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HIV-1 RT Enhances the Activity of a Tethered Dimer of HIV-1 Proteinase

Laura Goobar-Larsson,*,[1] Per Tomas Larsson,† Christine Debouck,‡ and Eric M. Towler‡

Microbiology and Tumorbiology Center, MTC, Karolinska Institute, S-171 77 Stockholm, Sweden; †STFI, Box 5604, S-114 86 Stockholm, Sweden; and ‡SmithKline Beecham Pharmaceuticals, Department of Molecular Genetics, King of Prussia, Pennsylvania 19406

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HIV-1 reverse transcriptase (RT) can specifically enhance HIV-1 proteinase activity *in vitro* and in eukaryotic cells (1). To determine if the effect of RT on proteinase activity was due to changes in the equilibrium dimerization constant of the proteinase or the stability of the proteinase dimer, we studied the effect of RT on a genetically engineered covalent dimer (tethered dimer) of the proteinase. RT was found to increase the activity of the tethered dimer independent of pH and ionic strength. The effect of RT on the kinetic constants (K_M and k_{cat}) of the wild type HIV-1 proteinase and its tethered dimer were also determined. These results show that RT can increase the enzymatic catalytic efficiency and substrate affinity of the proteinase, by a mechanism independent of promoting dimer formation. © 1996 Academic Press, Inc.

HIV-1 proteinase is a homodimeric enzyme (2–6), essential for the life cycle of the virus (7–9). The proteinase is expressed as an inactive monomer as part of the Gag-Pol polyprotein together with, among other proteins, the reverse transcriptase (RT) (10–13). Its function is to cleave the virus precursor polyproteins (Gag and Gag-Pol) into functional proteins (13–17). In order to process the precursor polyproteins the proteinase must first be activated through dimerization.

We have recently shown that RT can specifically enhance proteinase activity in a dose dependent manner (1), and that maximal RT effect is reached at equimolar concentrations of RT and proteinase. This effect was shown for all natural cleavage sites of the proteinase both *in vitro* and in eukaryotic cells.

In this work we wanted to determined the mechanism of enhancement of proteinase activity by RT. For this purpose the k_{cat} and K_M of the proteinase were determined in the presence and absence of RT. To determine if the effect of RT on proteinase activity was due to stabilisation of the proteinase dimer, or a change in the dimerization constant of the enzyme, we studied the effect of RT on the kinetics of a genetically engineered tethered dimer of the proteinase.

EXPERIMENTAL PROCEDURES

Recombinant wild type HIV-1 proteinase, HIV-1 proteinase tethered dimer, consisting of two copies of the monomeric coding sequence of the proteinase genetically linked in frame by the amino acids GGSSG (18), and HIV-1 RT, kindly provided by Dr. T. Unge, were expressed and purified to homogeneity as previously described (14,19,20). Peptide substrate SQNYPIVQ-NH_2 was purchased from Skandinavian Peptide Synthesis AB, Köping, Sweden.

Proteinase activity of the wild type enzyme and the tethered dimer, was measured in 50 mM Na-acetate buffer pH 5.5, 10% glycerol, 1 mM DTT, 1 mM EDTA and 185 mM or 1 M NaCl as indicated. For measurements of proteinase activity at neutral pH a buffer containing 75 mM sodium phosphate pH 7.0, 10% glycerol, 1 mM EDTA, and 1 mM DTT was used.

Proteinase (wild type or tethered dimer), was preincubated in 30 μl for 6 min at 37°C with or without RT prior to addition of 20 μl of various concentrations of the substrate SQNYPIVQ-NH_2. Samples were then incubated for 15 min at 37°C and reactions were stopped by the addition of 50 μl of 0.2% trifluoroacetic acid and heating to 100°C for 5 min. Reaction products were analysed and quantified by reversed phase HPLC using a 15 min gradient of 0 to 30% acetonitrile in water as previously described (20).

Wild type proteinase concentrations ranged from 10 to 25 nM for experiments run at pH 5.5, 1 M NaCl, and from 30 to 100 nM for experiments run at pH 5.5, 185 mM NaCl, or at pH 7.0. The concentrations of the tethered dimer ranged between

[1] Corresponding author. Fax: +46-8-331399; E-mail: Laura.Goobar-Larsson@mtc.ki.se.

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50–90 nM for both high and low salt concentration and pH 5.5. RT concentration was 250 nM. Measurements of proteinase activity in the presence and absence of RT where always run in parallel and with the same proteinase concentration.

Final substrate concentrations in the reaction mixtures ranged from 0.5 mM to approximately 12 mM, which is the highest concentration possible due to limited solubility of the peptide. Substrate cleavage did not exceed 20%. A linear dependence in proteinase activity was observed up to 50% cleavage of this substrate peptide (data not shown). Reactions were run for 15 min, but proteinase activity was linear for at least 1 hr after the addition of substrate.

Curves of activity (V) vs. substrate concentration (S) were fitted to the classical Michaelis-Menten kinetic model by non-linear least squares regression using the computer program Microsoft Excel.

RESULTS AND DISCUSSION

In a previous work we found that HIV-1 RT increases wild type HIV-1 proteinase activity at high and low salt concentration, and at low or neutral pH (1).

The enhancement of proteinase activity by RT could be an effect of an increased catalytic efficiency of the proteinase in the presence of RT, and/or an increase in the affinity of the enzyme for its substrate. Alternatively, RT could have an effect on proteinase dimer formation and/or stability. To elucidate which mechanism(s) were responsible for RT's enhancement of proteinase activity two questions were asked: 1) How does RT affect the kinetic constants k_{cat} and K_M of the proteinase, 2) Can RT enhance the activity of a tethered dimer of the proteinase.

At high salt concentration (1 M NaCl, pH 5.5), RT was found to both increase the k_{cat} ($p<0.0001$) and decrease the K_M ($p = 0.009$) of the wild type proteinase for the peptide substrate used. k_{cat}/K_M was approximately twice that found in the absence of RT (Fig. 1, Table 1).

At low salt concentration and low pH (185 mM NaCl, pH 5.5), or at pH 7.0 (75 mM sodium phosphate), curves of wild type proteinase activity vs. substrate in the presence and absence of RT could not be fitted to the Michaelis-Menten model (data not shown). A possible explanation for these results is that preincubation of a diluted solution of the proteinase at 37°C and at low ionic strength, leads to dissociation of proteinase dimers. Subsequent addition of substrate could lead to a reassociation of monomers into dimers. In other words, the concentration of active proteinase (dimers) would not be constant over the substrate concentration range used. The equilibrium dissociation constant of the proteinase has been shown to increase with decreasing ionic strength (21,22), and substrates and inhibitors have been proposed to induce dimerization of the proteinase *in vitro*, when added to a diluted proteinase solution that has been preincubated at 37°C at low ionic strength (23,24).

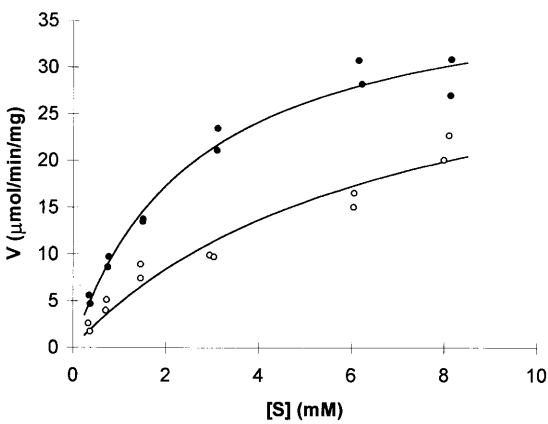

FIG. 1. Substrate concentration dependent activity of wild type HIV-1 proteinase at 1M NaCl, pH 5.5. Activity was measured as described in "Experimental Procedures". Proteinase activity was measured in the absence (○) or presence (●) of 250 nM HIV-1 RT. Proteinase concentration was 15.3 nM. Lines represent the non-linear least-squares fit of the Michaelis-Menten equation to the experimental data. The data is from one of six independent experiments showing similar results.

TABLE 1
Kinetic Parameters for Wild-Type and Tethered Dimer Proteinase in the Presence and Absence of HIV-1 RT

Proteinase	RT[a]	[NaCl] (M)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}s^{-1}$)	N[b]	P[c]
Wild Type	−	1.0	3.9 (0.7)	13.1 (0.7)	3.4 (0.7)	8	
Wild Type	+	1.0	2.8 (0.6)	16.7 (0.8)	6.0 (1.4)	6	0.0004
Tethered Dimer	−	1.0	2.0 (0.2)	5.8 (0.1)	2.9 (0.3)	3	
Tethered Dimer	+	1.0	1.7 (0.2)	9.9 (0.3)	5.8 (0.6)	3	0.0013
Tethered Dimer	−	0.185	9.4 (1.1)	10.3 (0.8)	1.1 (0.2)	3	
Tethered Dimer	+	0.185	6.2 (0.4)	17.0 (0.6)	2.7 (0.2)	5	<0.0001

K_M, k_{cat} and k_{cat}/K_M values are given as the mean of N independent determinations. Values in parantheses are the standard error of mean.

[a] + with RT, − without RT.

[b] N = number of independent determinations.

[c] P = level of significance for the difference in k_{cat}/K_M with and without RT.

The fact that RT increased wild type proteinase activity even at high ionic strength, and that the effect of RT and salt where roughly additive (1), suggested that the effect of RT and salt were independent of each other, and that RT was not primarily affecting proteinase dimer formation.

To test this hypothesis we studied the effect of RT on a tethered dimer of the proteinase (18). If RT only affected the dimerization of the proteinase it would not affect the activity of a tethered dimer. RT was found to enhance the activity of the tethered dimer at high and low concentrations of NaCl and pH 5.5 (Fig. 2, Table 1). At high ionic strength (1 M NaCl), the effect of RT on the k_{cat} of the tethered dimer was even higher than that on the wild type enzyme ($p<0.0001$), (Table 1), though K_M was unaltered in the presence of RT under these conditions (Table 1).

At low ionic strength (185 mM NaCl), RT significantly increased the k_{cat} of the tethered dimer ($p<0.0001$) and decreased its K_M ($p = 0.001$) (Table 1).

Although k_{cat} and K_M for the wild type proteinase at low ionic strength and pH 5.5 could not be accurately determined due to non Michaelis-Menten behavior, the effect of RT on the activity of

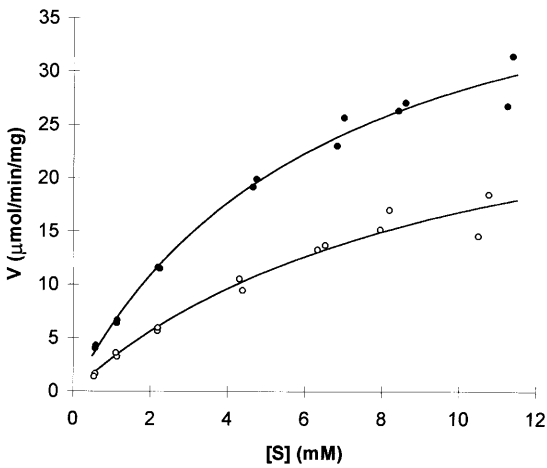

FIG. 2. Substrate concentration dependent activity of the tethered dimer of HIV-1 proteinase at 185 mM NaCl, pH 5.5. Activity was measured as described in "Experimental Procedures". Proteinase activity was measured in the absence (○) or presence (●) of 250 nM HIV-1 RT. The concentration of tethered dimer was 63 nM. Lines represent the non-linear least-squares fit of the Michaelis-Menten equation to the experimental data. The data are from one of three independent experiments showing similar results.

the wild type proteinase and the tethered dimer under these experimental conditions were comparable, the effect of RT on the wild type being slightly higher (data not shown).

Interestingly, the k_{cat} of the tethered dimer was lower at high salt than at low salt (Table 1). This is in contrast to what has been reported for the wild type proteinase, where k_{cat} was found to be independent of the ionic strength (25,26).

The K_M of the tethered dimer at high ionic strength was significantly lower than that at low ionic strength (Table 1), in agreement with what has been previously shown for the wild type enzyme both for similar and different substrates (25,26), and for the same tethered dimer with a different substrate (27). At pH 7.0 RT was also found to increase the activity of the tethered dimer (data not shown), but saturation curves could not be obtained due to the low affinity of the dimer for the substrate under these conditions, and the limited solubility of the substrate. The increase in K_M with increasing pH observed for the tethered dimer, has been also reported for the wild type enzyme (26,28), and for other tethered dimers of HIV-1 proteinase (29,30).

In summary, we have shown that: 1) RT can increase the k_{cat} and decrease the K_M of wild type proteinase under conditions where the proteinase obeys normal Michaelis-Menten kinetics (high ionic strength and low pH). 2) RT enhances the activity of a tethered proteinase dimer by increasing its k_{cat} (high and low ionic strength) and decreasing its K_M (low ionic strength). Since the effect of RT on k_{cat}/K_M for the wild type proteinase and the tethered dimer were approximately the same (Table 1), we can conclude that the main effect of RT on proteinase activity is that of increasing the catalytic efficiency of the proteinase dimer and its affinity for the substrate. The effect of RT on proteinase dimer stability and/or formation, if any, is marginal. These results indicate that *in vivo*, RT may regulate the activity of the proteinase dimer, rather than contribute to proteinase activation by promoting dimer formation.

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