Conformational state of a 25-mer peptide from the cyclophilin-binding loop of the HIV type 1 capsid protein

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Recently a 25-residue part of Gag polypeptide from HIV type 1 (HIV-1) was reported to bind to the cytosolic 18 kDa cyclophilin (Cyp18) with an IC_{50} value of 180 μ M. This peptide corresponds to the Cyp18-binding domain of HIV-1 Gag. A replacement of Gly with Ala in the cyclophilin-binding loop of HIV-1 Gag polypeptide results in the prevention of the packaging of Cyp18 into virions. We found only two conformers of this peptide among 16 possible expected conformers, owing to *cis/trans*

isomerization of four peptidyl-prolyl bonds. Although this finding implicates the existence of a stabilizing structure, we were not able to detect secondary structure formation by ¹H-NMR and CD spectroscopy. We characterized the peptide as a substrate for Cyp18 by two-dimensional exchange ¹H-NMR spectroscopy. Surprisingly, we found similar binding characteristics for a peptide corresponding to 25-mer peptide containing the above-mentioned Gly to Ala substitution.

INTRODUCTION

For replication of HIV type 1 (HIV-1) virions an interaction of the Gag polypeptide precursor Pr55^{gag} with human recombinant cytosolic cyclophilin with a molecular mass of 18 kDa (Cyp18) is necessary [1–6]. The Gag polypeptide of HIV-1 plays an important role in the assembly, infection and disassembly of the virions [7]. Pr55^{gag} constitutes three major components: p17 matrix, p24 capsid and p7 nucleocapsid [8]. Pr55^{gag} interacts with host cell cyclophilins [1–6]. Cyclophilins are ubiquitously occurring peptidyl-prolyl *cis/trans* isomerases (PPIases; EC 5.2.1.8). PPIases were found to guide protein folding by the catalytic acceleration of rate-limiting peptidyl-prolyl *cis/trans* isomerizations [9]. PPIases probably have an important role in protein folding, as proved in folding assays *in vitro* [10,11]. Within cells there are several proteins known to bind tightly to PPIases [12]. Because of known difficulties in measuring the catalysis of peptidyl-prolyl *cis/trans* isomerization in native proteins [1], the ability of such proteins to function as a substrate for Cyp18 is unknown. In most cases the enzymic activity of PPIases is assayed with an isomer-specific protease as helper enzyme [13]. These measurements are limited to specially designed short model peptide derivatives, because proteins might be cleaved by the proteases.

These difficulties have so far prevented the complete characterization of the binding of Cyp18 by Pr55^{gag}. A proline-rich region with an indispensable Gly-Pro segment in Pr55^{gag} was identified to be important for Cyp18 binding [3,6,14]. When one of these residues is changed to Ala the interaction of Pr55^{gag} with cyclophilin is abolished, resulting in a loss of virus infectivity [14]. Alteration of the other proline residues of the proline-rich array to alanine shows no detectable effect on the binding of Pr55^{gag} to Cyp18 [3]. Recently we used fragments from this array to investigate the interaction of those peptides with cyclophilin [15]. We demonstrated an unexpected strong and selective binding of a peptide containing 25 amino acid residues ranging from position 213 to position 237 of Pr55^{gag} with an IC_{50} of 180 μ M (peptide 1) (Figure 1). The IC_{50} for a model compound Ala-Ala-Pro-Ala-Ala is 20 mM (M. Schutkowski, M. Jakob and G. Fischer, unpublished work).

We used two-dimensional exchange ¹H-NMR spectroscopy to study whether the peptide acts as substrate or inhibitor for Cyp18. We investigated the binding behaviour of a corresponding 25-mer peptide including a Gly⁹ → Ala substitution to learn whether the physiological effects of this substitution are mirrored *in vitro*.

For the investigation of the structure of peptide 1 we carried out NMR and CD measurements. Besides we analysed the binding of peptides 1 and 2 to Cyp18. Peptide 2 is distinguished from 1 by the replacement of Gly⁹ with Ala and a conservative change of Met¹⁶ to norleucine (Nle).

MATERIALS AND METHODS

Peptide synthesis and sample preparation

Peptides 1 and 2 were synthesized as described [15]. The purity of each final product was assessed by analytical reversed-phase HPLC and by capillary electrophoresis. Both peptides were characterized by matrix-assisted laser desorption/ionization-time-of-flight MS.

For NMR measurements 5–8 mg of freeze-dried peptide were dissolved in 550 μ l of H₂O/²H₂O (9:1). All pH value adjustments were performed by adding NaOH or HCl. For kinetic measurements the same amount of peptide was dissolved in 20 mM phosphate buffer pH 7 (10% deuterated). For measurements with PPIase 75 μ l of a Cyp18 stock solution (10% deuterated, pH 7) was added, yielding a final enzyme concentration of 30 μ M. For inhibition we added 1 mg of cyclosporin A and incubated the sample for 10 h.

NMR measurements

All NMR measurements were performed on a Bruker ARX 500 NMR spectrometer. Temperature calibration was performed with an 866 thermometer (Keithley). Spectra were referenced to internal sodium [2,2,3,3-²H₄]3-trimethylsilylpropionate at 0 p.p.m. For assignment and detection of secondary structure we used common two-dimensional NMR methods such as double-

Abbreviations used: Cyp18, human recombinant cytosolic cyclophilin with molecular mass of 18 kDa; HIV-1, HIV type 1; Nle, norleucine; NOE, nuclear Overhauser effect; PPIase, peptidyl-prolyl *cis/trans* isomerase; ROESY, rotating-frame NOESY.

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Ac-Asp¹-Arg²-Val³-His⁴-Pro⁵-Val⁶-His⁷-Ala⁸-
Gly⁹-Pro¹⁰-Ile¹¹-Ala¹²-Pro¹³-Gly¹⁴-Gln¹⁵-
Met¹⁶-Arg¹⁷-Glu¹⁸-Pro¹⁹-Arg²⁰-Gly²¹-Ser²²-
Asp²³-Ile²⁴-Ala²⁵-NH₂

Figure 1 Sequence of peptide 1

The fragment corresponds to residues 81–105 of HIV-1 p24 capsid protein [7].

quantum-filtered COSY, TOCSY and rotating-frame NOESY (ROESY). For ROESY spectra we used mixing times of 50–300 ms. The water signal was suppressed by irradiation during the relaxation delay of three seconds. Full assignment was possible for the major all-*trans* isomer. The poor separation of signals from the major and minor conformers made an assignment of the minor isomer impossible. For the measurement of catalysis we recorded phase-sensitive NOESY spectra to observe the existence or lack of exchange peaks between the *cis* and *trans* conformers. We used a mixing time of 300 ms for these NOESY experiments. The spectral width of spectra was typically 5050 Hz; 512 t_1 increments were recorded with 32–96 scans per increment.

CD spectroscopy

CD data were collected on a Jasco J-710 spectropolarimeter (Tokyo, Japan). All measurements were performed in a thermostatically controlled quartz cell of 0.1 cm pathlength with 10 mM sodium phosphate buffer, pH 7.5, at 10 °C. Spectra were usually accumulated 15–20 times.

RESULTS

Solution conformation of peptides 1 and 2 as revealed by ¹H-NMR

The one-dimensional ¹H-NMR spectra of peptides 1 and 2 displayed in Figure 2 exhibit only a weak isomer-related signal splitting. All signals are found in the range expected from data of random coil peptides [16,17]. Because peptide 1 contains four proline residues, we expected a much more complex ¹H-NMR spectrum because of the 16 possible different isomers due to

cis/trans isomerization. Each isomer has to show dispersed signals at least for amino acids adjacent to the rotating peptide bond. We were able to detect only two isomers above the detection limit of 1 %. The main isomer, which could be assigned to the all-*trans* isomer as described below, was present at 93 %.

Complete evaluation of the spectra was performed only for the major all-*trans* isomer. No assignment of the signals of the minor isomer was possible owing to the low population of the minor isomer and no isomer-related signal splitting for the *cis* and *trans* isomers in most signals. Therefore all further explanations on secondary structure refer to the all-*trans* isomer.

We performed all measurements at three different pH values: 3, 6 and 7 [18,19]. We found no alteration of the spectra, except for the change of the chemical shift for residues, where side chain carboxylic or imidazolic groups were protonated. All measured pK_a values (Glu¹⁸, 4.5; Asp¹, Asp²³, 3.9; His⁴, His⁷, 6.5) were near the published values for random-coil peptides [20], even if the lack of the expected *cis/trans* conformational multiplicity suggests the stabilization of two preferred conformations by structure formation.

We used three parameters from ¹H-NMR spectroscopy to obtain information about the secondary structure in the peptide 1. First, we collected data of the temperature dependence of chemical shifts of amide protons, which are indicative of the formation of hydrogen bonds. Secondly, we measured coupling constants giving information about the backbone torsion angle, ϕ . Thirdly, we used NOE measurements to gain data about the spatial neighbourhood of protons.

We evaluated the chemical shifts of NH resonances at at least two different temperatures from two-dimensional spectra and calculated the temperature coefficients. To prove the validity of these values we used the chemical shifts for non-overlapping NH signals in one-dimensional spectra at seven different temperatures from 5 to 35 °C. These non-overlapping NH signals were assigned to Val³, His⁷, Gln¹⁵, Ser²², Asp²³ and Ile²⁴. The error between the two data sets was within 10 %. We found low-temperature coefficients for all amide protons between –8 and –12 p.p.b./K (Table 1). The only exceptions were the temperature coefficients of Gln¹⁵ and Asp²³, at –7.6 and –7.8 p.p.b./K respectively.

The HN-C²H coupling constants were in the range 6–11 Hz (Table 1). We found strong alterations from one residue to the next along the sequence, although not all coupling constants

Figure 2 500 MHz ¹H-NMR spectra of peptide 1 (bottom trace) and 2 (top trace)

The overview spectra are drawn in (a). The water resonance has been removed. Singlets at 2.85 and 3.01 p.p.m. in the upper spectrum were caused by solvent impurities. (b) Portion from spectra below with higher scaling of the ordinate and the same abscissa as in (a). The arrow indicates the C^αH resonance of Pro⁵ for the minor isomer.

Table 1 Coefficients for temperature-dependent shift of NH signals and HN-C²H coupling constants (³J_{HNz}) for the all-*trans* isomer of peptide 1

Residue	No.	$\Delta\delta/\Delta T$ (p.p.b./K)	³ J _{HNz} (Hz)
Asp	1	-8.7	6.3
Arg	2	-8.5	8.1
Val	3	-9.1	6.9
His	4	-9.3	11.2
Pro	5	-	-
Val	6	-10.5	8.0
His	7	-10.7	6.5
Ala	8	-11.0	9.3
Gly	9	-8.7	4.3
Pro	10	-	-
Ile	11	-11.5	7.7
Ala	12	-12.1	6.9
Pro	13	-	-
Gly	14	-10.1	*
Gln	15	-7.6	7.6
Met	16	-9.6	7.1
Arg	17	-8.5	6.8
Glu	18	-10.1	*
Pro	19	-	-
Arg	20	-10.4	7.6
Gly	21	-8.7	*
Ser	22	-8.0	10.1
Asp	23	-7.8	8.0
Ile	24	-8.5	7.6
Ala	25	-9.8	6.5

* No coupling constants were measured because of peak overlapping.

were measured owing to strong peak overlapping even in two-dimensional spectra.

The observation of nuclear Overhauser effect (NOE) signals from two-dimensional ROESY spectra showed all the expected sequential $d_{\alpha N}$ NOEs but only three sequential d_{NN} NOEs and intraresidual NOEs. No connectivities were observed between two successive residues in positions i and $i+2$ or even any long-range NOEs between more distant protons. All peptidyl-prolyl bonds are considered to be in the *trans* conformation in the major conformer because $d_{\alpha\delta}$ NOE cross peaks of the four residues preceding a proline had medium to strong intensity [21]. These NOE cross peaks are shown in Figure 3. As discussed below, we could not confirm the formation of secondary structure elements in peptide 1 from these results.

We were not able to detect directly which peptidyl-prolyl bond occurred in the *cis* conformation owing to strong overlapping and a small amount of *cis* isomer. We found no NOEs for the *cis* isomer in the C²H region or between the C²H and Pro-C²H region. Exchange peaks in NOESY spectra of peptide 1 with Cyp18 showed an interesting pattern of peaks (see below). We observed only a few exchange peaks, indicating that there is an isomer-related signal splitting between the *cis* and *trans* signals. We know that isomer-related signal splitting for His⁷-NH, Ala⁸-NH, Ile¹¹-NH and Ala¹²-NH is evident from the assignment of the exchange peaks. The separated peaks are found N-terminally (His⁷, Ala⁸) and C-terminally (Ile¹¹, Ala¹²) to Pro¹⁰. This indicates that the peptidyl-prolyl bond of Pro¹⁰ is in the *cis* conformation in the minor isomer.

Comparison of peptides 1 and 2

To avoid the difficulties we had in the synthesis of peptide 1 that were caused by Met¹⁶, we used Nle in the corresponding position

Figure 3 Portion of the 500 MHz phase-sensitive ¹H-ROESY spectrum of peptide 1 at pH 7 and 23 °C

All (Xaa C^α-H)-(Pro C^δ-H) NOE cross peaks are surrounded by broken boxes.

of peptide 2. This substitution shows no alteration in the behaviour of the respective peptides, as reported [22–24]. Both peptides have very similar CD spectra, as shown in Figure 4. We detected a negative extremum at 198 nm and a shoulder in the range 220–240 nm for both peptides. Our spectra showed no evidence of the formation of a polyproline II helix, but rather characteristics of spectra of random-coil peptides [25].

In the ¹H NMR spectrum of peptide 2 we found one additional methyl resonance at 1.36 p.p.m. for Ala⁹ and at 1.72 p.p.m. for Nle¹⁶. Methylene resonances appeared at 0.88, 1.3 and 1.8 p.p.m. for Nle¹⁶ in the spectra of peptide 2. The resonances assigned to Met¹⁶ in peptide 1 at 1.9, 2.09 and 2.53 p.p.m. disappeared in the spectra of peptide 2. The C²H resonances of residues 9 and 16 were changed from 4.10 and 4.48 p.p.m. in peptide 1 to 4.58 and 4.23 p.p.m. respectively in peptide 2. The acetyl methyl group appeared as a doublet with a coupling constant of 5.1 Hz owing to the geminal ¹³C-¹H coupling. Apart from that we obtained almost the same spectrum for both peptides (Figure 2a). We can

Figure 4 CD spectra of peptides 1 and 2 in 10 mM phosphate buffer, pH 7.5

see from the C_{δ} signals of Pro⁵ that the state of isomers is the same in both peptides, although the spectra are lacking in well-separated signals for the major and minor isomers (Figure 2b).

For comparing the binding properties of both peptides to Cyp18 we estimated IC_{50} values by the method of Schutkowski et al. [15]. For peptide 1 we confirmed the known value of $180 \pm 10 \mu\text{M}$ [15]. For peptide 2 we obtained a similar value of $230 \pm 14 \mu\text{M}$.

Exchange spectroscopy

NOESY was used to study the substrate or inhibitor properties of peptides 1 and 2. Figure 5 shows portions of three NOESY spectra of peptide 1, which were measured in turn. The first measurement was a NOESY spectrum of the peptide at 23 °C at pH 7. *Cis/trans* isomerization of peptidyl-prolyl bonds is slow on the NMR timescale, with values in the region of 10^{-3} s^{-1} [26]. We observed two sets of signals for some residues in NMR spectra and in two-dimensional NOESY spectra exchange cross peaks were not observable at room temperature. Looking at the spectrum in Figure 5(a) we see no NOE connectivities at all. For the second spectrum we added catalytic amounts of recombinant human Cyp18. A new set of NOESY cross peaks arising from the faster interconversion of *cis* and *trans* isomers (more than 0.1 s^{-1}) appeared. In Figure 5(b) these cross peaks are clearly seen. After these measurements we added cyclosporin A, a cyclophilin-specific tight binding inhibitor to the mixture. If the exchange cross peaks in Figure 5(b) originated from cyclophilin-mediated catalysis, they would disappear; we observed this effect, as shown in Figure 5(c).

The same measurements were performed for peptide 2. We obtained the same results as for peptide 1: on addition of Cyp18, cross peaks between the minor and the major isomers in NOESY spectra appeared and disappeared after the addition of cyclosporin A (results not shown).

DISCUSSION

The minimal binding region in Pr55^{gag} for Cyp18 was identified as including a proline-rich array [3]. Inside the array a proline residue is situated that is indispensable for the interaction between Cyp18 and Pr55^{gag}. This proline corresponds to proline 90 in the capsid protein, which is a product of HIV protease cleavage of Pr55^{gag} [7]. We synthesized the proline-rich array including the

Figure 5 Portions of the NH regions of NOESY spectra showing the catalytic effect of Cyp18 on the 25-mer peptide 1 from HIV-1 capsid protein

Sample and conditions were as described in the legend to Figure 2: (a) without Cyp18, (b) in the presence of $30 \mu\text{M}$ Cyp18. The exchange peaks are surrounded by broken boxes and marked at the right. (c) The same sample as in (b) but incubated with an excess of cyclosporin A for 10 h.

flanking sequences from Asp⁸¹ to Ala¹⁰⁵ of capsid protein. (For the corresponding numbering of our peptides see Figure 1.) For a structural study of this peptide we performed NMR investigations. We found two isomers: a major all-*trans* isomer present at 93% and a minor isomer. From measurements of pH dependence, temperature dependence of NH chemical shifts, NOEs and coupling constants (Table 1) we were able to exclude the formation of α - and 3_{10} -helices, β -sheets and turns for the all-*trans* isomer.

Left-handed polyproline II helices, one structure element that moved into focus as a binding motif in protein/protein interactions [27], are hard to detect in NMR spectra owing to the lack of hydrogen bonds and NOEs beside sequential NOEs [28]. However, the formation of PPII helices in peptides 1 and 2 is not supported by the CD measurements performed [25]. CD spectra can easily be interpreted as spectra of random coil peptides

(Figure 4). Although these facts highlight the lack of known secondary structure elements in peptides 1 and 2 in solution, we found indications for a populated structure too. The existence of only two out of sixteen theoretically possible isomers (Figure 3) suggests the formation of a structure stabilizing just these two conformers. Multiple proline-containing random coil peptides usually occur as a mixture of all possible *cis/trans* isomers ([29–31], and U. Reimer and G. Fisher, unpublished work). In addition we found a temperature dependence of the CD spectra in peptide 1 in buffered solution. We measured the temperature dependence under denaturing conditions as well by adding 6.0 M guanidinium chloride [15]. By plotting the CD signal against temperature we obtained different shapes of the graph, indicating a structural difference in the two solvents, pointing to the presence of a stabilizing conformation in peptide 1 in buffered solution [15,32].

We compared our data with recently published results from NMR studies of the N-terminal domain of HIV-1 capsid protein [7]. It was shown that the corresponding sequence to peptide 1 as the Cyp18-binding domain forms a loop that is well exposed to the solvent. The only peptidyl-prolyl bond, which occurs in a *cis/trans* equilibrium, is the bond of Pro⁹⁰. A crystal structure of complete capsid protein complexed with the Fab fragment of a monoclonal antibody shows a similar shape for the molecule [33]. In the structure derived from NMR data the Cyp18-binding loop includes a β II-turn from Ala⁹² to Gln⁹⁵ and an α -helix from Arg¹⁰⁰ to Ala¹⁰⁵. From a comparison with our data we are not able to find any indication of the formation of a helix, either in the NMR data or in the CD spectra. However, we find a relatively low temperature coefficient for the NH signal of Gln¹⁵ in our peptide 1. This could be taken as a weak indication of the existence of small amounts of a β -turn at positions Ala¹² to Gln¹⁵ (34). Data extracted from protein structures show a consensus sequence for β II-turns of Pro-Gly-Gln [35]. Support comes from the crystal structure of the complex of peptide 1 with Cyp18, where a β -turn from Ala¹² to Gln¹⁵ is observed [36].

The replacement of Gly with Ala within the proline-rich array (corresponding to the change from peptide 1 to peptide 2) abolishes the incorporation of Cyp18 into HIV-1 and decreases infectivity [14]. In tetrapeptide derivatives Suc-Ala-Xaa-Pro-Phe-pNA the peptide with Xaa = Ala shows 3-fold higher k_{cat}/K_m values for the cyclophilin-mediated catalysis of *cis/trans* isomerization than the peptide with Xaa = Gly [37]. This raises the question of why the change of Gly⁹ to Ala leads to such strong physiological effects.

The comparison of peptide 1 and peptide 2 shows very similar behaviour in CD and NMR spectroscopy (Figures 2 and 4), a comparable peptidyl-prolyl bond isomerism and similar IC_{50} values for the binding to Cyp18. Both peptides were shown to act as substrates for Cyp18 (Figure 5). One reason for the physiological effect of the Gly89 → Ala mutation in capsid protein could be the ability of glycine to adopt exceptional backbone conformations. However, because peptides 1 and 2 show no remarkable difference in binding behaviour and substrate character towards Cyp18 this explanation seems to be unlikely. Nevertheless it is not clear which structural effects are caused by this amino acid substitution in the whole capsid protein. Therefore studies of proteins as binding partners for cyclophilin and the solution of the three-dimensional structures of the complexes

of cyclophilin with both the capsid protein of HIV-1 and the modified (Gly89 → Ala) capsid protein are necessary.

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