Site-directed mutagenesis of the conserved Asp-443 and Asp-498 carboxy-terminal residues of HIV-1 reverse transcriptase

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ABSTRACT

Substitution of the conserved Asp-443 residue of HIV-1 reverse transcriptase by asparagine specifically suppressed the ribonuclease H activity of the enzyme without affecting the reverse transcriptase activity, suggesting involvement of this ionizable residue at the ribonuclease H active site. An analogous asparagine substitution of the Asp-498 residue yielded an unstable enzyme that was difficult to enzymatically characterize. However, the instability caused by the Asn-498 mutation was relieved by the introduction of a second distal Asn-443 substitution, yielding an enzyme with wild type reverse transcriptase activity, but lacking ribonuclease H activity.

INTRODUCTION

The ribonuclease H (RNase H) activity associated with a retroviral reverse transcriptase (RT) is responsible for catalyzing several key ribonucleolytic reactions in the course of replication of the genomic single-stranded RNA to a double-stranded DNA form (1–3). Proviral mutants of Moloney murine leukemia virus (M-MuLV) bearing defective RNase H activities have been shown to prevent the production of infectious viral particles, highlighting the essential role of this activity in the replication cycle of the virus (4). The RT-associated RNase H activity of human immunodeficiency virus type I (HIV-1), the etiologic agent of acquired immune deficiency syndrome (AIDS), has been the subject of several recent studies primarily aimed at facilitating the search for novel therapeutic strategies against AIDS, based upon inhibition of the viral RNase H activity (5–9). Further development of this therapeutic approach requires a more detailed knowledge of the structure-function relationships within the RT/RNase H molecule, and identification of the key amino acid residues within the RNase H active site.

On the basis of extensive amino acid homology between the COOH-terminal 160 amino acids of M-MuLV RT and *E. coli* RNase H, the RNase H activity of M-MuLV RT was proposed to reside in this portion of the molecule (10). This proposal, in conjunction with the separateness of the RT and RNase H active sites, was subsequently confirmed by the work of Tanese and Goff (11), Kotewicz et al. (12), and Repaske et al. (4). By analogy, the RNase H domain of HIV-1 RT was proposed to reside in the COOH-terminal domain of the molecule (10), the 120 amino acids of which are present in the p66, but absent in the p51 polypeptide (13). The linker insertion mutagenesis data of Prasad and Goff (14) were partially consistent with this view. Unambiguous confirmation of this hypothesis was recently obtained by Schatz et al. whose site-directed mutagenesis studies led to the specific repression of the RNase H activity, and implication of the Glu-478 as a critical residue in the RNase H active site (15). In addition, the mutagenesis data of Kanaya et al. of selected amino acid residues of *E. coli* RNase H indicated that the three conserved ionizable residues Asp-10, Glu-48 and Asp-70 (the homologues of HIV-1 RT residues Asp-443, Glu-478, and Asp-498 respectively) were all implicated at the active site of the enzyme, since substitution by the non-ionizable amide analogues Asn and Gln respectively, resulted in complete loss of RNase H activity (16).

In this paper, we report on the use of site-directed mutagenesis to probe the structural and/or functional importance of the highly conserved Asp-443 and Asp-498 residues of HIV-1 RT.

The results presented herein suggest that Asp-443 plays an important catalytic role in the mechanism of RNase H cleavage of RNA.DNA hybrid substrates, and thereby assist in further defining the active site of this key viral activity. Moreover, the Asp-498 residue appears to have an important function in directing correct folding of the heterodimeric RT enzyme.

MATERIALS AND METHODS

Bacteria and Plasmids

Recombinant HIV-1 RT was expressed in *E. coli* AR120/ (pRT1 + pDPTPRO4), as previously described (13). The *E. coli* strain CJ236 (*dut*$^-$, *ung*$^-$) used for oligonucleotide-directed mutagenesis was kindly provided by Dr. T. Kunkel. The M13 subclone M13-RT carrying the coding region of HIV-1 RT was

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prepared by cloning the 2.43-kb *Bam*HI/ *Xba*I fragment of pRT1 (13), in which the RT coding region was located between nucleotide positions 196 and 1875 of the *Bam*HI/ *Xba*I fragment, in M13mp11. The transcription vector pGEM-ΔGAG-C, used for the preparation of (+)-GAG^{345} RNA by run-off transcription, was previously described (8).

Materials

T4 DNA polymerase, T4 polynucleotide kinase, and restriction enzymes were from Boehringer-Mannheim, T7 RNA polymerase, *E. coli* RNase H and RNase inhibitor were from Promega. NTPs and dNTPs were from Sigma, [α-^{32}P]UTP (600 Ci/ mmol), [α-^{35}S]dATP S (800 Ci/ mmol), and [γ-^{32}P]ATP (>3,000 Ci/ mmol) were from Amersham. The mutagenic oligonucleotides D443N (5'-TTCTATGTAAATGG-GGCAG-3'), and D498N (5'-ATAGTAACAAACTCACAA-T-3'), the DNA sequencing primer SP (5'-ATTCCTGAGTG-GGAGTT-3'; nucleotide positions 1426−1442 of the *Bam*HI/ *Xba*I fragment), and the hybrid substrate oligonucleotide component P1 (5'-GGTCTACATAGTCTCTAAAA-3'; ref. 8) were synthesized using a Beckman System 1 Plus DNA synthesizer, and were HPLC-purified prior to use. Other reagents were as previously described (7,8,13).

Oligonucleotide-directed mutagenesis

The D443N and D498N single mutations, and the D443N/ D498N double mutation were introduced in the Asp codons 443 and 498 of the RT coding region in M13-RT according to the mutagenesis procedure of Kunkel et al. (17). Mutations were confirmed by DNA sequencing (18) using the primers SP, D443N and D498N to sequence the region of M13-RT between nucleotides 1468 and 1835 of the *Bam*HI/ *Xba*I insert, which flank the mutated Asp-443 (nucleotides 1522−1524) and Asp-498 codons (nucleotides 1687−1689). The mutagenized portion of M13-RT was excised as a 328-bp *Kpn*I fragment [the *Kpn*I sites were located at nucleotide positions 1476 and 1804 respectively]. The mutagenized *Kpn*I fragment was re-cloned in a wild type M13-RT vector from which the original wild type 328-bp *Kpn*I fragment had been deleted. Recombinant phages were screened by restriction analysis and by oligonucleotide hybridization using 5'-end-labeled D443N and/or D498N to identify a clone bearing a single copy of the mutagenized *Kpn*I-fragment inserted in an anticlockwise orientation (M13-RT^{M}). The nucleotide sequence of M13-RT^{M} flanking the two *Kpn*I sites was re-confirmed by DNA sequencing. This additional cloning step obviated the need to sequence the entire RT coding region to check for second-site mutations elsewhere in the RT coding region. The mutagenized expression vectors were constructed by ligating the 2.43-kb *Bam*HI/ *Xba*I fragment of M13-RT^{M} to the 5.82-kb *Bam*HI/ *Xba*I fragment of wild type pRT1 to create the 8.25-kb pRT1^{M} vector, which was used to transform AR120/ pDPTPRO4 to ampicillin resistance. Following the induction of AR120/ (pRT1^{M} + pDPTPRO4), total plasmid was isolated from the induced cells. The 2.43-kb *Bam*HI/ *Xba*I fragment of pRT1^{M} was isolated from the plasmid preparation and was cloned into M13mp11. Recombinant phages were again sequenced through the 328-bp *Kpn*I region to re-confirm the mutations.

RT Assay of bacterial extracts

Polyethylene glycol precipitation supernatants from 7-mL nalidixic acid-induced cultures were prepared as previously described (13), and 2.5 μL aliquots of 10−500-fold dilutions were assayed for RT activity as previously described (13). Uninduced cultures of equivalent cell density were lysed and assayed in parallel to correct for the RT level contributed by *E. coli* DNA polymerase I, which ranged from 3% (D443N/ D498N) to 23% (D498N) of the total RT activity in the lysate.

Enzyme purification

The wild type and mutant enzymes were purified from 5 g of induced cells by tracking RT activity, according to the purification procedure of (13), with the modifications described in (8). The D443N and D443N/ D498N mutant enzymes were purified to homogeneity in similar overall yield to the wild type enzyme (13). In contrast, the expression level and the stability during purification of D498N was significantly lower than the wild type enzyme, preventing purification of this mutant protein.

RNase H Assay

The RNase H activities of the enzymes were assayed by denaturing gel electrophoretic analysis of the cleavage products of the RNA.oligonucleotide hybrid (+)-GAG^{345}/P1, as previously described for the wild type enzyme (8). Assay mixtures (15 μL) containing 15 nM uniformly ^{32}P-labeled hybrid (prepared as described in (8) using a 1:16 ratio of (+)-GAG^{345}:P1), 58 mM Tris. HCl (pH 7.9), 58 mM KCl, 8.7 mM $MgCl_2$, 5 mM DTT and either 5−20 nM wild type RT, 5−160 nM D443N RT, 5−160 nM D443N/ D498N RT, or 0.02−0.1 nM *E. coli* RNase H were incubated at 37°C. Aliquots (3 μL) were withdrawn after 0−30 min and were quenched with 6 μL of sample loading buffer. Quenched samples (3 μL) were electrophoretically analyzed as previously described (7,8).

RESULTS

Effects of the Mutations on RT Activity

The positions of the mutations introduced into the COOH-terminal domain of HIV-1 RT are indicated in Fig. 1. The effects of the mutations on the RT expression level and specific activity are illustrated in Fig. 2. Whereas the D443N single mutation affected neither the RT expression level nor the RT specific activity, the D498N single mutation resulted in a 4-fold decrease in the overall expression level. Moreover, the latter mutant protein underwent rapid RT activity loss during purification, and all attempts to purify it to homogeneity were unsuccessful. In contrast, the expression level of the D443N/ D498N double mutant was 2-fold higher than the wild type and D443N mutant, and approximately 8-fold higher than the D498N mutant enzyme. The RT specific

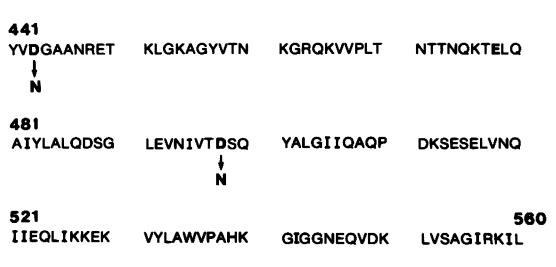

Figure 1. Amino acid sequence of the COOH-terminal 120 amino acids of the p66 subunit of HIV-1 RT. The positions of the Glu-478 residue previously identified as being essential to the RNase H activity (15), and the mutated Asp residues are shown in bold face.

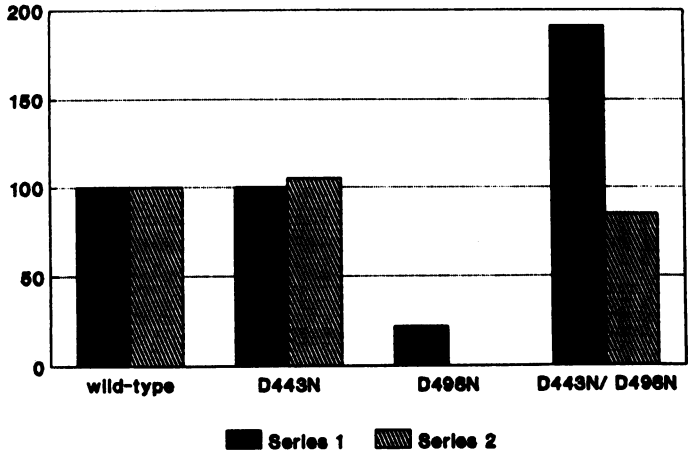

Figure 2. Effects of the mutations at aspartic acids 443 and 498 on the RT expression level (series 1), and on the RT specific activity (series 2). Percentage RT activity is shown on the ordinate. RT expression levels of crude lysates were measured as described under 'Materials and Methods'. Comparative RT specific activities were measured for the purified proteins.

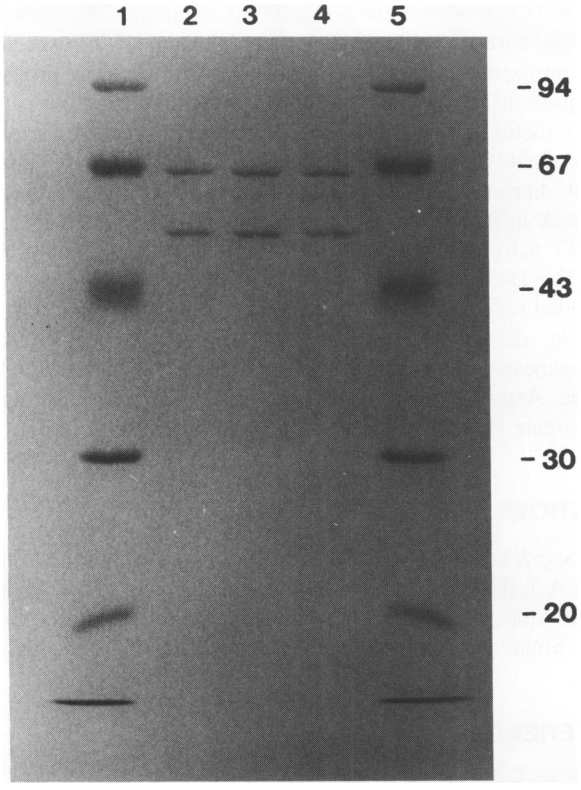

Figure 3. SDS-polyacrylamide gel analysis of the purified enzymes. Lanes 1 and 5: molecular weight markers; lanes 2–5: Sephadex G-75 fractions of wild type (lane 2), D443N (lane 3) and D443N/ D498N (lane 4) HIV-1 RT. The enzymes were purified according to the procedure of (13), with the modifications of (8). The marker sizes are indicated in kilodaltons.

activity of the purified D443N/ D498N double mutant was similar to that of the wild type and D443N mutant enzymes (±20%). The D443N and D443N/ D498N mutant enzymes were purified to homogeneity according to the procedure developed for the wild type enzyme (8, 13). As illustrated in Fig. 3, both proteins were

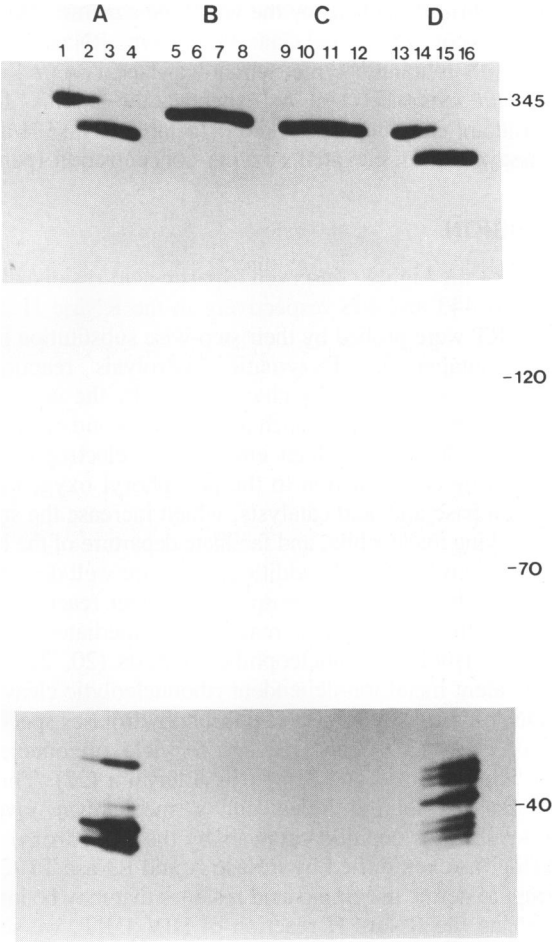

Figure 4. Comparative RNase H activities of wild type and mutant enzymes. Denaturing gel electrophoretic analysis of the RNA cleavage products of the hybrid (+)-GAG^{345}/P1 (8) formed by wild type (panel A), D443N (panel B), D443N/ D498N (panel C) HIV-1 RT, and *E. coli* RNase H (panel D) cleavage. Reactions (as described under 'Materials and Methods') were initiated with either 9 nM wild type RT (panel A), 90 nM D443N RT (panel B), 90 nM D443N/ D498N RT (panel C), or 0.03 nM *E. coli* RNase H (panel D), and were incubated at 37°C for 0 (lanes 1, 5, 9, and 13), 2 (lanes 2, 6, 10, and 14), 10 (lanes 3, 7, 11 and 15), and 30 min (lanes 4, 8, 12 and 16). Samples were quenched and electrophoresed on a 20 cm×40 cm×0.4 mm 8% polyacrylamide gel containing 7 M urea, as previously described (7,8). The approximate lengths of the oligonucleotide products are indicated. For the sake of clarity, the small oligonucleotide products (<50 nt) in panels A–D were visualized by exposure for 40 h compared to 4 h for the larger oligonucleotides.

isolated as an equimolar p66/p51 polypeptide mixture (lanes 3 and 4), indistinguishable from the purified wild type (lane 2).

Effects of the Mutations on RNase H Activity

The time courses for RNase H cleavage of the (+)-GAG^{345}/P1 RNA.DNA hybrid catalyzed by wild type (panel A), D443N (panel B), and D443N/ D498N (panel C) RT enzymes, and *E. coli* RNase H (panel D) are illustrated in Fig. 4. The marked difference in the cleavage patterns generated by the three RT enzymes (panels A–C) compared to that formed by *E. coli* RNase H cleavage (panel D) convincingly argued against detectable bacterial enzyme contamination of the RT preparations. Comparison of the cleavage patterns in panels A and B suggested that the D443N mutant enzyme lacked RNase H activity, even at a 10-fold higher enzyme concentration than that resulting in

complete hybrid hydrolysis by the wild type enzyme. This result is a correction of our original claim of wild type RNase H activity of the D443N mutant enzyme, which was based on preliminary experimental evidence (19). As expected, the D443N/ D498N double mutant enzyme also lacked detectable RNase H activity when assayed at an elevated enzyme concentration (panel C).

DISCUSSION

The roles of the highly conserved aspartic acid residues located at positions 443 and 498 respectively in the RNase H domain of HIV-1 RT were probed by their step-wise substitution by site-directed mutagenesis. Enzymatic hydrolysis reactions of phosphodiesters are typically characterized by the involvement of several types of catalysts, such as metal ions and electrophilic amino acid side chains which enhance the electrophilicity of phosphorus by co-ordination to the phosphoryl oxygen atoms, and general base and acid catalysts, which increase the strength of the attacking nucleophile, and facilitate departure of the leaving group, respectively (20). In addition, there are well documented examples of phosphodiester phosphoryl transfer reactions which proceed via the formation of reactive intermediates involving intra- or intermolecular nucleophilic catalysts (20, 21).

The divalent metal ion-dependent ribonucleolytic cleavage of RNA.DNA hybrids by RNase H phosphohydrolases specifically results in P−O (3′) bond cleavage to yield oligonucleotides bearing 5′-phosphoryl and 3′-hydroxyl termini (22). This type of product distribution rules out a mechanism involving intramolecular nucleophilic catalysis by the 2′-hydroxyl of the ribose ring, as exemplified by RNase A and RNase T1 (23). In an attempt to define the amino acid residues that may be involved in catalyzing the RNase H reaction of HIV-1 RT, we selected the aspartic acid residues at positions 443 and 498 respectively, on the grounds that they were both highly conserved (10), and possessed the necessary chemical functionality to participate in general acid/ base catalysis, in nucleophilic catalysis, and in the binding of divalent metal ions.

The aspartic acid residues were substituted by the related amino acid asparagine, which was specifically selected since it represented a structurally conservative substitution which should not affect the protein folding (according to prediction by the Chou-Fasman algorithm (24)), but which is non-ionizable and weakly nucleophilic, and hence incapable of acting as a general acid/ base or covalent catalyst. The single D443N mutation specifically suppressed the RNase H activity without affecting the RT activity, suggesting an important role of the ionizable Asp-443 side-chain in the RNase H active site. However, this mutation, which was located only two amino acids away from the HIV-1 protease p51/p15 cleavage site within the p66 polypeptide of RT (Phe-440/ Tyr-441 (13)), did not affect the proteolytic processing of the precursor *pol* protein to yield the mature p66/p51 heterodimer. The equally conservative single D498N substitution yielded a highly unstable enzyme which was difficult to enzymatically characterize. The lability of this mutant protein suggested that the D498N substitution adversely affected the folding of the protein. In this regard, Hizi et al. have found that a linker insertion mutation at the nearby Gln-501 position (QYALG to QYGIRLG) resulted in a large decrease in the RT activity, which was also ascribed to a folding defect in the RT domain (25, 26).

The D443N/ D498N double mutation relieved the adverse effects of the single D498N mutation to yield a stable, well expressed, and correctly processed enzyme with wild type RT activity, but lacking RNase H activity. Since the D498N mutation was only stable in the context of the D443N/ D498N double mutation, the effect of this mutation on the RNase H activity could not be directly evaluated against the RNase H×-‘ background provided by the D443N mutation. During the course of this study, Schatz et al. reported that an equally conservative glutamic acid to glutamine substitution at position 478 specifically repressed the RNase H activity of HIV-1 RT (15). In conjunction, these data suggested that the Glu-478 and Asp-443 carboxylates may function either as nucleophilic, general acid, or general base catalysts in the RNase H reaction. Alternatively, these ionizable residues may be involved in positioning a divalent metal ion at the active site. Although the role of Asp-498 could not directly be determined in this study, it is likely that it forms part of a ‘catalytic triad’ motif of acidic active site residues (Asp-Glu-Asp) that is common to both bacterial and retroviral RNase H enzymes, based on the analogous *E. coli* RNase H mutagenesis data of Kanaya et al. (16). However, elaboration of the specific catalytic functions of each residue in the triad awaits further structural and mechanistic investigation.

The long-range stabilization of the D498N single mutation provided by the second distal D443N mutation suggested that the effects of the two individual substitutions on the RT and RNase H activities were not additive. Although the carboxylic acid and carboxyamide side chains of Asp and Asn respectively are approximately isosteric, their hydrogen bonding properties are quite different (27). Moreover, Asp is specifically capable of interacting electrostatically with positively charged moieties in its vicinity. It is possible that the D443N mutation caused a slight disruption in a hydrogen bonding and/or electrostatic network in its vicinity which did not in itself, adversely affect the RT activity of the enzyme. However, in the context of the D443N/ D498 double mutation, the altered network may have provided sufficient conformational flexibility to accommodate the folding defects caused by the D498N single mutation. Mutagenesis experiments involving other amino acid substitutions at the Asp-443 position are currently underway to further investigate this interesting second-site substitution effect.

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