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Conformational Study of a Putative HTLV-1 Retroviral Protease Inhibitor

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Abstract

The crystal structure of prolyl-glutaminyl-valyl-statyl-alanyl-leucine (Pro-Gln-Val-Sta-Ala-Leu, $C_{32}H_{57}N_7O_9.5H_2O$, M_r = 683.9 + 90.1), a putative HTLV-1 protease inhibitor based on one of the consensus retroviral protease cleavage sequences, and containing the statine residue [(4*S*,3*S*)-4-amino-3-hydroxy-6-methylheptanoic acid], has been determined by X-ray diffraction. The same molecule has been modelled in the active site of the HTLV-1 protease and both conformations have been compared. The peptide crystallizes as a pentahydrate in space group $P2_1$ with a = 10.874(2), b = 9.501(2), c = 21.062(5) Å, β = 103.68 (1)°, Z = 2, V = 2114.3 Å^3, D_x = 1.21 g cm^{-3}, μ = 8.02 cm^{-1}, T = 293 K, λ(Cu $K\alpha$) = 1.5418 Å. The structure has been refined to an R value of 0.070 for 2152 observed reflections. The peptide main chain can be described as extended and adopts the usual zigzag conformation from the prolyl to the statyl residue. The main difference in conformation between the individual observed and modelled molecules is located on the Sta, Ala and Leu residues with the main chain of the modelled molecule rotated by about 180° as compared to the observed conformation in the crystal state.

Introduction

HTLV-1, human T-cell leukaemia virus type 1, contains genes coding for the *gag* and *pol* polyproteins. During the maturation of retrovirus, these polyproteins are processed by a virally encoded protease to yield structural proteins and enzymes including the protease itself. Structural studies of RSV (Rous sarcoma virus) (Jaskolski, Miller, Mohana Rao, Leis & Wlodawer, 1989; Miller, Jaskolski, Mohana Rao, Leis & Wlodawer, 1990) and HIV (human immunodeficiency virus) (Navia *et al.*, 1989; Wlodawer *et al.*, 1989; Lapatto *et al.*, 1989) retroviral proteases suggested that such a protease should be a dimer structurally related to the aspartyl-protease family of enzymes. Like all other aspartyl proteases, HTLV-1 protease (HTLV-1 PR) is inhibited by pepstatin A (isovaleryl-Val-Val-Sta-Ala-Sta), where statine (Sta) is (4*S*,3*S*)-4-amino-3-hydroxy-6-methylheptanoic acid, at a relatively high drug concentration (Katoh, Yasunaga, Ikawa & Yoshinaka, 1987). Analysis of cleavage sites in the *gag* and *pol* polyproteins indicates that HTLV-1 PR shows a primary specificity for oligopeptides including the naturally occurring Leu-Pro cleavage site. Among the strategies for the design of protease inhibitors, one strategy consists of the replacement of the scissile peptide bond of a substrate with other non-hydrolyzable moieties. Another strategy could be the substitution of a normal endogenic amino acid with an unusual one. In the present paper, the synthesized peptide is a statine-containing derivative based on the *gag* sequence Pro-Gln-Val-Leu-Pro-Val-Met with the Leu-Pro cleavage site generating the P15 and P24 proteins of the virus core. The peptide has been successfully crystallized and its crystal structure conformation is reported along with a conformation modelled in the active site of the HTLV-1 PR.

Experimental

Synthesis and purification

The Boc-Sta residue was prepared as described by Rich, Sun & Boparai (1978). The peptide, Pro-Gln-Val-Sta-Ala-Leu, was synthesized according to Merrifield's solid-phase procedure using an original automatic apparatus (Merrifield, 1963; Mellado & Geoffre, 1983). The *p*-chloromethyl-poly(styrene-*co*-divinylbenzene) resin (*ca* 1 mol chloride g^{-1}) was purchased from Bio Rad Laboratories; the protected *N*-Boc amino acids and HOBT (1-hydroxybenzotriazole) were supplied by Nova Biochem, Switzerland. Removal of the N^{α}-Boc protecting group was accomplished using 30% (*v*/*v*) trifluoroacetic acid in dichloromethane, followed by neutralization of the intermediate peptide-resin trifluoroacetate salt by 10% diisopropylethylamine. The coupling reactions were effected using *N*,*N*′-dicyclohexylcarbodiimide as an *in situ* condensing agent (Sheenan & Ness, 1955). The combination of dicyclohexylcarbodiimide with hydroxybenzotriazole was used with Boc-Gln to prevent dehydration of glutamine. Completion of the coupling reaction was monitored by the ninhydrin test (Kaiser, Colescott & Bossinger, 1970). The peptide was cleaved (60 min at 273–277 K) from the resin by 10% anisole–90% hydrogen fluoride (HF) which simultaneously removed the *N*-im-tosyl and dichlorobenzyl protections. After removal of HF, the peptide was washed with ethyl ether, the crude peptide extracted with 10% acetic acid–90% water and lyophilized to yield amorphous powder. The purification was achieved by gel permeation chromatography on a Sephadex G15 column followed by semi-preparative reverse-phase HPLC using an isocratic

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Table 1. *Crystal data*

Chemical formula	$C_{32}H_{57}N_7O_9.5H_2O$
Molecular weight	683.9 + 90.1
Space group	$P2_1$
Z	2
Cell parameters (Å)	
a	10.874 (2)
b	9.501 (2)
c	21.062 (5)
β (°)	103.68 (1)
V (Å^3)	2114.3
D_x (g cm^{-3})	1.21
R	0.070
wR	0.071
Radiation, λ (Å)	Cu $K\alpha$, 1.5418
μ (Cu $K\alpha$) (cm^{-1})	8.02

methanol–water–trifluoroacetic acid (40:60:0.1) solvent as mobile phase.

X-ray crystallography

Colourless crystals were obtained in Corning glass depression plates by the vapour-diffusion method (McPherson, 1982). All diffraction data were collected from one plate crystal of dimensions 0.2 × 0.1 × 0.03 mm grown from a solution containing 30 m*M* hexapeptide, 30 m*M* sodium cacodylate (pH = 6) and 20% (*w*/*v*) 1,6-hexamethylenediol by equilibration with a reservoir of 100% 2-methyl-2,4-pentanediol. The space group and preliminary unit-cell parameters were determined from Weissenberg photographs. The final values were refined from the setting angles of 25 reflections ($19 < 2\theta < 58°$) measured on an Enraf–Nonius CAD-4 diffractometer with graphite-monochromated Cu $K\alpha$ radiation. The crystallographic data are summarized in Table 1. A total of 2866 unique reflections were collected using the ω–2θ scan technique within the Cu sphere up to $\theta = 55°$: $-11 < h < 11$; $0 < k < 10$; $0 < l < 22$; $\omega = (2 + 0.15\tan\theta)°$. The two reference reflections monitored every 100 reflections did not show significant variation in intensity over the data collection period ($\Delta I/I < 0.03$). Among all the reflections, 2152 with $I > 2\sigma(I)$ were considered as observed and selected for the structural analysis. The intensities were corrected for Lorentz and polarization effects, but not for absorption.

The structure was solved by direct phase determination using the random-start multi-solution procedure of the *SHELXS*86 computer program (Sheldrick, 1986). Initially, the positions of 22 atoms, mainly in the backbone, were assigned. The positions of the remainder of the C, N and O atoms were found by means of Fourier recycling procedures. Although peaks for many H atoms were found in difference maps, idealized calculated positions were used. Subsequently, their coordinates were refined by least squares, the isotropic thermal parameters being fixed at a value slightly larger than that of the equivalent isotropic thermal parameter of the atom to which they are covalently bonded. The H atoms of the water molecules were not detected. Full-matrix least-squares refinement using *SHELX*76 (Sheldrick, 1976) [function minimized: $\Sigma w(F_o - F_c)^2$] with anisotropic thermal factors for the C, N and O atoms converged to $R = 0.070$ and $wR = 0.071$; $w = 1/[\sigma^2(F) + 0.000861F^2]$. Maximum Δ/σ in the final cycle refinement 0.2; maximum variations in the final difference Fourier map within ±0.3 e Å^{-3}. Atomic scattering factors were those incorporated in *SHELX*76. Fractional coordinates and thermal parameters for the C, N and O atoms are listed in Table 2. A stereoview of the molecule is shown in Fig. 1.

Table 2. *Positional* (× 10^4) *and thermal* (Å^2 × 10^3) *parameters of non-*H *atoms*

	Residue	x	y	z	U_{eq}
N	Pro	6797 (7)	8646 (13)	7475 (4)	37 (9)
C*A*	Pro	6763 (9)	7851 (14)	8071 (4)	33 (10)
C′	Pro	6047 (9)	8758 (13)	8467 (5)	32 (11)
O	Pro	6005 (7)	10018 (1)	8432 (3)	45 (8)
C*B*	Pro	8157 (9)	7605 (16)	8404 (5)	39 (11)
C*G*	Pro	8699 (11)	7283 (15)	7798 (6)	53 (13)
C*D*	Pro	7877 (9)	8061 (17)	7228 (5)	49 (12)
N	Gln	5481 (7)	7964 (13)	8873 (3)	33 (8)
C*A*	Gln	4765 (9)	8702 (14)	9285 (4)	29 (10)
C′	Gln	4936 (9)	7955 (15)	9934 (5)	34 (11)
O	Gln	5097 (8)	6645 (12)	9961 (3)	51 (9)
C*B*	Gln	3319 (9)	8754 (16)	8968 (5)	41 (11)
C*G*	Gln	3001 (9)	9477 (17)	8310 (5)	49 (13)
C*D*	Gln	1671 (9)	9951 (16)	8065 (6)	45 (12)
N*E*2	Gln	1353 (9)	10447 (16)	7470 (5)	67 (13)
O*E*1	Gln	916 (7)	9905 (14)	8427 (4)	67 (10)
N	Val	4858 (7)	8720 (12)	10426 (3)	28 (8)
C*A*	Val	4813 (9)	8189 (15)	11074 (4)	37 (11)
C′	Val	3641 (10)	8854 (17)	11228 (5)	38 (12)
O	Val	3445 (7)	10098 (12)	11190 (4)	50 (9)
C*B*	Val	6050 (11)	8513 (16)	11593 (5)	48 (13)
C*G*1	Val	6426 (14)	9986 (20)	11615 (7)	83 (18)
C*G*2	Val	5919 (11)	8011 (18)	12262 (5)	63 (15)
N	Sta	2843 (7)	7882 (12)	11399 (4)	35 (8)
C*A*	Sta	1597 (8)	8270 (15)	11504 (5)	40 (11)
C1	Sta	1364 (10)	7453 (15)	12091 (5)	36 (11)
OH	Sta	1457 (6)	5960 (12)	11988 (3)	41 (8)
C2	Sta	2265 (9)	7848 (15)	12726 (5)	41 (11)
C′	Sta	2064 (10)	6965 (14)	13293 (5)	40 (12)
O	Sta	988 (7)	6584 (13)	13329 (3)	57 (9)
C*B*	Sta	635 (9)	7960 (18)	10857 (5)	60 (14)
C*G*	Sta	−690 (13)	8477 (25)	10802 (7)	93 (23)
C*D*1	Sta	−719 (18)	10077 (30)	10713 (13)	196 (46)
C*D*2	Sta	−1549 (14)	8015 (33)	10194 (7)	188 (45)
N	Ala	3097 (8)	6626 (15)	13739 (4)	55 (11)
C*A*	Ala	3085 (11)	5764 (17)	14326 (5)	53 (14)
C′	Ala	3029 (10)	6763 (19)	14884 (6)	53 (15)
O	Ala	3213 (11)	8014 (15)	14870 (5)	96 (14)
C*B*	Ala	4265 (13)	4846 (20)	14472 (6)	86 (19)
N	Leu	2825 (8)	6102 (15)	15414 (4)	57 (11)
C*A*	Leu	2901 (10)	6864 (19)	16028 (5)	64 (16)
C′	Leu	4288 (12)	7047 (21)	16418 (5)	61 (17)
O′	Leu	4493 (7)	8163 (15)	16748 (4)	69 (11)
O″	Leu	5064 (9)	6104 (16)	16405 (5)	92 (13)
C*B*	Leu	2232 (11)	6042 (19)	16460 (6)	71 (16)
C*G*	Leu	775 (13)	6013 (25)	16206 (8)	96 (22)
C*D*1	Leu	248 (15)	5072 (26)	16638 (9)	131 (27)
C*D*2	Leu	206 (14)	7428 (25)	16171 (9)	115 (27)
O1	*W*	3055 (9)	10519 (15)	16571 (4)	91 (12)
O2	*W*	6982 (10)	5746 (17)	15703 (6)	115 (16)
O3	*W*	5436 (8)	8126 (15)	13764 (4)	85 (12)
O4	*W*	7568 (12)	7754 (19)	14795 (6)	137 (18)
O5	*W*	567 (16)	1459 (28)	15702 (7)	224 (32)

*Molecular modelling of the HTLV-*1 *protease-inhibitor complex*

The superposition and structural alignment of two protease crystal structures (RSV PR and HIV-1 PR) were used

to update the previously published sequence alignments (Pearl & Taylor, 1987; Blundell *et al.*, 1988). In the RSV PR crystal structure, there is no electron density detectable for the region of the flap and, in addition, there is no structural information about the RSV PR complexed with any inhibitor. The recently determined crystal structures of HIV-1 PR complexed with inhibitors (Miller *et al.*, 1989; Fitzgerald *et al.*, 1990; Erickson *et al.*, 1990) show that binding of an inhibitor leads to substantial movement of the flaps. Consequently, HIV-1 PR complexes were used to build a model of the structure of the HTLV-1 PR complexed with the statine-containing inhibitor. Although the HTLV-1 PR is longer than the HIV-1 PR by 16 residues, this excess occurs in sequences corresponding to external loops. These loops were rebuilt to correspond to the lengths predicted using the molecular modelling program *TOM FRODO* (Jones, 1985; Cambillau & Horjales, 1987) and the RSV PR conformation, except for the flap, was taken into account. The amino acids were mutated with the program *MMS* (Dempsey, 1986) in order to recover the HTLV-1 sequence, and the side chains were reorientated so that they occupy the same regions as observed in the crystallographic structures. Using the active-site pseudo-twofold axis from HIV-1 PR complexed to a substrate-based inhibitor, Ac-Thr-Ile-Nle-Nle-Gln-Arg amide (Miller *et al.*, 1989), a dimer of the three-dimensional model of the HTLV-1 PR was generated. Having produced a tentative model for the HTLV-1 PR, the next step was to model the interactions between the inhibitor and the enzyme. For this task, we made use of the crystal structure of HIV-1 PR complexed with acetyl pepstatine (Fitzgerald *et al.*, 1990). The crystal structure clearly indicates hydrogen-bond interactions between the inhibitor and the enzyme. Similar interactions were used to find the binding conformation of Pro-Gln-Val-Sta-Ala-Leu. As observed in all other aspartyl proteases, one of the two aspartyl residues in the active site was protonated for the energy calculations. The minimization process was performed using the *X-PLOR* program (Brünger, Kuriyan & Karplus, 1987). In a first step consisting of about 1000 cycles, the main-chain atoms of ten well conserved residues of the active site were forced to remain in close proximity to the starting structure using harmonic coordinate restraints. In the next step, the system was allowed to relax. The minimization required about 15 000 iterations to bring the system to a stable minimization defined by a maximum derivative of the potential function < 0.01. The conformation of the modelled HTLV-1 PR inhibitor is illustrated in Fig. 1. The modelling of several HTLV-1 complexes will be published in detail elsewhere.

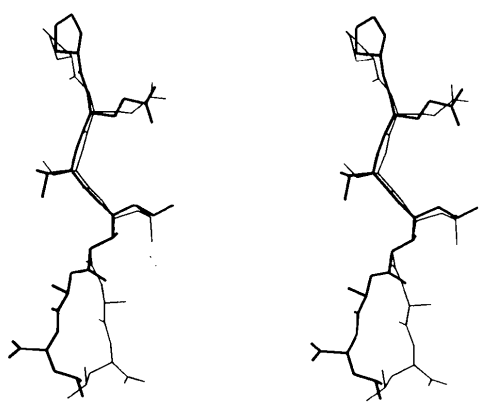

Fig. 1. Stereoview of the observed (thick lines) and modelled (thin lines) conformations (when complexed to the HTLV-1 PR) of the molecule (best least-squares fit of the first four C*A* atoms).

Results and discussion

X-ray conformation of the molecule

The backbone of Pro-Gln-Val-Sta-Ala-Leu, in which all residues but glutamyl have hydrophobic side chains, adopts an extended conformation. A β-strand is initiated by the Pro residue at the N-terminal end. The breaking of the β-strand is coincident with the occurrence of the Sta residue. The value of the C*A*(Sta)—C*A*(Ala) interatomic distance, 6.25 Å, approaches the usual value observed between the CA_i and CA_{i+3} atoms of normal residues in a β-strand (6.5–7 Å). Thus the length of the statine main chain mimics that of two normal residues. The structure of the peptide molecule, viewed along the *a* axis is displayed in Fig. 2. The main conformational angles for the backbone and lateral chains are listed in Table 3 and the hydrogen bonds are listed in Table 4.* The hexapeptide molecule exists as a zwitterion, with terminal NH_2^+ and COO^- groups. The first, third and fourth peptide units are quite planar while the second and the fifth show significant deviations from planarity ($\Delta\omega$ = 8–9°).

As a result of the negative value of $\chi^4 = -18°$, the conformation of the prolyl ring can be described as a distorted conformation of type *A* (Balasubramanian *et al.*, 1971). The Gln, Sta and Leu residues possess the most probable side-chain conformations (Benedetti, Morelli, Nemethy & Scheraga, 1983), with $\chi^1 \simeq -60$ and $\chi^2 \simeq 180°$.

Intermolecular hydrogen bonding

In the direction of the long axis of the cell, the molecules are connected by head-to-tail hydrogen bonding involving the NH_2^+ and COO^- polar groups. The shortest interatomic distance, 2.65 Å, is observed between $N(NH_2^+)$ in one peptide molecule and $O'(COO^-)$ in a translated peptide molecule. On the N-terminus side, three NH···O=C bonds are formed between peptide molecules related by a twofold screw axis. Fig. 2 shows this hydrogen-bonding scheme and the incomplete antiparallel sheet so formed. In addition, three direct hydrogen bonds are observed between the symmetry-

* Lists of structure factors, anisotropic thermal parameters and H-atom parameters have been deposited with the British Library Document Supply Centre as Supplementary Publication No. SUP 55875 (19 pp.). Copies may be obtained through The Technical Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England.

related peptides which engaged the glutamyl side-chain N and O atoms and/or the backbone hydroxyl group. Additional indirect hydrogen bonds *via* water molecules are located in the vicinity of the C terminus. The water molecules serve to bond adjacent peptide molecules by forming bridging hydrogen bonds through one water molecule: N*E*2(Gln)···O1(*W*)···O′(Leu), O(Ala)···O2(*W*)···O′(Leu) and N(Ala)···O3(*W*)···O(Leu), or through two water molecules: N(Leu)···O4(*W*)···O5(*W*)···O(Sta).

Comparison between the observed and modelled conformations

The individual observed and modelled molecules are shown in Fig. 1. The molecules have been superimposed using the least-squares fit of the first four C*A* atoms to illustrate similarities and differences in the backbone conformations. In the two molecules, the greatest analogies in conformation lie in the backbone at the N-terminal end, whereas the greatest differences are observed at the C-terminal end. As far as the N-terminal part is concerned, from Pro to Sta, the main chains adopt very similar conformations of β-strand type and the side chains are alternatively extended on the right and left sides. For the C-terminal end, from Sta to Leu, the main chain of the modelled molecule is rotated by about 180° as compared to the observed conformation in the crystal state. As shown in Table 3, the main differences in the dihedral angle values are observed for the θ_1 and θ_2 angles of the statine flexible main chain. Such a rotation leads to conformations with the two Ala and the two Leu side chains respectively extended in opposite directions. With respect to the usual β-strand conformation, where the lateral chains are alternatively extended on one side and on the other side of the main chain, the statine of the modelled molecule mimics two usual residues whereas the statine of the crystallized molecule mimics only one residue.

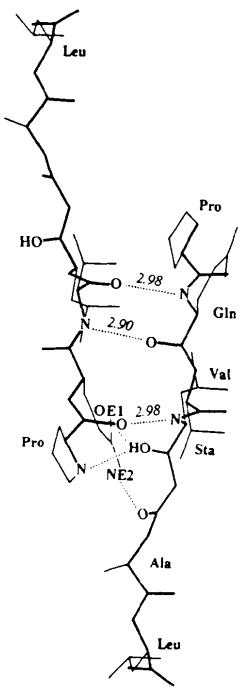

Fig. 2. Hydrogen-bonding scheme viewed along **a**.

Table 3. *Main conformational angles* (°) *for the observed and modelled molecules*

The torsion angles for rotation about bonds of the peptide backbone (φ, ψ and ω) and about bonds of the amino side chains (χ) are as defined by the IUPAC–IUB Commission on Biochemical Nomenclature (1970). For the statyl main chain, the torsion angles are described by Précigoux (1991) and for the prolyl side chain by Balasubramanian *et al.* (1971). Conformational angles for the modelled molecules are in italics.

	φ	ψ	ψ^*	θ_1	θ_2	ω	χ^1	χ^2	χ^3	χ^4	θ
Pro	—	154	—	—	—	180	−40	29	−7	−18	37
	—	*175*	—	—	—	*−179*	*−47*	*40*	*−20*	*−8*	*34*
Gln	−144	151	—	—	—	171	−59	−163	−173	—	—
	−124	*155*	—	—	—	*179*	*63*	*−135*	*−68*	—	—
Val	−127	127	—	—	—	−174	176	—	—	—	—
	−139	*121*	—	—	—	*178*	*−72*	—	—	—	—
Sta	−140	—	65	183	144	180	−171	−174	—	—	—
	−141	—	*81*	*82*	*84*	*−179*	*−135*	*−58*	—	—	—
Ala	−93	171	—	—	—	172	—	—	—	—	—
	−151	*−166*	—	—	—	*176*	—	—	—	—	—
Leu	−80	—	—	—	—	—	−71	175	—	—	—
	−156	—	—	—	—	—	*−155*	*−167*	—	—	—

Table 4. *Hydrogen bonds* (*e.s.d.'s are approximately* 0.03 Å)

A···*B*	Symmetry code for *B*	*A*···*B* (Å)
O1(*W*)···O3(*W*)	$-x+1, y+\frac{1}{2}, z+3$	3.14
O1(*W*)···O5(*W*)	$x, y+1, z$	3.02
O1(*W*)···N*E*2(Gln)	$x, y, z+1$	2.95
O1(*W*)···O′(Leu)	x, y, z	2.70
O2(*W*)···O4(*W*)	x, y, z	2.88
O2(*W*)···O(Ala)	$-x+1, y-\frac{1}{2}, z+3$	2.85
O2(*W*)···O″(Leu)	x, y, z	2.85
O3(*W*)···O4(*W*)	x, y, z	2.80
O3(*W*)···N(Ala)	x, y, z	2.91
O3(*W*)···O″(Leu)	$-x+1, y+\frac{1}{2}, -z+3$	2.88
O4(*W*)···O5(*W*)	$-x+1, y+\frac{1}{2}, -z+3$	2.78
O4(*W*)···N(Leu)	$-x+1, y+\frac{1}{2}, -z+3$	3.22
O5(*W*)···O(Sta)	$-x, y-\frac{1}{2}, -z+3$	2.95
N(Pro)···O′(Leu)	$x, y, z-1$	2.65
N(Pro)···OH(Sta)	$-x+1, y+\frac{1}{2}, -z+2$	2.95
O(Pro)···N(Sta)	$-x+1, y+\frac{1}{2}, -z+2$	2.98
N(Gln)···O(Val)	$-x+1, y-\frac{1}{2}, -z+2$	2.98
N*E*2(Gln)···O(Sta)	$-x, y+\frac{1}{2}, -z+2$	2.91
O*E*1(Gln)···OH(Sta)	$-x, y+\frac{1}{2}, -z+2$	2.71
O(Gln)···N(Val)	$-x+1, y-\frac{1}{2}, -z+2$	2.90

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