomer unit from the β -sheet interface that is crucial for dimer stability.⁶ The drastically lower dimer stability consistent with weakened terminal β -sheet conformations of the precursor provides a structural basis for possible attempts to perturb autoprocessing.

Numerous studies have focused on disrupting the dimeric structure of mature PR primarily by targeting the terminal β -sheet;^{7–14} for reviews see⁴ (page 279). Proposed dimerization inhibitors that bind to the mature PR monomers must compete with dimer formation, and this is possible only at low nM concentrations of enzyme (the most prevalent HIV-1 group M)¹⁵ where partial dimer dissociation may occur.^{4,7,16,17} Thus, characterization of such inhibitors of PR has depended mainly on kinetic measurements,⁷ and mechanistic studies including isolation of resultant complexes to permit structural studies have not been performed using optimized PR constructs.^{7,11,12} In addition many proposed dimerization inhibitors are hydrophobic or amphiphilic and promote precipitation of PR or its precursor mimetic TFR-PR even at low μ M concentrations.

To circumvent the poor solubility of many proposed chemical inhibitors of dimerization, we used an antibody as a model dimerization inhibitor to study its effects on PR autoprocessing. A mouse monoclonal antibody (mAb1696) investigated by Lescar et al.,¹⁸ was shown to inhibit both the mature HIV-1 and HIV-2 proteases, and it was suggested that mAb1696 binding shifts the monomer-dimer equilibrium of PR to a monomer. The epitope recognized by mAb1696 was mapped to N-terminal residues 1–7 of PR and the amino terminal Pro1 was shown to contribute significantly to binding by deleting the Pro1 residue.¹⁹ Interestingly, it was also stated without providing data that mAb1696 recognizes the mature PR and not a PR precursor containing 20 amino acids of the N-terminally flanking TFR.¹⁸

An engineered single-chain Fv fragment of mAb1696 (scFv) was shown by Rezacova et al.¹⁹ to retain the specificity of mAb1696. No crystal structures of the mAb or scFv bound to the mature PR or to a PR monomer are available. However, a crystal structure of the scFv/peptide PQITLWQRR (non native C-terminal Arg added to enhance solubility) complex revealed the interactions between the antibody and the first six residues of the peptide (Fig. 2A). The main chain conformation of the antibody-bound peptide residues PQITLW (1JP5)¹⁹ is shown in Figure 2B superimposed with the same residues in mature dimeric PR

complexed with a substrate analogue (3BVA)²⁰ and in the precursor mimetic ^{SNF}PR as a complex of the dimeric precursor with inhibitor DRV (4TKW)⁶ to visualize the varying conformations of the N-terminal residues. The 4TKW structure is characterized by a novel conformation in which the four N-terminal (PQIT) residues of one monomer detach from the β -sheet and form a loop with a new H-bond interaction between Q2 and L5. The conformations of all three peptide chains differ from each other, most strikingly in the bent conformation of the disengaged N-terminal residues⁶ of ^{SNF}PR in contrast with the extended conformations seen in mature PR and the antibody bound peptide (Fig. 2B and C). We speculated that disengagement of the N-terminal PQIT residues of PR as a precursor might facilitate antibody binding, albeit at a lower affinity because of flanking N-terminal sequences, to inhibit dimerization and subsequent self-cleavage of the PR at its N- or C-termini to release mature PR.

In the present study we explore in detail several aspects of the interaction of mAb1696 and scFv with 19 HIV protease constructs, to extend our understanding of the mechanisms of antibody mediated dissociative inhibition of the mature PR and its precursor. These include 1) time dependent inhibition of mature PR activity and its N- and C-terminal autoprocessing from precursor mimetics by antibody 1696, 2) effects of increasing antibody concentration on the kinetics of mature PR-mediated hydrolysis of substrate, 3) specificity and binding affinity of antibody 1696 to PR monomer, its precursor, and peptide analogues spanning the N-terminus of PR by isothermal titration calorimetry, Size exclusion chromatography with molecular mass analysis by Multiangle light scattering, Refractive index and UV measurements (SMR), and immunoblotting. As a proof of principle, these studies provide a comprehensive understanding of the mode of inhibition of the autoprocessing reaction by a dimerization inhibitor that fails to block the N-terminal cleavage but prevents the subsequent C-terminal cleavage carried out at μ M protein concentrations. In addition we have investigated the effect on catalytic activity of a human monoclonal antibody, selected from a recombinant HuCAL GOLD library,^{21,22} which binds to the flap region. Its loss of recognition of extreme multi drug-resistant mutants of PR may have practical applications for monitoring the appearance of such mutations during patient therapy.

RESULTS AND DISCUSSION

Inhibition of Catalytic Activity of Natural Variants and Multi Drug Resistant Mutants of Mature PR by mAb1696

Addition of a small (~1.2-fold) molar excess of pure mAb1696 to folded mature PR (0.5 μ M as monomers) at pH 6 (Fig. S1A) results in inhibition of activity as measured by hydrolysis of a chromogenic substrate. Only limited inhibition is observed within the 15–30 sec required to mix and add the substrate after addition of the antibody; however, inhibition is ~98% complete after 30 min incubation at room temperature, and no activity is detected after 1 h. When PR was folded in the presence of mAb (0.4 μ M, 1:1 ratio) and the enzyme was assayed immediately, negligible activity was observed. The time dependence of inhibition when mAb is added to the folded enzyme may be a consequence of slow and unfavorable dissociation of the dimeric PR to monomers, which bind to the antibody, thus shifting the dimer-monomer equilibrium to the monomer. This observation is consistent with the relatively slow rate of PR dimer dissociation (forward rate constant corresponding to a half-life of ~5 min at pH 7).²³ Addition of mAb1696 at a less than stoichiometric (0.6:1) ratio relative to PR monomers gives only marginally less inhibition than a 1.2:1 ratio (Fig. S1B), suggesting that one monomer can be sequestered at each site of the bivalent antibody. Consistent with the pH dependence of antigen-antibody interactions, little or no inhibition of activity by the mAb is observed at pH 5.

The engineered single chain Fv fragment of mAb1696 (scFv)¹⁹ provides a convenient alternative to the mAb because of its smaller size and the ability to express, purify and easily modify scFv for biochemical and biophysical studies. Higher concentrations of scFv relative to the mAb were required to achieve comparable levels of inhibition (e.g., compare the inhibition of PR by mAb and scFv in Fig. S1A, B and S1C, respectively). HIV-2 PR (PR2, N-terminal sequence PQFSLW; Fig. 1 and S1D) is also inhibited by mAb and scFv, consistent with kinetic observations.^{18,19} The natural variant of PR Group O (PR_O, N-terminal sequence PQIPLW; Fig. 1 and S1G) is also inhibited. Thus antibody binding is reasonably tolerant of conservative substitutions (PR2) as well as the non-conservative substitution of Pro for Thr at position 4 in PR_O. An extreme multidrug resistant mutant, PR_{M-20}, which has the same N-terminal hexapeptide sequence as PR, is also inhibited as expected by mAb and scFv (Fig. S1E and F). To test the possibility that apparent inhibition could result from aggregation of the PR-antibody complex, both PR and PR_{M-20} were incubated with scFv, assayed and compared with an aliquot of the same incubation mixture after centrifugation. Only a marginal decrease or no decrease of activity was observed in the supernatant solution (cf. Fig. S1F).

Maturation of the PR at its N-terminus is concomitant with the appearance of stable dimers with full catalytic activity, followed by cleavage at its C-terminus. Incubation of several precursor analogues during autoprocessing with either mAb or scFv resulted in inhibition of enzymatic activity (Fig. S2). However, this observation did not provide a clear distinction between inhibition of N-terminal autoprocessing and subsequent inhibition of the released intermediate containing the C-terminal flanking sequence or the mature PR, but biophysical studies of antibody binding to precursor and mature PR constructs, described below, have resolved this ambiguity.

Antibody Interaction Affects Both k_{cat} and K_{m} of PR Mediated Substrate Hydrolysis

As scFv binds primarily to the N-terminus of PR and has been postulated to act as a dimerization inhibitor, we initially expected that antibody binding would sequester the enzyme as an inactive monomer complex, thereby decreasing its apparent k_{cat} , while having little effect on K_{m} . scFv binds engineered PR monomers with dissociation constants (K_{Ab}) of ~ 100 nM (see below). However, examination of the effect of increasing concentrations of scFv on k_{cat} and K_{m} for hydrolysis of a chromogenic substrate (Fig. 3A) indicates a mixed mechanism of inhibition in which a 3.3 fold excess scFv over PR concentration increases the apparent K_{m} , but has little effect on k_{cat} ; however, at higher concentrations (~10 fold excess scFv) K_{m} continues to increase and k_{cat} decreases.

A peptide consisting of four C-terminal residues of HIV-1 PR has also been proposed as a possible dimerization inhibitor of PR.⁷ We took advantage of the more favorable solubility properties of HIV-2 PR (PR2)^{24,25} to investigate the inhibition of this protease by a corresponding C-terminal PR2 nonapeptide, T⁹¹ALGMSLNL⁹⁹, and to compare its kinetic mechanism with that of inhibition of PR by scFv. Interestingly this peptide, which is a half substrate of the PR2-RT natural cleavage site, clearly exhibits classical competitive inhibition (Fig. 3B), consistent with binding at the active site: increasing the inhibitor concentration increases the apparent K_{m} , with little or no effect on k_{cat} . This kinetic behavior is in distinct contrast to the mixed inhibition observed with scFv. As the antibody is known to bind at the N-terminus of PR, the apparent “competitive” binding at concentrations in small excess must have an explanation unrelated to competition for occupancy of the active site. This effect on K_{m} may result from partial reversibility of the scFv complex and the resulting competitive equilibria for formation of mutually exclusive antibody and substrate complexes, or possibly from an interaction between the antibody and substrate that decreases its affinity for the PR.

Antibody Binding Results in a Dissociated PR as a Monomer-Antibody Complex

We also considered that binding of scFv to one subunit of the PR dimer without fully dissociating it could elicit a long range conformational effect to perturb the active site and reduce its affinity for substrate. To assess this possible mechanism we used SMR to evaluate the properties of scFv /PR complex as dimers or monomers. We chose PR2 for this study because in addition to enhanced solubility it exhibits desirable chromatographic properties relative to PR,²⁵ which interacts non-specifically with column matrices. An active site mutation, D25N, which abolishes catalytic activity, was introduced to prevent self-degradation (autoproteolysis), which would interfere with analysis of the data, and also to increase the K_d of PR2 and thus to facilitate interaction with the antibody. In earlier studies we had shown that the K_d for PR_{D25N} is >100-fold higher (1–2 μ M) than that for PR (< 5–10 nM)²⁰.

In the absence of antibody, PR_{D25N} is clearly dimeric (Fig. 4A). At a 1:1 ratio of scFv to PR_{D25N} dimers, 2 peaks that are shifted to an earlier elution volume relative to the antibody or the PR dimer, respectively, are observed (Fig. 4B, orange trace and labels). Proteins were quantified by UV absorbance at 280 nM (Figure S3 and Table S1). One peak (mass 35660 $\pm$ 71, Fig. 4B) corresponds to a complex of one PR_{D25N} monomer + one scFv and shows the expected increase in area (Fig. S3B) relative to the scFv alone (Fig. S3A) resulting from complexation of 1 monomer unit per scFv (half of the PR_{D25N} initially present as dimers), and the PR peak area (Fig S3B) is decreased relative to Fig. S3A by an equivalent amount. This second peak exhibits a mass slightly higher than that of a PR2 dimer. Deconvolution of the data in Fig. 4B had indicated that that the monomer/scFv complex (dashed orange trace) does not significantly contribute to the PR2 dimer peak, and thus cannot account for its apparent increase in mass. The most reasonable interpretation is that this peak consists of an equilibrium mixture of PR2 dimer plus dimer/scFv complex in a 7:3 ratio, with an apparent mass corresponding to a weighted average of the two masses. Since this peak constitutes half of the PR2 in the overall mixture, we estimate that a small but significant fraction (~15%) of the total PR2 is present as a dimer/scFv complex under our experimental conditions.

We also examined the effect of DRV, which was expected to bind to PR_{D25N}, stabilizing it as a ternary dimer/DRV complex, albeit much less strongly than to wild type PR [$\sim 10^6$ -fold increase in DRV dissociation constant (low μ M range) based on our earlier comparison²⁰ of PR and PR_{D25N}, and a ~ 17 -fold increase in DRV dissociation constant for PR2 relative to PR²⁶]. A 1.1 to 1.0 ratio of DRV to PR_{D25N} dimers has little effect on the elution profile (Fig. 4B, green trace and labels) and almost no effect on the mass of the early-eluting PR monomer/scFv complex. The lack of a significant decrease in the peak area for this complex presumably reflects the weak binding of DRV to dimeric PR_{D25N}, which does not effectively compete with scFv binding to dissociated monomers. The apparent mass of the later-eluting peak (primarily PR2 dimer/DRV complex, calculated mass 22032) is less than that observed in the absence of DRV, consistent with a reduced contribution of dimer/DRV/scFv complex to this peak. We ascribe this effect to a small shift in equilibrium brought about by forming a dimer/DRV complex, thereby making scFv binding slightly less favorable. An analysis similar to that described above indicates an approximately 1:9 ratio of dimer/DRV/scFv to dimer/DRV complex, corresponding to 4.5% of the total PR_{D25N} in the mixture. These observations are consistent with lack of Fab'1696 binding to PR in the presence of an active site inhibitor.¹⁸ For very stable dimers (small K_d) and/or inhibitors with extremely high affinity (small K_i), binding of antibody to the dimer may not be observable. Thus, unlike PR monomers, mature PR, stabilized as a tight dimer by formation of its DRV complex (K_i 5–10 μ M)²⁷ gave no thermal response above control when titrated with scFv (Fig. S4A). The present results provide the first definitive physical demonstration that, although scFv exhibits much greater affinity for PR monomers, an equilibrium between

the predominant antibody/monomer complex and a small fraction of antibody/dimer complex is possible. We propose a mechanism shown in the scheme to account for the dissociative inhibition of PR2 (Fig. 4C). This equilibrium may account for the observed increase in K_m of PR by forming a dimer/scFv complex with reduced substrate affinity.

Antibody Selectively Inhibits the C-terminal Intermolecular Autoprocessing of PR Precursor

Release of mature PR from its Gag-Pol precursor is an ordered process comprising several steps (Fig. 1). Here we focus on the N- and C-terminal cleavages represented by steps 3 and 4; namely at the TFR-PR site concomitant with appearance of mature-like catalytic activity, and subsequent cleavage at the PR-RT site to produce the mature PR. To test the effects of scFv on these two sequential cleavages, a construct, TFR-PR-RT²⁰, was expressed and purified as an intact precursor containing the first 20 amino acids of RT fused to the C-terminus of the TFR-PR, thus making it possible to assess both the N- and C-terminal cleavage unambiguously by SDS-PAGE. The rapid appearance of PR-RT²⁰ (in 5–10 min) based on an N-terminal cleavage (TFR/PR) and its subsequent conversion to PR based on a C-terminal cleavage (PR/RT²⁰, Fig. 5A) in the absence of antibody is consistent with the stepwise mechanism described.^{2,4} We had shown previously, using a chimeric model PR precursor, that the appearance of a PR-RT²⁰ equivalent (PR-RT¹⁹) superimposes on the appearance of mature-like catalytic activity, and the sum of the products of PR-RT¹⁹ and PR superimposes on the disappearance of the full-length starting material.²⁸

Figure 5B shows the effect of scFv on the N- and C-terminal cleavages of TFR-PR-RT²⁰. There is no significant accumulation of PR-RT²⁰ in the absence of antibody after 2.5 or 18 h (lanes 3 and 4), indicative of rapid cleavage of PR-RT²⁰ by itself (dimer cleaving another dimer) and possibly later by the released mature PR, such that essentially all the cleaved products consist of TFR and PR. The RT²⁰ is not visible due to its small size and also possibly to its very weak or no staining. By contrast, in the presence of scFv virtually no mature PR is formed, and the intermediate PR-RT²⁰ (intensity comparable to PR in the control) accumulates (lanes 6 and 7). This leads us to conclude that scFv permits N-terminal cleavage but almost completely prevents active PR-RT²⁰ from catalyzing subsequent C-terminal cleavage.

Although processing of TFR-PR-RT²⁰ is essentially complete after 18 h in the absence of antibody, a significant amount of this full length precursor remains when antibody is present. This is likely a result of the inhibition of catalytic activity of PR-RT²⁰ such that its further processing to mature PR cannot occur. The intramolecular mechanism, initially the exclusive mode of N-terminal cleavage, is drastically slowed by depletion of the precursor as the reaction progresses and the consequent inability to form the required transient dimers; thus in the absence of intermolecular cleavage by functional mature PR, significant amounts of unprocessed TFR-PR-RT²⁰ remain (Fig. 5B). These observations are consistent with the interpretation that the inhibition of catalytic activity after incubation of TFR-PR or ^{SFNF}PR with antibody (Fig. S2) results from binding to the mature protease as monomers concomitant with or subsequent to its N-terminal cleavage.

Selective High Affinity of mAb1696 Requires a Free N-terminus but not Necessarily N-terminal Proline

To demonstrate the generality of this interpretation we further investigated the effect of N-terminal flanking sequences on antibody binding to various precursor and mature PR constructs by immunoblotting. Thus a series of PR constructs with and without the TFR or segments thereof at the N-terminus (listed in Fig. 1) were probed with mAb1696 and detected using an anti-mouse secondary antibody (Fig. 6; data summarized in Table 1).

Notably, none of the precursor analogues with an N-terminal TFR (Fig. 6A, 1, 2, 3, 5, and 7) are recognized by mAb1696, although the antibody binds to three proteins (4, 6, and 8) with a free N-terminus. Even short N-terminal extensions prevent the binding of mAb1696 (Fig. 6C, 9 and 10) whereas PR constructs bearing a C-terminal deletion or substitutions remote from the N-terminus are recognized by the mAb (Fig. 6A and C, 6, 8 and 11–13, and Fig. 6D). Among these are four DRMs and the natural variant, PR_N which possess the same conserved N-terminal PQITLW sequence (Fig. 1). PR_{M-20}²⁹ and PR_N³⁰, whose crystal structures have been solved, exhibit β -sheet interfaces that are essentially identical to PR (Group M). These results confirm the conclusion that antibody binding to PR is possible only subsequently to N-terminal autoprocessing, thus accounting for the differential effects (Fig. 5B and C) of scFv on stepwise N- and C-terminal cleavages. A C-terminal RT extension does not perturb binding as evidenced in Fig. 5C that mAb1696 binds to PR-RT²⁰, the end product, when autoprocessing of TFR-PR-RT²⁰ is conducted in the presence of the antibody for 20h.

The effect of N-terminal modifications on antibody binding was quantitatively assessed by isothermal titration calorimetry. In these studies it was necessary to use inactive mature PR constructs or an inhibitor to preclude autoprocessing of the precursors during titrations. Thus two precursor mimetics, inactive TFR-PR₁₋₉₅ (monomeric) and active TFR-PR^{PISP} in the presence of inhibitor DRV (dimeric as its inhibitor complex), as well as two inactive constructs, PR_{T26A} and PR₁₋₉₅, corresponding to the mature PR (as monomers), were titrated with scFv. The monomeric models were chosen because our observation of monomer-antibody complex by SMR, and the observed time dependence of PR inhibition, suggests slow, partial dissociation of PR to monomers that are recognized by the antibody, a mechanism that could complicate assessment by ITC. Essentially no thermal change is observed on titration of two constructs incorporating an N-terminal TFR (upper panels, Fig. 7A and B), consistent with very weak or no antibody binding to these proteins based on the immunoblotting experiments. By contrast, mature PR monomers (lower panels) exhibit substantial thermal changes indicative of antibody binding. Titration curves shown in Figure 7C and D correspond to dissociation constants (K_{Ab}) of 116 and 72 nM for mature PR_{T26A} and PR₁₋₉₅ antibody complexes, respectively (see also Table 2). These values, measured at pH 5.8, are ~100-fold larger than 0.6 nM measured kinetically for inhibition of mature PR in PBS at pH 7.4¹⁹ likely due to weaker binding at the lower pH (cf. Fig. S1B for mAb).

We also systematically examined the binding of several modified N-terminal peptides to the antibody to assess quantitatively its specific interactions with the N-terminal sequence. Titration of scFv with the N-terminal PR hexapeptide PQITLW in PBS gives an estimated K_{Ab} 30 nM (Fig. 7E and Table 2). A truncated peptide, QITLW (Fig. S4B), exhibits at least 20-fold weaker binding to the antibody than PQITLW, consistent with a moderate contribution of the N-terminal residue to antibody binding. That this contribution is not specific for Pro as had been suggested¹⁹ is indicated by the observation that mAb binds to PR and PR_{P1A} on western blots with similar intensity (Fig. 6D and Table 1, compare 4 and 14).

By contrast, blocking the imino nitrogen of Pro by acetylation (Fig. S4C) causes a drastic ~400-fold decrease in affinity relative to PQITLW. Titration of scFv with FPQITLW gave a curve that qualitatively resembled that with AcPQITLW (Fig. S4D); however, binding was so weak that only a partial titration was obtained, and the parameters derived for this peptide by curve fitting (Table 2) are rough estimates only. These results are consistent with a requirement for a free imino or amino nitrogen at the N-terminus. Addition of a single Phe N-terminal to the Pro compromises antibody binding more than acetylation, probably in part as a result of unfavorable steric interactions with the “partially closed” binding pocket of the

antibody.¹⁹ These observations clarify the structural basis for the inability of the antibody to interact tightly with PR precursors prior to their N-terminal cleavage.

Interestingly, binding of all four peptides to scFv is characterized by negative entropy, presumably resulting from their immobilization and loss of conformational flexibility. By contrast, the entropy of binding of the two PR monomers, PR_{T26A} and PR₁₋₉₅ is positive, contributing ~40% of the total free energy of binding at 28 °C, and may be related to desolvation effects on forming hydrophobic contacts between the antibody and the PR. The observation that the association constants and related thermodynamic parameters are very similar for a full length and a C-terminally truncated monomer (PR₁₋₉₅) indicates that the C-terminal sequence has no effect on antibody binding, consistent with the observed absence of structure at the C- and N-termini of folded PR monomers.⁴

Binding of an Antibody to the PR Flap Residues 41–47 does not Perturb Autoprocessing or Resultant Catalytic Activity

A human monoclonal antibody, PRM1 (heavy and light chain masses of 31620 and 22628, respectively as determined by ESI-MS) used as a control for the binding studies with mAb1696, exhibits a different binding pattern as shown by the western blots in Figure 8 and Table 1. We initially observed that although mature PR (Fig. 8A, lane 4) binds to PRM1, one of our constructs of TFR-PR, bearing a fortuitous K43E substitution mutation which does not affect autoprocessing or resultant catalytic activity, failed to bind (lane 3). Subsequent experiments revealed that natural variant PR_N, which has an R41K substitution, also is not recognized by the human antibody. Furthermore, the four DRMs examined, all of which bear resistance mutations at either M46 or I47, bind very weakly or not at all to the antibody. These observations indicate that the epitope is located in the region of residues 41–47. Since mutations in or near the flap region (residues 49–54) are frequently associated with resistance to multiple PIs,³¹ we suggest that western blots with PRM1 could provide a rapid and simple screen probing for such highly drug resistant mutants. Despite binding of the large antibody protein to the flaps, whose mobility is associated with substrate binding, PRM1 has no apparent effect on autoprocessing (Fig. 8B) or mature PR activity (Fig. 8C). In the absence of structural information the explanation for this observation is unclear. Interestingly, the epitope recognized by this human antibody is in the same region (residues 36–46) as identified for a mouse monoclonal antibody F11.2.32, which elicits inhibition.^{32,33}

Concluding Remarks

The present findings constitute a proof of principle that an inhibitor, which specifically binds to the N-terminus of PR and disrupts the dimer interface by forming a complex with the monomer, can effectively interfere with the function of the enzyme at nanomolar to low micromolar concentrations, provided that the inhibitor binds to the monomer tightly enough to compete with dimerization. A previously characterized antibody, scFv, provides the requisite tight binding with a K_{Ab} of ~100 nM for binding to a PR monomer, and possesses solubility properties that easily permit physical studies. On the basis of its 10-fold difference in affinity relative to PR monomer association (K_d 10 nM),⁴ an approximately 10-fold excess of scFv relative to PR monomers was expected to result in effective inhibition, consistent with kinetic experiments (Fig. 3) in which decreases in k_{cat}/K_m of 64% and 90% are observed at ratios of scFv to PR monomer of 3.3:1 and 10:1 respectively. We have obtained conclusive biophysical evidence for dimer disruption by formation of a PR_{2D25N} monomer/scFv complex using molecular mass analysis of the complex in the presence and absence of an active site inhibitor that is expected to stabilize the dimer (although weakly because of reduced affinity to the D25N mutant).

Precursor models consisting of TFR or partial TFR sequences fused to the N-terminus of PR lack the tight dimer interactions that are formed upon N-terminal cleavage to give the mature PR. On the basis of our recent crystal structures⁶ that reveal partial disengagement of one N-terminal strand from the dimer interface in a precursor mimetic, we initially speculated that this detached strand might be more available for antibody binding, albeit weakly, and thus provide a model for dimerization inhibition of N-terminal autoprocessing. However several lines of evidence including ITC and immunoblotting are consistent with the conclusion that the antibody binds to mature PR constructs but not to precursor analogues with an N-terminal extension of the PR. Furthermore, although the antibody binds tightly to a peptide comprising the N-terminal PR sequence ($K_{Ab} \approx 30$ nM), it binds only very weakly to the same peptide modified by a sterically minimal N-terminal acetyl group, consistent with a requirement for polar interactions between the antibody and a free N-terminus. In contrast, however, the antibody effectively blocks C-terminal autoprocessing of TFR-PR-RT²⁰ after its N-terminal cleavage, resulting in accumulation of both uncleaved starting material and the intermediate PR-RT²⁰. This observation suggests that inhibition of PR autoprocessing immediately following N-terminal cleavage deserves consideration.

Development of dimerization inhibitors directed to the N-terminus and adjacent TFR may be useful, because transient dimer formation needed to initiate autoprocessing (TFR/PR cleavage) constitutes an early step in the polyprotein processing cascade. Interactions between the C-terminal residues 96–99 also play a role in transient dimerization because deletion of these residues in a precursor construct abolishes N-terminal autoprocessing.⁴ Thus, sequestering the monomer through interactions with small molecules at the interior of the C-terminal interface may also effectively abrogate N-terminal autoprocessing. It is likely that dimerization inhibitors of autoprocessing will act synergistically with current PIs, which bind tightly to the active site of the mature PR dimer but exhibit 3–5 orders of magnitude lower affinity for the precursor.⁵ Thus these antibody studies provide insights and techniques for the design or identification of small-molecule inhibitors of dimerization with similar properties. Selective binding of mAb1696 or scFv may permit screening for inhibitors to block folding and dimerization of the PR precursor prior to its N-terminal autoprocessing. Furthermore, PRM1 antibody which was matched roughly to an epitope spanning the region 41–47 of the PR could be used as a control in screens using antibody 1696 to recognize the precursor as well as to probe for some natural and drug resistant variants of PR.

EXPERIMENTAL PROCEDURES

Protease Constructs and Protein Preparations

Gene manipulations were carried out as described. Constructs used in this study are listed in Figure 1. DNA inserts were cloned either in pET11a vector between NdeI and BamHI sites or in pET15b between NcoI and BamHI sites, and expressed in *E. coli* BL21(DE3). Mature and inactive PR proteins were expressed by following a typical protocol of inducing protein expression at an optical density of 0.6–0.7 (600 nm) using IPTG for 4 h. To allow isolation of sufficient quantities of purified intact active precursor proteins, 100 mL cultures were grown in the presence of DRV to prevent their autocatalytic maturation during expression as described recently.⁵ Proteins were purified from inclusion bodies and folded as described.^{25, 30}

Antibodies

Purified mAb1696 was purchased from Abcam Inc. scFv (1JP5) was expressed from a synthetic gene, purified and folded as described.^{19,34} PRM1 was generated from the HuCAL

GOLD (AbD Serotec, Germany) collection of human antibody genes using several rounds of selection (panning) on immobilized mature HIV-1 protease.^{21,22}

Monitoring Time Dependent Enzyme Inhibition

PR and PR precursor constructs were folded to a total volume of 110 μ L, by addition of 45–48 μ L of buffer A (5 mM sodium acetate, pH 6) to the enzyme (typically 2–5 μ L) in 12 mM HCl, immediately followed by 60 μ L of 100 mM sodium acetate buffer, pH 6, containing 500 mM NaCl. PR²⁵ and HIV-1 group O mature PR (PR_O, in 50 mM Tris-HCl buffer, pH 7.6, containing 50 mM NaCl) were added to the diluted reaction buffer (50 mM sodium acetate, pH 6, 250 mM NaCl) as folded enzymes. To monitor time dependent inhibition, mAb1696 (in 3–10 μ L of 1 $\times$ PBS) or scFv (in 3–5 μ L of 1 $\times$ PBS) was added and the solutions were incubated for 30–60 min at room temperature. Controls contained 1 $\times$ PBS in place of the antibody. Assays were initiated by addition of 10 or 12 μ L of 4.3 mM chromogenic peptide substrate IV [Lys-Ala-Arg-Val-Nle-(4-NO₂Phe)-Glu-Ala-Nle-NH₂], and were monitored by the absorbance decrease at 310 nm.³⁵

Determination of Kinetic Parameters

Sufficient PR for each series of kinetic assays was folded by addition of 510–524 μ L of 5 μ M sodium acetate, pH 6, to 9.4 μ L of PR (0.41 mg/mL [19 μ M as dimers] in 12 mM HCl), immediately followed by 540 μ L of 100 mM sodium acetate, pH 6, containing 2M NaCl. Immediately following folding, scFv was added to give final concentrations of 1.0 or 3.0 μ M. After a minimum of 30 min incubation at ambient temperature, 108- μ L aliquots were assayed by addition of varying amounts of substrate IV as described (final concentrations of 0.15 μ M PR dimers in a total volume of 120 μ L). No difference in rate was observed between selected samples assayed after 30 and 60 min incubation.

Folded mature PR2 (in 20 mM sodium phosphate buffer, pH 6, containing 50 mM NaCl)²⁵ was added to reaction mixtures containing 16 or 32 μ M PR_{291–99} (TALGMSLNL) in 50 mM sodium formate, pH 4, containing 50 mM NaCl, to achieve a final concentration of dimeric enzyme of 0.2 μ M. Assays were performed as described between 50–390 μ M substrate concentrations.

Molecular Mass Analysis

Molecular masses were estimated by analytical SEC with in-line multiangle light scattering (DAWN EOS, Wyatt Technology Inc., Santa Barbara, CA), refractive index (Optilab T-rx, Wyatt Technology Inc.) and UV (Waters 2487, Waters Corporation, Milford, MA) detectors. PR_{2D25N} (60 μ L in 12 mM HCl) was folded by mixing with 440 μ L of 20 mM sodium phosphate and 50 mM NaCl (protein folding [PF] buffer) to achieve a final concentration and pH of ~11 μ M and 5.8, respectively. The sample was centrifuged at 12800 rpm for 4 min in an Eppendorf 5415 centrifuge and the supernatant applied to a pre-equilibrated Superdex-75 column (1.0 $\times$ 30 cm, GE Healthcare) at a flow rate of 0.7 mL/min at room temperature and eluted in PF buffer. For chromatograms containing scFv (and DRV), PR_{2D25N} was folded in the presence of scFv (and DRV) in the PF buffer. Molecular masses were calculated from RI and light scattering data using the Astra V (version 5.3.4.20) software provided with the instrument.

Autoprocessing of TFR-PR-RT²⁰

The control autoprocessing reaction in the absence of antibody was carried out as described below, for the indicated times. Reactions with scFv contained 19.7 μ M antibody. The precursor (7 μ L at 1 mg/mL in 40 mM Tris-HCl, pH 7.5, and 2M urea) was mixed with 63 μ L of 0.5 $\times$ PBS, pH 6.5, to give a final concentration of precursor monomer of 5.17 μ M.

Samples (20 μ L) were withdrawn at 2.5 and 18 h, mixed with 8 μ L of SDS-PAGE sample buffer (2.6 $\times$), subjected to electrophoresis on 10–20% gradient Tris-tricine gels (Invitrogen) and Coomassie stained. Reactions of the precursor with PRM1 were carried out following the same procedure.

Western Blotting

Proteins were separated on 10–20% Tris-tricine gels and immunoblotted either using the mouse antibody mAb1696 according to the One-Hour Western Detection System provided by Genscript or as described previously³⁶ using the human antibody PRM1.

Isothermal Titration Calorimetry

Proteins TFR-PR^{PISP}, TFR-PR_{1–95}, PR_{T26A} and PR_{1–95} (20–36 μ L of a ~2 mg/mL solution in 12 mM HCl) were folded by addition of 5 mM sodium acetate buffer, pH 6 (buffer A), to give 175 μ L followed immediately by 175 μ L of 50 mM sodium phosphate buffer, pH 5.8, containing 300 mM NaCl, to give a final buffer concentration of 25 mM sodium phosphate and 150 mM NaCl. With TFR-PR^{PISP}, buffer A contained DRV to give a final concentration of 20 μ M in the titration mixture. As mild precipitation was observed upon folding this sample, the protein concentration in the supernatant after centrifugation was estimated by UV prior to titration. Samples were titrated with fifteen 2.45 μ L aliquots of 80 or 100 μ M scFv in a buffer of the same composition using a MicroCal high-precision iTC₂₀₀ microcalorimeter (GE Healthcare). Data were analyzed by use of the Origin software provided with the instrument. Concentrations of PR_{T26A} and PR_{1–95} were estimated from the number of sites titrated (N-value) and a binding stoichiometry of 1:1 for scFv:PR monomer.

ScFv (~22 μ M) in PBS containing 0.5% DMSO was titrated as above with 300 μ M PQITLW in the same solvent. Similarly, scFv (~10 μ M) was titrated with 100 μ M peptides QITLW, AcPQITLW, and FPQITLW in the presence of 0.2% DMSO. For titrations that did not reach a constant end point, data were corrected for the thermal response obtained on buffer titration with the peptides. An N-value of 0.68 was observed for the titrations with both PQITLW and QITLW, and was used to adjust the concentration of scFv to 6.8 μ M (with N fixed at 1) for the titrations with very weak binders which gave insufficient data for accurate calculation of N by curve-fitting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HIV-1 and HIV-2	human immunodeficiency virus type 1 and 2, respectively
PR	mature HIV-1 protease
PR2	mature HIV-2 protease
mAb or mAb1696	mouse monoclonal antibody to PR

ScFv	single chain Fv fragment of mAb1696
TFR	transframe region of the Gag-Pol precursor
PIs	clinical inhibitors of HIV-1 protease
DRMs	drug-resistant mutants or drug-resistance mutations
DRV	darunavir
ITC	isothermal titration calorimetry
SEC	size exclusion chromatography
SMR	SEC coupled with multiangle light scattering and refractive index measurements
PRM1	human monoclonal antibody to PR

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HIV protease (PR) catalyzes its own release from the viral Gag-Pol polyprotein
N-terminal cleavage essential for dimer stability precedes C-terminal cleavage
Upon cleavage at the N-terminus, antibody binds and blocks C-terminal cleavage
Thermochemistry and isolated antibody/PR complex reveal sequestration of PR monomer
Transient precursor dimerization could be an effective target to block PR function

Figure 1.

Structural organization of the HIV-1 Gag-Pol polyprotein, and sequences of constructs used in this study. Individual domains are MA, matrix; CA, capsid; NC, nucleocapsid; RT, reverse transcriptase; RN, RNase H; IN, integrase. Black arrows indicate specific sites of cleavage by PR. The green (TFR) and red (PR) bars denote a protease precursor mimetic, TFR-PR. N-terminal extensions of PR derived from TFR used in this study are shown in green letters and C-terminal extensions (PISP and RT²⁰) derived from RT are shown in blue letters. N-terminal autoprocessing occurs stepwise in the order as listed (red arrows denoting sites 1, 2, and 3) at pH < 5; in the present study at pH > 5 only the site 3 cleavage of TFR-PR is observed. The pH dependence of these N-terminal cleavages has been described previously.² C-terminal autoprocessing (site 4) of a precursor model TFR-PR-RT²⁰ occurs after N-terminal cleavage to give fully active PR-RT²⁰, and is catalyzed either by itself or subsequently by the mature PR. The wild-type PR sequence of group M (subtype B-HXB2) without Q7K, L33I, L63I, C67A, and C95A mutations optimized for biochemical and structural studies (the present construct, hereafter designated as PR) is aligned with HIV-2 protease (PR2)²⁵ and proteases of natural variants HIV-1 groups N and O (PR_N³⁰ and PR_O³⁷) and four drug-resistant mutants (DRMs): PR_{M-20}³⁸, PR_{M-14}³⁹ and PR_{M-11}^{40,41}, bearing 20, 14 and 11 amino acid substitutions, respectively, and PR_{MP-51}, a DRM derived from a mixture of clinical isolates by selection in cell cultures grown with increasing concentrations of darunavir (DRV).⁴² Only the amino acid differences relative to PR are indicated. Gold and white backgrounds indicate highly conserved regions in drug treated patients and areas of greatest natural variability in the PR domain respectively.⁴³ Green background indicates regions where most major drug resistance mutations occur.³¹

Figure 2.

A. Interactions between scFv and the N-terminal PQITLW sequence of PR. The structure of the antibody bound to a peptide containing the 6 N-terminal residues of PR is from¹⁹. The scFv is shown as a semi-transparent surface representation with opaque residues contacting the hexapeptide. Red and blue atoms correspond to oxygen and nitrogen, respectively. One monomer of the protease (3BVA)²⁰ represented as a green ribbon with its N-terminal Pro residue indicated, was oriented relative to the scFv by alignment of its N-terminal sequence with the antibody bound PQITLW peptide. B. Ca alignment of PQITLW residues of the peptide bound to scFv (1JP5, white), mature dimeric PR_{D25N} complexed with a substrate analogue (3BVA, green), and a precursor mimetic with an N-terminal 4-residue extension as its ternary complex of dimer plus DRV (4TKW, orange). C. Conformations of the main chain and side chains of PQITLW in the antibody-hexapeptide complex, in mature PR_{D25N} and in the precursor mimetic; colors correspond to those in (B).

Figure 3.

(A) Effect of scFv on the kinetics parameters for hydrolysis of substrate IV catalyzed by $0.15 \mu\text{M}$ of PR (as dimer). Measurements were made in 50 mM sodium acetate buffer, pH 6, containing 1 M NaCl. The antibody was added to folded PR and incubated at room temperature for at least 30 min before assays were begun. Open circles, control without antibody; closed circles and triangles, with 1 and $3 \mu\text{M}$ antibody, respectively. For comparison, (B) shows plots for competitive inhibition of PR2 by a peptide consisting of PR2 residues 91–99 ($K_i = 17 \mu\text{M}$). Measurements were made in 50 mM sodium formate buffer, pH 4, containing 50 mM NaCl at an enzyme dimer concentration of $0.2 \mu\text{M}$. Open circles, control; closed circles and triangles, with 16 and $32 \mu\text{M}$ inhibitor, respectively. In

(A) the apparent K_m is increased in the presence of low antibody concentrations without significant change in k_{cat} , but at higher antibody concentrations k_{cat} also decreases, unlike the classical pattern for competitive inhibition (B) in which only the apparent K_m is affected by increasing inhibitor concentration.

Figure 4.

Molecular mass analyses of PR/antibody complexes by SMR. Proteins were fractionated on a Superdex-75 column at room temperature equilibrated in 20 mM sodium phosphate, pH 5.8, 50 mM NaCl. (A) Calculated masses of PR_{2D25N} (monomer) and scFv are 10718 and 26908, respectively. Experimental masses match closely to that of the calculated values indicating the elution of PR_{2D25N} as a dimer (red) and scFv as a monomer (black). (B) Values in parentheses denote molar ratios of PR_{2D25N} dimer (11 μ M), DRV and scFv mixed prior to SMR. The masses determined for the PR_{2D25N} and scFv mixture (orange) correspond to the PR_{2D25N} monomer/scFv (35660 ± 71) complex and the PR_{2D25N} dimer (29840 ± 597 , mass increase is likely due to a very small amount of scFv bound to the dimer). The latter

peak may also contain a minimal amount of free scFv due to equilibration between the dimer and dimer/scFv complex. The orange dashed line, derived from peak deconvolution, indicates that the monomer/scFv complex does not significantly overlap with the PR_{2D25N} dimer. In the PR_{2D25N}, DRV and scFv mixture (green), masses correspond to mainly PR_{2D25N} monomer/scFv (34750 ± 104) and PR_{2D25N} dimer/DRV (24500 ± 245) complexes. (C) Equilibria involved in dimer disruption of mature wild-type PR. Perturbing dimer formation is practical only when the dissociation constant of a putative dimerization inhibitor (K_{Ab}) is comparable to or smaller than the dimer dissociation constant (K_d) of PR. An inhibitor (I) which binds tightly to the active site (low nM or pM, K_i) further stabilizes the PR dimer making the dimer/I complex less accessible for antibody binding. Brackets indicate a possible minor contribution to the equilibria shown.

Figure 5.

Inhibition of autoprocessing of TFR-PR-RT²⁰ by scFv. (A) Time course (in hours) of the reaction (5.17 μM precursor) at pH 6.5 in 0.5x PBS and 0.2 M urea. M denotes molecular weight standards in kDa. (B) Autoprocessing in the absence and presence of ~4-fold molar excess of scFv. Reactions and gel analysis were carried out as described in Experimental procedures. Proteins were visualized by Coomassie staining. (C) Inhibition of autoprocessing using mAb1696 monitored by western blotting. Lanes 1 and 2 are controls without scFv; lanes 3 and 4, with same excess of scFv as in B. Note that the mAb does not recognize the precursor containing the TFR (time zero lanes) but recognizes the precursor only after the cleavage at its N-terminus. The scheme accounts for the experimental results shown in panels B and C. The stepwise maturation of PR from a model precursor, comprising the PR (red) flanked by the transframe region (TFR, green) and a partial reverse transcriptase sequence (ΔRT, black), and its inhibition by scFv are shown.

Figure 6.

Western blotting of mAb1696 binding to mature PR and its precursor mimetics. Lane identifications and notation are described in Table 1. Proteins (500 ng/lane) were subjected to electrophoresis on 10–20% gradient Tris-Tricine gels, transferred to nitrocellulose membrane and probed with mAb1696 as described in the Experimental procedures. Only those proteins lacking an N-terminal flanking sequence are recognized by mAb1696. Proteins ranging in concentration from 0.28–0.6 mg/mL are shown in panels B and E by Coomassie staining to validate the western blotting results (a and c). Arrows in panel A correspond to stained band positions seen in panel B.

Figure 7.

ITC analysis of scFv binding to mature PR and its precursors at 28 °C. Panels A–D, experiments in 25 mM sodium phosphate buffer, pH 5.8, containing 150 mM NaCl. Thermal responses on titration of (A) active precursor TFR-PR^{PISP}/DRV complex (upper panel) and mature PR_{T26A} with scFv (lower panel) and (B) inactive precursor model TFR-PR₁₋₉₅ (upper panel) and mature PR construct PR₁₋₉₅ (lower panel). Titration curves of (C) PR_{T26A} (8 μM) with 100 μM scFv and (D) PR₁₋₉₅ (9 μM) with 80 μM scFv. (E) Titration of scFv (22 μM) with 300 μM hexapeptide PQITLW corresponding to the N-terminus of PR in 1 $\times$ PBS. Equilibrium constants and thermodynamic parameters are listed in Table 2.

Figure 8.

Interaction of human mAb (PRM1) with mature PR and its precursor mimetics. (A) Western blotting of PRM1 binding. Lane identifications and notation are listed in Table 1. Proteins (500 ng/lane) were subjected to electrophoresis on 10–20% gradient Tris-Tricine gels, transferred to nitrocellulose membrane and probed with PRM1 as described in Experimental Procedures. (B) Lack of inhibition of autoprocessing of TFR-PR-RT²⁰ by PRM1. Samples were withdrawn and quenched with SDS-PAGE sample buffer at the indicated times (in h), subjected to electrophoresis on 10–20% gradient Tris-Tricine gels and stained as described in Experimental Procedures. (C) Lack of inhibition of mature PR. The antibody was added

to the folded enzyme in 50 mM sodium acetate buffer, pH 6, containing 250 mM NaCl and the enzyme was assayed after incubation at room temperature for 30 min.

Table 1

Binding of mAbs to various precursor and mature protease constructs of HIV protease

Construct number ^a	Designation and form	Binding to mAb	
		1696 (mouse)	PRM1 (human)
1	TFR-PR-RT ²⁰ (precursor)	– (X)	+
2	TFR-PR ^{PISP} (precursor)	– (X)	+
3	TFR-PR _{K43E} (precursor mutant)	– (X)	– (X)
4	PR (mature, pseudo WT)	+	+
5	TFR-PR _{D25N} (precursor mutant)	– (X)	+
6	PR _{T26A} (mature mutant)	+	+
7	TFR-PR _{1–95} (precursor mutant)	– (X)	+
8	PR _{1–95} (mature mutant)	+	+
9	⁺⁸ PR (precursor)	– (X)	+
10	SFNFPR (precursor)	– (X)	+
11	PR _{D25N/T26A} (mature mutant)	+	+
12	PR _{M-20} (mature mutant)	+	– (X)
13	PR _N (mature natural variant)	+	– (X)
14	PR _{P1A} (mature mutant)	+	+
15	PR _{M-11} (mature mutant)	+	– (X)
16	PR _{M-14} (mature mutant)	+	+(x)
17	PR _{M-P51} (mature mutant)	+	+(x)

+, – (or capital X) and small x denote binding, no binding and very weak binding, respectively.

^a protein designations correspond to the same numbering in this table and in the western blots (Fig. 6 and 8).

Table 2

ITC of scFv with (A) peptides corresponding to the N terminus of PR in 1X PBS, pH 7.4 and (B) folded PR monomers using scFv as titrant in 25 mM sodium phosphate, 0.15 M NaCl, pH 5.8

(A)				
Titant (cal/mol/deg)	K_{assoc} (M^{-1})	K_{Ab} (nM)	ΔH (kcal/mol)	ΔS
PQITLW	$(3.3 \pm 0.1) \times 10^7$	30	-13.1 ± 0.1	-9.12
QITLW	$(1.59 \pm 0.09) \times 10^6$	630	-9.96 ± 0.13	-4.72
AcPQITLW	$(8.21 \pm 0.66) \times 10^4$	~12,200	-14.7 ± 8.0	-26
FPQITLW	$(2.53 \pm 0.21) \times 10^4$	~39,000	$-(31.4 \pm 1.8)$	-84.4
(B)				
PR monomer (cal/mol/deg)	K_{assoc} (M^{-1})	K_{Ab} (nM)	ΔH (kcal/mol)	ΔS
PR _{T26A}	$(8.56 \pm 2.69) \times 10^6$	116	-5.42 ± 0.16	13.7
PR ₁₋₉₅	$(1.39 \pm 0.20) \times 10^7$	72	$-(5.58 \pm 0.06)$	14.2