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Mutational analysis of two conserved sequence motifs in HIV-1 reverse transcriptase

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Two conserved sequence motifs, occurring in HIV-1 reverse transcriptase at residues 110–116 and 183–190, have been studied using site-directed mutagenesis of the cloned gene. In particular, aspartates at positions 185 and 186 have each been mutated to either asparagine or glutamate. The resulting mutant proteins were catalytically inactive but still able to bind the template-primer complex, poly rA-oligo dT. Other mutations in these regions resulted in reduced reverse transcriptase activity but the mutation of tyrosine-183 to serine caused a significant increase in the K_m for dTTP and the K_i for inhibition by 3'-azidothymidine-triphosphate, 2',3'-dideoxythymidine-triphosphate and phosphonoformic acid.

Reverse transcriptase (HIV-1); Site-directed mutagenesis; Conserved sequence motif; Enzyme inhibition

1. INTRODUCTION

Several reports detailing amino acid sequence comparisons between polymerases highlight a conserved sequence motif containing an Asp-Asp doublet flanked by hydrophobic residues [1–7]. This Asp-Asp motif is observed in many RNA-dependent RNA- and DNA-polymerases [4,6], and some DNA-dependent RNA- and DNA-polymerases [5,7]. Additionally, another conserved region has been identified which also features an invariant Asp [2,3,6,7]. The strict conservation of these acidic residues suggests an important role in polymerase function.

The reverse transcriptase (EC 2.7.7.49) from HIV-1 is responsible for converting the RNA genome of the virus into a double-stranded DNA copy. The Asp-Asp motif has been identified at residues 185 and 186 and the third invariant Asp is at residue 110 (Fig. 1). In a previous site-specific mutagenesis study of the HIV-1 RT [8], Larder et al. showed that mutation of either Asp^{110} or Asp^{185} abolished reverse transcriptase activity. In order to study the role of the Asp-Asp motif, we have mutated each Asp to the residues Asn or Glu such as to introduce minimum disruption. These, and other mutants with mutations in these conserved regions (denoted B and E in Fig. 1 as in the study by Larder et al. [8]), were analyzed in detail. The effect on K_m for the substrate, dTTP, and K_i for the inhibitors AZT-TP, ddTTP and PFA are presented. The results confirm that only the conserved aspartate residues in these regions are essential for polymerase activity but they are not essential for template-primer binding, suggesting a catalytic or substrate binding role in the polymerization process.

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Abbreviations: RT, reverse transcriptase; AZT-TP, 3'-azidothymidine triphosphate; ddTTP, 2',3'-dideoxythymidine triphosphate

2. MATERIALS AND METHODS

2.1. *Mutant construction*

Mutants were prepared by site-directed mutagenesis of the coding sequence for HIV-1 RT cloned in the M13 expression vector, mptac 18 [8,9]. Selection was achieved in some cases using the mutagenesis methods of Kunkel [10] or Eckstein [11]. For high-level expression of some of the mutant proteins, the mutated gene was sub-cloned into a derivative of the plasmid pKK233-2 [9] in which the kanamycin resistance gene had been inserted for increased plasmid stability (P.Ertl, unpublished).

2.2. *Purification of HIV-1 reverse transcriptase*

Wild-type and mutant proteins were expressed in *E. coli* and cell-free extracts prepared as described by Larder et al. [9]. The preparation was passed through a Q-Sepharose column (10 × 1.5 cm) (Pharmacia) equilibrated in 50 mM-diethylamine-HCl buffer (pH 8.8), 100 mM-NaCl, 10% (v/v) glycerol, under which conditions the RT is not retained by the column and is collected in the flow-through. This material was then loaded directly onto a RTMAb 8 immunoabsorbent column [12] equilibrated in the same buffer. After washing the column with diethylamine buffer, the RT was eluted by 1 M NaI dissolved in diethylamine buffer (pH 8.8). The NaI was subsequently removed by extensive dialysis in 50 mM Tris, pH 7.5, 0.2 mM dithiothreitol and the purified protein was stored frozen at −70°C.

2.3. *Assay for reverse transcriptase activity*

Assays were performed exactly as described by Lowe et al. [13], following the incorporation of [^{3}H]dTTP (2 Ci/mmol) into DNA using poly rA-oligo dT (18-mer) as template-primer. Kinetic data were analyzed by non-linear least-squares methods. PFA was obtained from Dr. B. Oberg, AZT-TP from Dr. P.A. Furman and ddTTP from Boehringer Mannheim Biochemicals.

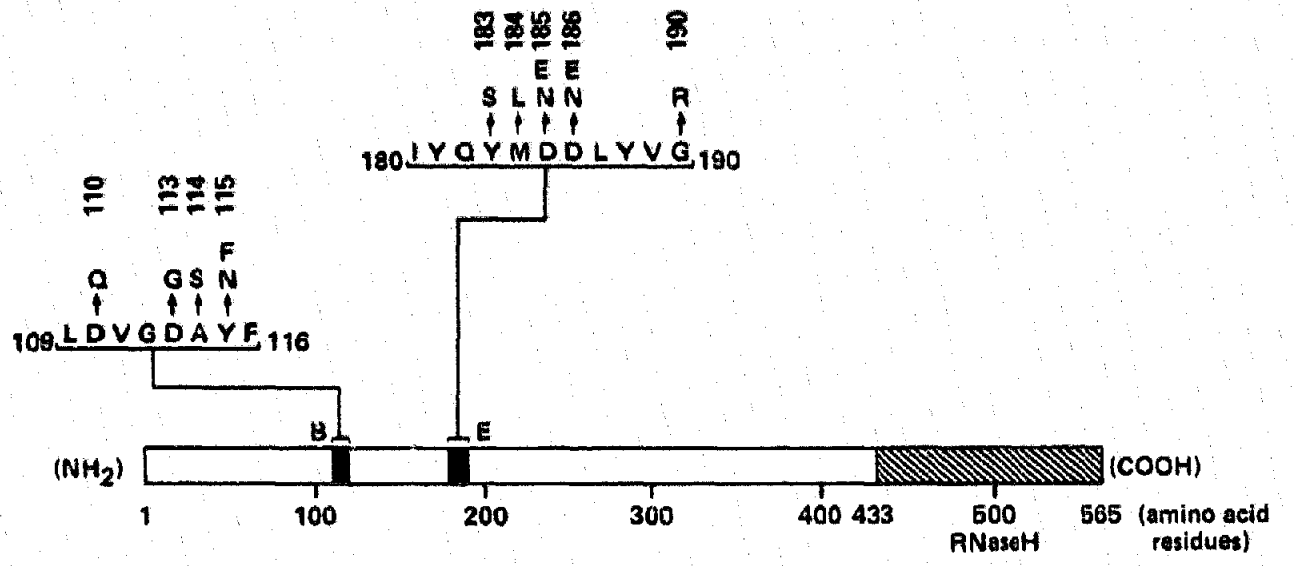

Fig. 1. Position of two conserved regions in HIV-1 RT with indication where single amino-acid substitutions have been made. Adapted from Larder et al. [8].

A competition assay was performed using aliquots of catalytically inactive RT mutants (10-100 nM protein) added to a RT assay containing 5 μM [^{3}H]dTTP, 10 nM poly rA-oligo dT and approximately 10 nM wild-type RT protein. At this limiting concentration of poly rA-oligo dT, binding of poly rA-oligo dT by a mutant protein results in inhibition of the wild-type RT activity.

2.4. Preparation of poly [^{3}H]rA-oligo dT

Poly [8-^{3}H]adenylic acid, Na salt (Amersham International plc) (range 38-137 nucleotides; average spec. act. 50 Ci/mmol) was dissolved in water and mixed with synthetic oligo dT (18-mer) in approximately equimolar amounts. After 5 min at 42°C, the mixture was annealed by cooling slowly to room temperature.

2.5. Binding of poly [^{3}H]rA-oligo dT to RT

RT (approx. 50 nM protein) was incubated at 37°C with poly [^{3}H]rA-oligo dT, 5-100 nM, in 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, before filtering onto wetted nitrocellulose disks (Schleicher & Schuell, BA 85) and washing with 8 ml ice-cold 50 mM Tris, pH 7.5, 5 mM $MgCl_2$. The discs were air-dried before counting in liquid scintillant. In blank reactions containing no protein, boiled RT protein or bovine serum albumin, approximately 20% of the applied radioactivity remained bound to the filter. This amount was subtracted from the amount of radioactivity bound in the presence of RT protein. The binding of poly [^{3}H]rA-oligo dT to wild-type RT was not dependent on $MgCl_2$ and occurred even in the presence of 0.5 mM EDTA and no $MgCl_2$.

3. RESULTS AND DISCUSSION

The mutations that we have introduced are shown in Fig. 1 and the kinetic parameters determined for the purified mutant proteins are presented in Table I. Inhibition by AZT-TP and ddTTP was competitive with respect to dTTP, whereas PFA, a pyrophosphate

Table I

Kinetic and inhibitor constants determined for mutants of reverse transcriptase

Enzyme	RT activity (% wild-type)	K_m for dTTP (μM)	K_i for AZT-TP (μM)	K_i for ddTTP (μM)	K_i for PFA (c,u) (μM)	Binding of poly rA-oligo dT
Wild-type	100	5	0.013	0.05	0.2, 0.3	+
Asp→Gln^{110}	<0.01*	-	-	-	-	ND
Asp→Gly^{113}	59*	5	0.23	1.8	0.7, 0.0	ND
Ala→Ser^{114}	80*	11	0.04	0.09	0.7, 0.9	ND
Tyr→Asn^{115}	12	50	5.0	12.4	Non-linear	ND
Tyr→Phe^{115}	90	5	ND	ND	ND	ND
Tyr→Ser^{183}	1*	385	3.3	14.0	32.0, 92.0	ND
Met→Leu^{184}	8	7	ND	ND	ND	ND
Asp→Asn^{185}	<0.01	-	-	-	-	+
Asp→Glu^{185}	<0.01	-	-	-	-	+
Asp→Asn^{186}	<0.01	-	-	-	-	+
Asp→Glu^{186}	<0.01	-	-	-	-	+
Gly→Arg^{190}	23*	5	0.028	0.13	0.3, 2.4	ND

The standard deviations for these results were typically about 10%.
*From Larder et al. [8]. ND, Not done.

analogue, showed mixed inhibition with dTTP as the variable substrate.

In region B (Fig. 1), mutation of the conserved Asp at position 110 to Gln reduced reverse transcriptase activity to undetectable levels [8]. The other Asp in this region (Asp^{111}) is not highly conserved amongst polymerases outside the retrovirus group [6,7], and its mutation to Gly only has a limited effect on the level of enzyme activity while the K_m for dTTP is not significantly affected (Table I). The same is true for the neighbouring mutation of Ala^{114} to Ser (Table I). Both

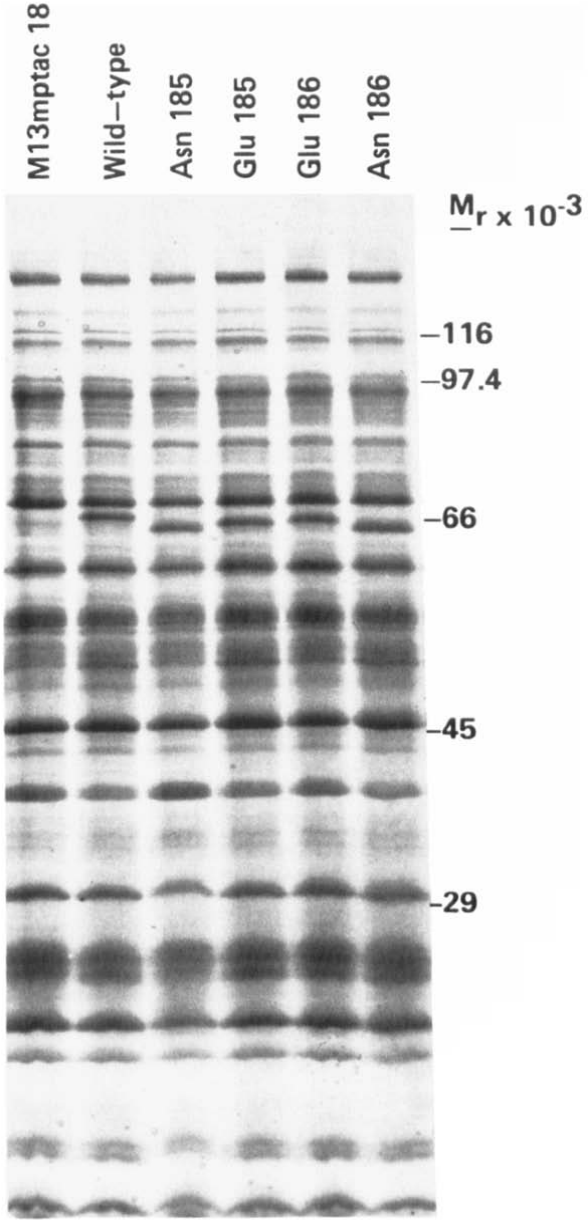

Fig. 2. SDS-polyacrylamide gel electrophoresis of *E. coli* extracts after infection with M13mptac 18 containing no insert or expressing the gene for wild-type or mutated RT proteins. The samples were analyzed on a 12.5% SDS-polyacrylamide gel (Tris-Glycine) and stained with Coomassie Brilliant blue.

these mutated enzymes show reduced sensitivity to the inhibitors tested, which is particularly marked for the inhibition of the RT mutant ($Asp \rightarrow Gly^{113}$) by AZT-TP and ddTTP. The Tyr at position 115 is moderately well conserved [6,7] and its mutation to Asn results in a 10-fold increase in the K_m for dTTP with a resulting drop in RT activity (Table I), suggesting a degree of disruption at the active site. This mutant also displayed raised K_i's for AZT-TP and ddTTP and the kinetics with PFA were non-linear, giving incomplete inhibition even at high PFA concentrations (only 60% inhibition was achieved at 1.5 mM PFA). However, when the more conservative mutation to Phe was made, the resulting protein had wild-type activity and K_m for dTTP. This is consistent with Phe appearing at this position in other RNA-dependent polymerases [6], and suggests that the hydrophobic ring structure is important at this position and not the presence of a hydrogen-bonding group.

The role of the Asp-Asp doublet in region E (Fig. 1) was probed by making the conservative changes $Asp \rightarrow Asn^{185}$, $Asp \rightarrow Glu^{185}$, $Asp \rightarrow Asn^{186}$ and $Asp \rightarrow Glu^{186}$. After induction of the mutant proteins, activity measurements made on crude extracts failed to detect any reverse transcriptase activity above background (Table I). Analysis of the extracts by SDS-polyacrylamide gel electrophoresis confirmed that proteins of M_r about 66000, equivalent to wild-type RT, were being expressed (Fig. 2). The mutant proteins were subsequently purified to near homogeneity (Materials and Methods), but still failed to show any significant activity in a standard reverse transcriptase assay. Their ability to bind template primer was tested using the nitrocellulose filter binding assay (see Materials and Methods) which gave titratable binding of poly rA-

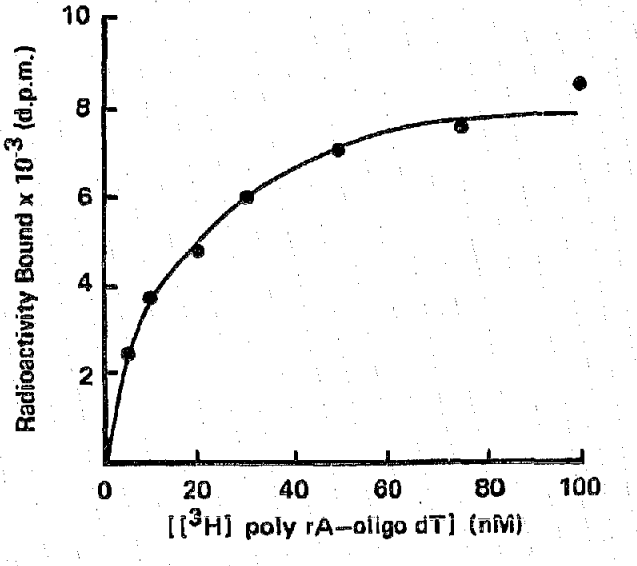

Fig. 3. Binding of poly [^{3}H]rA-oligo dT to mutant RT protein (Asp→Asn at residue 186).

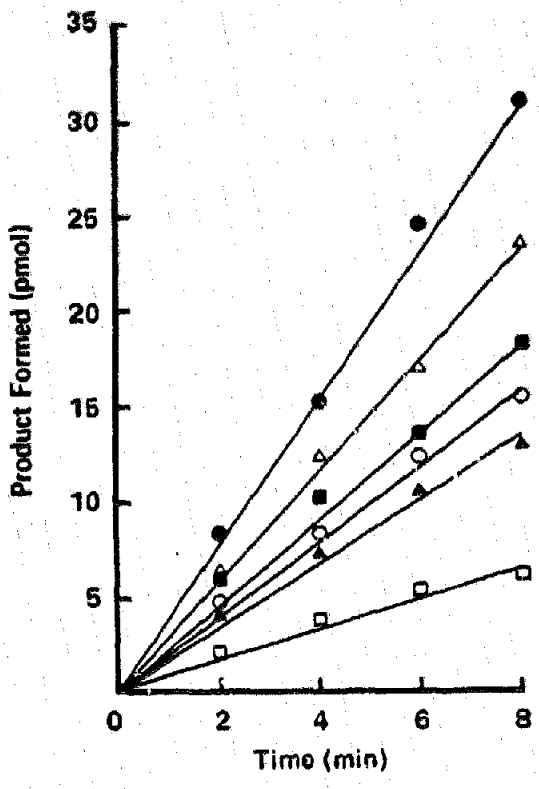

Fig. 4. Inhibition of wild-type RT activity at limiting template-primer concentration by the presence of RT protein with mutation of Asp→Glu at residue 186. Concentration of mutant RT protein (Asp→Glu 186) was as follows; ●, no protein; △, 30 nM; ■, 45 nM; ○, 52.5 nM; ▲, 60 nM; □, 90 nM.

oligo dT for each mutant (example in Fig. 3). Hence, although these mutants are not catalytically active reverse transcriptases, they still retain the ability to bind template-primer.

As a further test for template-primer binding, proteins with Asn^{185} and Glu^{186} mutations were found to behave as inhibitors in a competition assay with wild-type RT, run at limiting template-primer concentration (Materials and Methods) (example in Fig. 4).

Other mutations previously prepared in region E of RT include $Tyr \rightarrow Ser^{183}$, $Gly \rightarrow Arg^{190}$ [8] and $Met \rightarrow Leu^{184}$. None of these residues prove to be essential for catalytic activity but their mutation results in a significant reduction of detectable enzyme activity (Table I). In the case of the Ser^{183} mutant, this is largely explained by a 77-fold increase in the K_m for dTTP, and there is a corresponding increase in the K_i values for inhibition by AZT-TP, ddTTP and PFA, whereas the K_m and K_i values for RT mutants Leu^{184} and Arg^{190} are virtually unchanged relative to wild-type, suggesting an effect on V_{max}. All three mutants retain the same requirement for $MgCl_2$ as wild-type, with an optimum of about 10 mM.

These results confirm that an intact Asp–Asp doublet at positions 185–186 in HIV-1 RT is absolutely essential for reverse transcriptase activity, but that conservative mutations of these residues do not prevent RNA–DNA binding. This would imply that the structural integrity of the mutant RT proteins is not seriously affected, which is reinforced by the stability of these mutant proteins on purification. (In contrast, some mutants, in particular those with 6–13 amino acid deletions, where the protein structure was likely to be disrupted, tended to degrade during purification (D. Lowe, unpublished)).

Mutation of surrounding residues in region E and also in region B did not indicate any further absolutely essential residues, apart from the previously described Asp^{110} [8]. The most significant kinetic effect resulted from mutation of $Tyr \rightarrow Ser^{183}$, where the large increase in K_m for dTTP suggests a functional role in catalysis. This could involve pyrophosphate exchange, since the binding of PFA is reduced in line with that of the substrate analogues, AZT-TP and ddTTP.

Thus, the structure of the active site is likely to bring the three Asp's at positions 110, 185 and 186 into a spatial arrangement whereby they can perform a catalytic role or, possibly, bind Mg^{2+} which, in turn, acts catalytically or in the binding of phosphates. Various structure predictions, based on the known tertiary structure of the Klenow fragment of Pol I [14], have placed these Asp's at the bottom of a cleft [7,15], but their precise location must await the solving of the X-ray crystallographic structure for HIV-1 reverse transcriptase.

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