Extended binding sites of cyclophilin as revealed by the interaction with HIV-1 Gag polyprotein derived oligopeptides

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Abstract **Oligopeptides derived from the gag polyprotein ($Pr55^{gag}$) of human immunodeficiency virus type 1 (HIV-1) segment were used to evaluate the extension of the putative binding region for the complex of $Pr55^{gag}$ and the human cytosolic peptidyl prolyl *cis/trans* isomerase (PPIase) 18 kDa cyclophilin (Cyp18). Five N-terminally acetylated, C-terminally amidated oligopeptides containing one (HIV-1 $Gag^{218-224}$; 1), two (HIV-1 $Gag^{218-226}$ and HIV-1 $Gag^{217-224}$; 2 and 3, respectively), three (HIV-1 $Gag^{217-226}$; 4) or four (HIV-1 $Gag^{213-237}$; 5) proline residues were synthesized. Using competition experiments with a standard substrate the binding affinities to Cyp18 of the synthesized peptides were determined. The IC_{50} value of 184 μM for the 25-mer peptide 5 was fivefold or more lower than those of the peptides 1–4 lacking one or more prolines. Failure of competition in assays containing enzymes of other PPIase families by millimolar concentrations of 5 revealed a Cyp18 specific interaction involving the active site of the enzyme. In its far UV circular dichroism, aqueous solutions of 5 display properties of random coil conformation, but spectra were also consistent with a small contribution of proline specific secondary structures. However, a proline-rich peptide typical of forming left-handed polyproline II helices did not compete for the active site of Cyp18. The results demonstrate that the putative binding region of HIV-1 gag polyprotein has a certain degree of binding affinity to the PPIase site of Cyp18, and may add a previously unrecognized topological component to the known subsite specificity of cyclophilins.**

Key words: Cyclophilin; HIV-1 Gag polyprotein; Peptide; CD spectroscopy; Selective binding

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Abbreviations: Cyp18, human recombinant cytosolic cyclophilin with molecular mass of 18 kDa (according to Fischer, G. (1994) Angew. Chem. Int. Ed. Engl. 33, 1415–1436); PPIase, peptidyl prolyl *cis/trans* isomerase; FKBP, FK506 binding protein; CsA, cyclosporin A; HIV-1, human immunodeficiency virus type 1; SIV, simian immunodeficiency virus; NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DMAP, *N,N*-dimethylaminopyridine; Fmoc, 9-fluorenylmethoxycarbonyl; GdmCl, guanidinium hydrochloride; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; HPLC, high performance liquid chromatography; MBHA, 4-methylbenzhydrylamine; NMP, *N*-methylpyrrolidone; tBu, *tert.*-butyl; Trt, trityl; HOBt, 1-hydroxybenzotriazole; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Ac, acetyl; CE, capillary electrophoresis; CA, capsid protein of HIV-1; GST, glutathione *S*-transferase

1. Introduction

Previous reports have shown the lower incidence of developing AIDS for HIV^+ patients who received immunosuppressive therapy with cyclosporin A (CsA) after organ transplantation [1–3]. Moreover, in response to the immunosuppressive drugs CsA and FK506, the growth of cells chronically infected with HIV-1 and HIV-2 was selectively inhibited [4]. After identifying two host cell cyclophilins (Cyp18 or Cyp23sec) in binary complexes with Gag polyprotein precursor $Pr55^{gag}$ of HIV-1 the molecular causality underlying the CsA effect became more obvious [5]. CsA enables complex dissociation by its high affinity to cyclophilins. Obviously, the antiviral effect of CsA has to be due to a pathway distinct from immunosuppression because CsA derivatives with negligible immunosuppressive activity but high affinities for the active site of Cyp18 retain potent anti-HIV activity [6–12].

Cyclophilins, the cellular receptors for CsA [13], are known to possess peptidyl prolyl *cis/trans* isomerase (PPIase) activity [14]. This enzyme activity seems to improve the correct timing of those protein folding events in which a *cis/trans* Xaa-Pro isomer distribution transiently does not correspond to the thermodynamic equilibrium but reflects the immediate surrounding of the polypeptide chain.

Recently, it was shown that Cyp18 is required for HIV-1 infectivity prior to reverse transcription but subsequent to receptor binding and membrane fusion [15]. Incorporation of Cyp18 into HIV-1 virions occurred via contact with a proline-rich segment of the capsid domain of $Pr55^{gag}$ [16]. This conserved array totalling four prolines occurs in the periodicity $(P(X)_4P^{222}(X)_2P(X)_5P)$. Prolines are preferentially flanked by glycine and charged amino acids. Mutant proteins of the $HIV\text{-}1_{HXB2}$, having site-directed mutations, P222A or G221A, failed to bind on GST-Cyp18. Virions equipped with these proteins cannot sequester Cyp18 into the released virions [15,16], emphasizing the importance of the Gly^{221}-Pro^{222} bond for $Pr55^{gag}$/Cyp18 complex formation. With respect to Cyp18 packaging, P225A behaves like wild type devoid of forming a defective viral phenotype. Similarly, protein variants P217A and P231A retain Cyp18 binding. Analogously, small in-frame deletions in the capsid domain of $Pr55^{gag}$ drastically decrease Cyp18 packaging into viral particles [7].

However, it is not known whether or not catalysis of prolyl bond isomerization in $Pr55^{gag}$ plays a role in the $Pr55^{gag}$/Cyp18 mediated enhancement of HIV-1 infectivity. Moreover, the finding of a stable $Pr55^{gag}$/Cyp18 interaction is at variance with the known weak binding of proline-containing peptides to the active center of Cyp18 [17].

In order to evaluate whether the four prolines module of

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HIV-1 $Pr55^{gag}$ represents a high affinity module for the active site of Cyp18, we synthesized five oligopeptides derived from the capsid domain of $Pr55^{gag}$ including throughout the essential Gly^{221}-Pro^{222} peptide bond:

1 Ac-VHAGPIA-NH_2
2 Ac-VHAGPIAPG-NH_2
3 Ac-PVHAGPIA-NH_2
4 Ac-PVHAGPIAPG-NH_2
5 Ac-DRVHPVHAGPIAPGQMREPRGSDIA-NH_2

In peptide **4**, three proline residues in central position are combined, and finally, peptide **5** assembled the conserved four prolines in conjunction with the N- and C-terminal flanking sequences, hydrophilic in their nature. The peptides were synthesized with both acetylated and amidated termini thought to mimic more likely the binding module of the $Pr55^{gag}$ polypeptide chain. All compounds were applied to an assay for PPIase activity of Cyp18 to determine their ability to compete with a substrate containing a single proline residue. These investigations were complemented by measuring proline-rich peptides known as typical ligands of SH3 or WW domains with K_d values in the μM range. For evaluating enzyme specificity of binding, we analyzed the interaction of the peptides **1–5** with members of other PPIase families. This major result implied an unexpected strong and selective binding of peptide **5** at or near the active site of Cyp18.

2. Materials and methods

2.1. Peptide synthesis

Fmoc-amino acids were purchased from Alexis (Grünberg, Germany). All chemicals and solvents were of analytical grade and were used without further purification. As an exception diethylether was distilled from sodium and stored over KOH.

All syntheses were carried out using a semiautomatic ACT 90 peptide synthesizer and Rink amide-MBHA resin (Novabiochem, Bad Soden, Germany; 0.55 mmol/g, 400 mg). HBTU/HOBt/DIEA activation of N^{α}-Fmoc-protected amino acids (5 equiv.) was employed for coupling in the case of peptides **1–4** and HATU/HOAt/DIEA activation in the case of peptide **5** (10 equiv.). The side-chain protecting groups used in all syntheses were tBu for Ser, Asp, Glu; Trt for His, Gln; and Pmc for Arg. The coupling reactions were performed in NMP. Initially, chain elongation was associated with the reaction time of 5 min for the Fmoc deprotection in 20% piperidine in NMP, but it gradually increased to 20 min when the number of amino acids of the chain bound to the resin was increased up to 10 residues. A double deprotection strategy was used at each step to ensure complete deprotection. Final acetylation was carried out using acetanhydride (10 equiv.), DIEA (10 equiv.) and DMAP (0.5 equiv.) in NMP. Following acetylation the resin was washed twice with NMP; DCM; *iso*-propanol and diethylether and dried carefully.

The peptides were cleaved from the resin using 95% TFA. The crude peptides were precipitated from *iso*-propanol solution using diethylether. The methionine containing peptide **5** was treated with trimethylsilylbromide to remove methionine *S*-oxide. All peptides were purified by preparative RP-HPLC using a Sycam apparatus equipped with a Merck LiChrosorb RP8 (250×25 mm, 7 μm, flow rate 20 ml/min, detection wavelength 215 nm) column or a Macherey-Nagel Nucleosil 300 RP4 (250×10 mm, 7 μm, flow rate 10 ml/min; detection wavelength 210 nm) column and 5–40% aqueous acetonitrile containing 0.05% TFA gradient. The product containing fractions were evaporated in vacuo to remove acetonitrile and lyophilized yielding white powders. The purity of each final product was assessed by analytical RP-HPLC using a Sycam Nucleosil 100 RP18 (250×4 mm, 5 μm, flow rate 1 ml/min; detection wavelength 210 nm) and by capillary electrophoresis (Applied Biosystems model 270A-HT, 30 kV, 30°C, hydrodynamic injection for 2 s/5″Hg, fused silica capillary, uncoated, 72 cm×50 μm, λ: 200 nm, 70 mM sodium citrate pH 2.3). All peptides were characterized by TOF-MALDI-MS and by ^{1}H-NMR spectroscopy (Bruker ARX 500 spectrometer at 500.13 MHz).

2.2. Determination of the IC_{50} values

All measurements were performed on a Hewlett-Packard 8452A diode array spectrophotometer with a thermostatted cell holder. Constant temperature was maintained within the cell (d = 1 cm) by water circulated from a Cryostat Haake D8 (Haake Fisons, Germany). The Hewlett-Packard 89531A MS-DOS-UV/VIS operation software or SigmaPlot Scientific Graphing System Vers. 2.00 (Jandel Corp., USA) was used for data analysis. The concentration of enzymatically active Cyp18 was determined using fluorometric titration with CsA assuming that inhibitor binding reflects an unperturbed active site [13]. Typical purity was above 70% active enzyme leading to a k_{cat}/K_m value of 1.5×10^7 s^{-1} M^{-1} for Suc-Ala-Ala-Pro-Phe-NH-Np. All stock solutions were filtered through a 0.45 mm PVDF membrane (AllTech, Unterhaching, Germany). Measurements of the bimolecular rate constants k_{cat}/K_m for the PPIase catalyzed isomerization in the absence and the presence of the proline-containing peptides were performed utilizing a solvent assisted shift in the *cis/trans* equilibrium [18] of Suc-Ala-Ala-Pro-Phe-2,4-difluoroanilide after jumping into the final buffer solution at 240 nm. Typically, a mixture of (1240−x) μl buffer, 5 μl BSA stock solution (5 mg/ml in 35 mM HEPES pH 7.8) and 5 μl PPIase stock solution was treated with x μl proline-containing peptide solution reaching final peptide concentrations up to 1.5 mM and incubated for 4 min at 10°C. Reactions were started by addition of 2 μl of the mentioned substrate stock solution (60 mM). Plot of residual PPIase activity versus peptide concentration was used to calculate the IC_{50} values of **1–5**. All measurements were performed in duplicate.

2.3. Determination of the steady-state parameters k_{cat} and K_m of Suc-Ala-Gly-Pro-Phe-NH-Np

The substrate Suc-Ala-Gly-Pro-Phe-NH-Np was purchased from Bachem (Heidelberg, Germany). The steady-state parameters became available by the improved protease coupled assay devised by Kofron et al. [19]. The measurements were performed by monitoring the absorbance of released 4-nitroaniline at 390 nm (ε = 11 814 M^{-1} cm^{-1}) on a Hewlett Packard 8452A diode array spectrophotometer according to Schutkowski et al. [20]. Absorbance data points from each progress curve were analyzed by nonlinear least-squares optimization of K_m and k_{cat} in the differential equation $d[C]/dt = -k_o[C] - V_{max}[C]/(K_m+[C])$ using the software TREND, version 1.2 (Martin-Luther-University, Halle-Wittenberg, Germany). The resulting initial rates were fitted by nonlinear least-squares optimization of K_m and V_{max} in the Michaelis-Menten equation $v_0 = V_{max}[C_0]/([C_0]+K_m)$.

2.4. CD spectroscopy

CD data were collected on a Jasco J-710 spectropolarimeter (Tokyo, Japan) interfaced to a personal computer. If not otherwise stated, far UV measurements were carried out in a thermostatted quartz cell of the cylindrical 0.1 cm path length using 10 mM sodium phosphate buffer pH 7.5 at 10°C. Baselines for all spectra were corrected by subtracting baselines for the appropriate solutions without peptide. Jasco software version 1,33.00 was used to calculate molar ellipticity based on the supplied sample molarity and cell path length. Usually, 15–20 transients were obtained to improve the signal to noise ratio. Temperature dependence of the CD signals was investigated using the AVIV CD-Spectrometer 62A DS. Data recording occurred in the range 15–85°C with a step width of 1°C. Equilibration time at each temperature was 0.7 min (heating rate: 100°C/min), the time constant for data sampling was 12 s.

3. Results

3.1. Conformational homogeneity

Due to the different number of proline residues the covalent structure of the peptides **1–5** provides the potential for 2–16 (2^n) *cis/trans* isomers. Slow interconverting *cis/trans* isomers were shown to lead to peak multiplicity in RP-HPLC [21,22] and capillary electrophoresis [23,24] since there is a difference in the migration time of the isomers. Differentiating minor

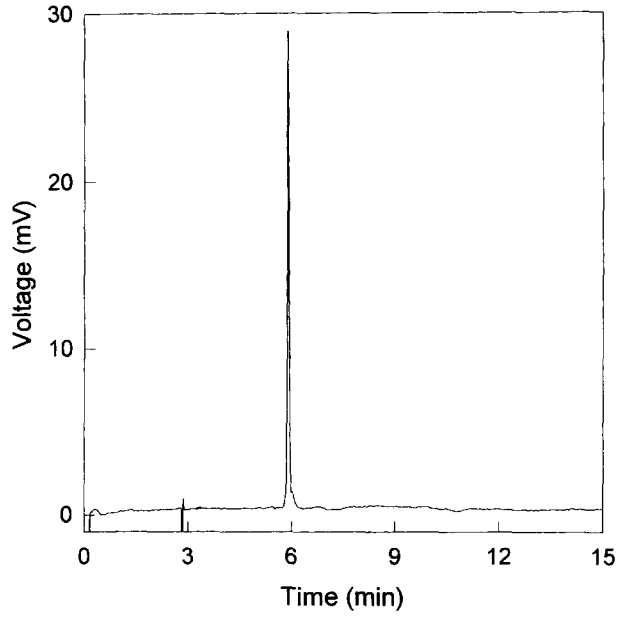

Fig. 1. Capillary zone electrophoresis of purified peptide **5** using an uncoated fused silica capillary (72 cm×50 μm) and 70 mM citrate buffer, pH 2.3. Conditions: constant voltage: 30 kV (resulting current: 21 μA), T: 30°C, λ: 200 nm.

peaks of *cis/trans* conformers from those resulting from impurities usually inherent in solid phase peptide synthesis becomes increasingly difficult when chain length increases. However, as could be shown by RP-HPLC and CE, even in the case of the 25-mer peptide **5** the purity of the crude products was surprisingly high (>80%). After a two-step purification by HPLC using an RP-8 and an RP-4 column peptide **5** was chemically homogeneous as could be demonstrated by both ^{1}H-NMR spectroscopy and capillary electrophoresis (Fig. 1). Remarkable is the fact that for peptides **1–5** at 25°C neither in HPLC (data not shown) nor in capillary electrophoresis analyses could any proline-mediated peak splitting or widening be observed. Given a difference in the migration time of most isomers theoretically possible, this result may indicate the predominance of a single proline isomer in solution due to energetic stabilization by structure formation. Alternatively, the results can be explained by isomerization rates larger than 0.05 s^{-1} at 25°C [23].

3.2. Kinetic competition experiments

Binding to the active site can be detected by incubating mixtures of peptides **1–5** and a PPIase substrate with Cyp18 with the consequent competition kinetics in an activity assay. However, in the standard PPIase assay the presence of a helper protease leads to rapid degradation of several proline peptides used here (data not shown), thus preventing any protease-coupled analytics [25]. To circumvent this limitation we used a protease-free assay system taking advantage of a solvent assisted shift in the *cis/trans* equilibrium [18] of Suc-Ala-Ala-Pro-Phe-2,4-difluoroanilide. After a solvent jump the time-course of *cis/trans* isomerization follows a first-order reaction characterized by the rate constant $k_{obs} = k_{cis \rightarrow trans} + k_{trans \rightarrow cis}$. The catalytic activity of Cyp18 and other PPIases toward this substrate was evaluated in the presence of different concentrations of peptides **1–5** by calculating first-order rate constants for the accelerated isomerization. Since the substrate concentration in the assay was 96 μM, it ranges well below the K_m value of ≥0.8 mM measured for this substrate composed by an equilibrated mixture of *cis/trans* conformers (unpublished data). In contrast to the sole presence of the *cis* isomer of the substrate in proteolytic assay, the non-proteolytic assay contains a mixture of *cis/trans* isomers for all peptides involved. Assuming zero enzyme activity toward the anilide substrate for Cyp18 completely saturated with the competitor peptides, IC_{50} values of peptides **1–5** were obtained by plotting residual PPIase activities versus peptide concentration (peptide **5**; see Fig. 2). The solid line in Fig. 2 represents the theoretical curve yielded from the rate equation for competitive inhibition and the IC_{50} value calculated.

For peptides **1–5** the IC_{50} values are summarized in Table 1. All peptides were able to compete with the assay peptide for the active site of Cyp18, irrespective of whether these compounds act as alternative substrates or as inhibitors. The tabulated IC_{50} values are significantly lower than the K_i values estimated for the uniform proline peptides AAPF, AAPA and AAPAA. Even in the presence of 5 mM of the latter tetra- or pentapeptides, inhibition of Cyp18 mediated isomerization of *cis* Suc-Ala-Ala-Pro-Phe-NH-Np was less than 10% [26]. In contrast, the binding affinity of the 25-mer peptide **5** for Cyp18 is particularly high, even if the number of prolines involved in contacts is still an open question. However, the number of prolines in the competing peptides does not play a major role for affinity because the proline-rich peptides Ac-RALPPLPRY-NH_2 and Ac-ATPPPLPPPL-NH_2 have no detectable effect on the isomerization kinetics in concentrations of up to 0.8 mM in our assay.

Furthermore, we measured whether peptide **5** would allow an even better fit into the active site of members of other PPIase families like human FKBP12, *E. coli* parvulin and *E. coli* trigger factor. The latter two enzymes remain completely unaffected by **5** in the assay, whereas the interaction with FKBP12 is characterized by the high IC_{50} value of 1.6 mM. The results shown in Table 1 gave rise to the assumption that for an effective and selective binding to the active site of

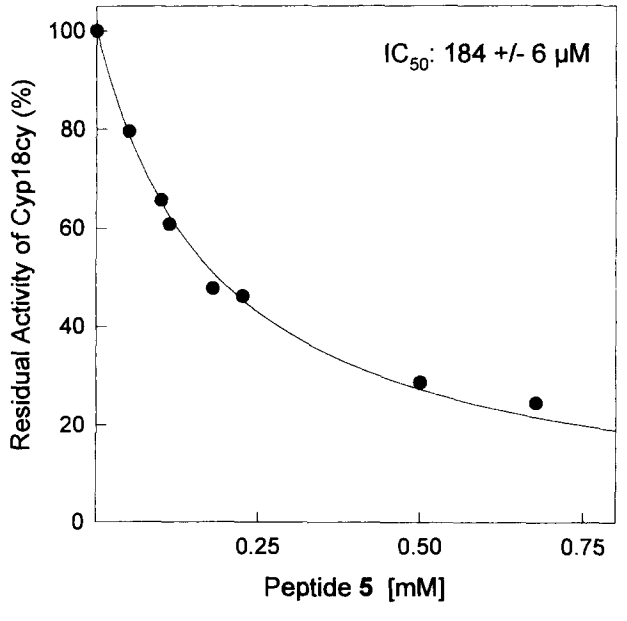

Fig. 2. Inhibition of Cyp18 by peptide **5**, measured using the protease-free assay described in Section 2.

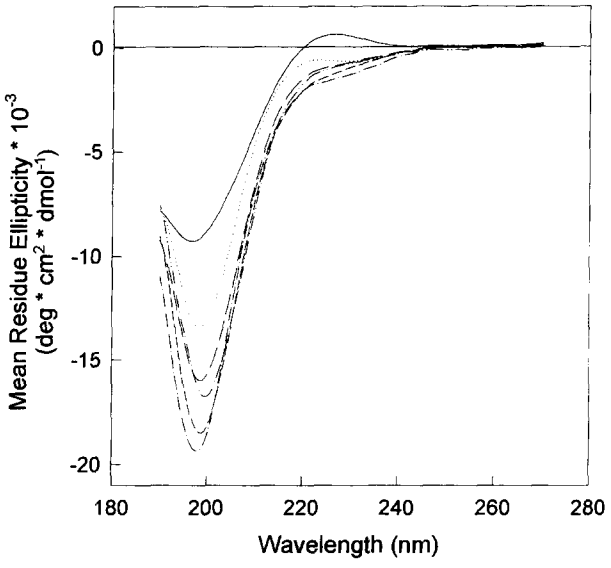

Fig. 3. Comparison of the CD spectra of the following peptides: Ac-RALPPLPRY-NH_2 (solid line), peptide **5** (dotted line), peptide **3** (dashed line), peptide **4** (-·-·-), peptide **2** (——) and peptide **1** (-··-··-) measured in 10 mM phosphate, pH 7.5 at 10°C, $d = 1$ mm, at a scan speed of 100 nm/min (response 0.5 s). Raw data of 16 accumulations were noise reduced by Fourier transformation.

Cyp18cy all four proline residues of the proline array of HIV-1 $Pr55^{gag}$ have to be combined within one molecule.

3.3. Structural properties of peptides **1–5**

At first glance, the circular dichroism (CD) spectra of peptides **1–5** in the far UV region at pH 7.5 and 10°C do not differ greatly from each other by expressing minima centered at 200 nm, having the 215 nm $n \rightarrow \pi_3^*$ slightly negative (Fig. 3). In contrast, the latter spectral transition gives rise to a weakly positive band at 225 nm for Ac-RALPPLPRY-NH_2, indicative of polyproline II helical conformation with *trans* Xaa-Pro bonds [27–32]. Similarly, Fig. 3 demonstrates a trend for decreasing negative molar ellipticities for peptide **1–5** in this spectral region when chain length increases.

A 10-fold decrease in peptide concentration did not alter the CD spectra indicating that peptide association is not significant at the concentrations used for CD measurements.

Temperature variations as well as denaturants may alter the relative amount of structured conformations contributing to the CD spectrum. A clear isodichroic point at 209 nm occurs recording the CD spectra of **5** at different temperatures from 10°C to 85°C. The temperature dependence of the CD signal at 220 nm exhibits a different shape when determined for peptide **5** either in buffer solution or in 6.0 M guanidinium hydrochloride (GdmCl). The strictly linear plot of Θ^{220} with increasing temperature in GdmCl contrasts with that obtained in buffer, which is bent downward (data not shown). Additionally, the difference between the CD signal at 220 nm in the absence and presence of 6.0 M GdmCl for peptide **1** is smaller than for peptide **5**. Reversibility was demonstrated by the recovery of the original spectra after cooling of the previously heated peptide solutions (Fig. 4).

Furthermore, we could demonstrate that in spite of the various side chain functions the influence of the pH value on the CD spectra of **5** is negligible. The peptide solution was titrated from 7.5 to 2.1 or 10.8. Only a subtle decrease or increase, respectively, of the signals in the 200 nm region is observable without any change in overall spectral shape (results not shown). Titration of these samples back to pH 7.5 reversed this minor effect.

^{1}H-NMR studies have shown that peptide **5** has no stable secondary structure in aqueous solution (Reimer et al., in preparation). However, the left-handed polyproline II helix often found in peptides of related structure is so extended [33] that no special NOEs are detectable allowing a clear distinction from unordered segments.

4. Discussion

The identification of a binding protein with the yeast two hybrid system requires quite tight interactions between the partner proteins. Similarly, co-purification or retardation in affinity chromatography may also function only for relatively stable complexes. The identification of the $Pr55^{gag}$/Cyp18 interaction by these techniques established a quite high binding affinity of the proteins to each other. This finding is at variance with the high K_m values that could be separately determined, in part, for *cis* and *trans* oligopeptide substrates covering either the millimolar range for *trans* isomers or slightly lower for the *cis* peptides [19,34]. Attempts to saturate partially Cyp18 in the enzyme catalyzed refolding of denatured RNase T1 were also unsuccessful even at the highest protein concentrations used [35]. This substrate carries many potential subsites for disposal because it presents a *trans* to *cis* isomerization at the Ser-Pro^{55} peptide bond in the context of a partially unfolded polypeptide chain.

The Pro^{222} residue that was found to be critical in a genetic screen [16] was invariantly positioned in peptides **1–5**. Obviously, by a knowledge-based model the heptapeptide **1** already fulfils the structural requirements to act as an alternative substrate within a Cyp18 assay. Indeed, ^{1}H-NOESY spectra of 1 show additional cross-peaks as the result of magnetization transfer in the presence of Cyp18 indicating enzymatic catalysis of *cis/trans* isomerization [36,37]. Considering the slightly different conditions, e.g. the ratio of *cis/trans* isomers and solvent composition, the IC_{50} value of 0.71 mM for **1** (Table 1) fits the K_m value of 1.2 ± 0.4 mM measured for another peptide with the Gly-Pro moiety, the optimized stand-

Table 1
IC_{50} values of peptides **1–5** interacting with peptidyl prolyl *cis/trans* isomerases

PPIase[a,b]	IC_{50} of **1** (mM)	IC_{50} of **2** (mM)	IC_{50} of **3** (mM)	IC_{50} of **4** (mM)	IC_{50} of **5** (mM)
Cyp18	0.71 ± 0.03	1.6 ± 0.08	1.3 ± 0.06	1.30 ± 0.05	0.18 ± 0.01
FKBP12	[c]	[c]	[c]	1.12 ± 0.04	1.6 ± 0.06

[a]All measurements using a protese-free assay in 0.05 mM HEPES pH 7.6 at 10°C, for details see Section 2.
[b]In the case of parvulin and trigger factor (both from *E. coli*) no detectable effect on peptidyl prolyl *cis/trans* isomerase activity was found up to final peptide concentrations of 0.6 mM.
[c]The enzymatic activity was not affected up to final peptide concentrations of 1.2 mM.

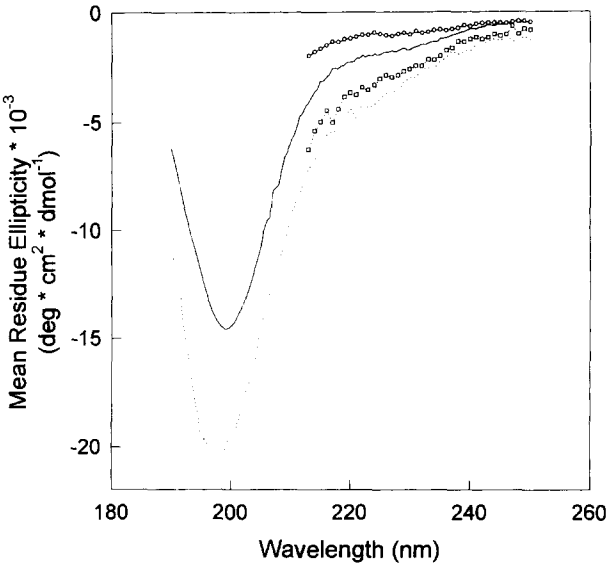

Fig. 4. Comparison of the CD-spectra of peptide **1** (dotted line), peptide **1** dissolved in 6 M GdmCl (□), peptide **5** (solid line) and peptide **5** dissolved in 6 M GdmCl (○).

ard substrate Suc-Ala-Gly-Pro-Phe-4-nitroanilide. Similar to many proteolytic enzymes, cyclophilins require secondary binding sites in the peptide chain of substrates to express catalytic activity. It was often found that the presence of an appropriate linear stretch of amino acids adjacent to the reaction center was sufficient to represent the structural prerequisite for lowering the activation energy for catalysis of both classes of enzymes. Whereas the nature of the amino acids in both positions N-terminally and C-terminally to proline does not contribute much to the second order rate constant k_{cat}/K_m of Cyp18 catalyzed reaction, chain length was found to be critical. Two amino acids preceding proline are required to establish minimal substrate properties, whereas a total of 5 peptide bonds was required to maximize the k_{cat}/K_m value for Cyp18 catalysis [38,39]. Substrate binding is reflected more precisely by the Michaelis constant but data for peptides with different chain length were lacking.

With respect to these data **1** has the characteristics of an optimal binder for Cyp18. Indeed, although the probability of coming across prolines is higher and chain lengths are increased, the IC_{50} values of peptides **2–4** demonstrate a slightly reduced level of affinity. Failure of the numbers of proline residues alone to create high Cyp18 affinity was further shown by the formin-derived peptide Ac-ATPPPLPPPL-NH_2 [40], which does not realize an efficient Cyp18 binder. This compound has a minor effect of 10% inhibition at 0.8 mM concentration. Similarly, the same concentration of Ac-RALPPLPRY-NH_2 did not affect the PPIase assay. Both compounds represent typical cognate peptides of WW [40,41] and SH3 domains [42,43], respectively, thought to be involved in mediating protein-protein interactions by polyproline recognition. In its non-acetylated state, the latter peptide (RALPPLPRY-NH_2) binds to the Src SH3 domain with a K_d value of 8 μM [43]. In addition, regiospecificity of Cyp18 catalysis caused by multiple proline sites was already found to occur [36].

Most interestingly, Cyp18 displays an enhancement in binding only for a peptide covering all the prolines around Pro^{222}, as could be demonstrated by an IC_{50} value of 0.18 mM for the 25-mer peptide **5**. Since the fractions of the individual *cis/trans* isomers occurring in aqueous solution of **5** are still unknown, the IC_{50} value determined represents the lower binding limit for a discrete isomer. Due to the considerable chain length of **5**, affinity is less likely to increase by utilizing a larger number of subsites in a conventional way. As an alternative possibility it may happen that any *cis/trans* isomer of peptide **5** will form a certain type of peptide structure in solution. Recently, the three-dimensional structure of a proteolytic fragment of the core domain of the HIV-1 capsid protein containing amino acid residues Pro^{133}-Leu^{283} was solved by NMR spectroscopy [44]. Apart from seven helices the monomeric domain contains an exposed loop. The essential Gly^{221}-Pro^{222} peptide bond is located on this loop, being specifically affected in its conformational state by a *cis/trans* equilibrium. Concerning the structural pattern of the protein segment excised, peptide **5** encompasses the entire loop region including the type II β-turn (Ala^{224} through Gln^{227}) and helix V (Arg^{232} through Ala^{237}).

However, substantial contributions of a random mixture of conformations may be inferred from the CD spectra of peptides **1–4**. Obviously, the CD spectrum of **5** is reminiscent of the truncated peptides lacking any indication for helical segments. Furthermore, in the case of peptide **5** the curved plot of Θ^{220} with increasing temperature is consistent with a number of partially ordered structures having different melting points.

Recently, it was shown by NMR spectroscopy and crystal structure data for a 23-mer peptide APTMTRVLQGVL-PALPQVVCNYR, corresponding to the 38–60 fragment of the β-subunit of human chorionic gonadotropin, that in aqueous solution the majority of the residues adopted an extended polyproline II conformation [45]. The similarity of the central proline pair with that of **5** may suggest structural relatedness. The polyproline II helix known to be formed from Ac-RALPPLPRY-NH_2 [43] is clearly indicated by a positive CD signal at 225 nm, but cannot be found as a major conformation in the spectra of **5** (Fig. 3).

Clearly, further studies will have to elucidate the high affinity prolyl bond conformation(s) of **5** by analyzing its composition in solution with respect to the ratio of *cis/trans* isomers, and whether this particular isomer will be recognized as a substrate by the active site of Cyp18.

As a recognition template this structure might be implicated in Cyp18 binding in vivo. Viral Cyp18 packaging shows that $Pr55^{gag}$ interacts via its native state. However, native state Xaa-Pro *cis/trans* isomerization, known to exist for many proteins, has not yet been reported for an enzyme catalyzed rate acceleration [46]. As binding to the active site of Cyp18 is a prerequisite of catalysis, it still remains an open question whether the accessibility of the active cleft may allow Cyp18 to accommodate a native protein. However, it is not possible to compare peptide **5** with both the $Pr55^{gag}$ and the fragment $Pr24^{CA}$ in terms of binding constants because data for the proteins are not yet known.

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