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HIV protease substrate conformation: modulation by cyclophilin A

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Abstract Cyclophilin A (CyPA), a cytosolic peptidyl-prolyl *trans-cis* isomerase can accelerate the *trans-cis* isomerization of Xxx-Pro peptide bonds. One- and two-dimensional ^{1}H-NMR spectroscopy were used to determine that the heptapeptide Ser-Gln-Asn-Tyr-Pro-Ile-Val, a model peptide of an HIV-1 protease cleavage site in the *gag* polyprotein of HIV-1, is a substrate for CyPA. Experiments revealed a slow exchange about the Tyr-Pro peptide bond with 30 ± 5% in the *cis* conformation (pH 1–9). While the interconversion rate is too slow to measure by kinetic NMR methods in the absence of CyPA, these methods, saturation transfer and NOE experiments, established that CyPA enhanced the rate of *trans-cis* interconversion, a process inhibited by cyclosporin A (CsA). With a substrate:CyPA ratio of 40:1, an interconversion rate of 2.5 s^{-1} at 25°C was observed.

Key words: HIV protease; Cyclophilin A; Cyclosporin A; Saturation transfer; Nuclear Overhauser effect; Peptidyl-prolyl *trans-cis* isomerase; *gag* polyprotein

1. Introduction

The mature virion of the human immunodeficiency virus type-1 (HIV-1) consists of viral proteins, the single stranded RNA genome, and enzymes required for replication. Subsequent to translation and incorporation into the immature virion, the precursor polyproteins are cleaved by HIV protease (Fig. 1). Cleavage of the *gag*, *gag-pol*, and *env* polyproteins produces the structural proteins: p17 (MA), matrix protein; p24 (CA), capsid protein; p7 (NC), nucleocapsid protein; and p6; the enzymes: HIV protease (PR); reverse transcriptase (RT); and integrase (IN); and the envelope proteins: gp120 (SU) and gp41 (TM), respectively [1–3]. Inhibition of cleavage results in the production of poorly infectious virions [3,4]. One site of proteolytic cleavage has been shown to occur at the Tyr^{132}/Pro^{133} peptide bond in the *gag* polyprotein (Fig. 1, Site 1). It has been shown that the heptapeptide corresponding to residues 129–135 of the *gag* polyprotein functions as a suitable active model substrate [3].

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Abbreviations: CsA, cyclosporin A; CyPA, cyclophilin A; CA, capsid protein; DQF-COSY, double quantum-filtered correlation spectroscopy; HIV-1, human immunodeficiency virus type-1; IN, integrase; KPi, potassium phosphate; MA, matrix protein; NC, nucleocapsid protein; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; ppm, parts per million; PR, protease; RT, reverse transcriptase; T_1, longitudinal relaxation time; TPPI, time-proportional phase incrementation

In addition to the enzymes encoded by the HIV-1 genome, CyPA is incorporated into the virion during assembly [5–8]. A member of the immunophilin family of enzymes possessing peptidyl-prolyl *trans-cis* isomerase activity [9], CyPA is the intracellular, cytosolic receptor for the immunosuppressive drug cyclosporin A, CsA [10]. The CyPA:CsA complex binds and inhibits calcineurin thereby inhibiting the gene transcription of several cytokines in T-cells [11–13]. The family of immunophilins also functions as chaperone proteins as they catalyze *trans-cis* interconversion about Xxx-Pro peptide bonds, often a rate-determining step in protein folding [14,15]. In addition, it has been proposed that CyPA may function as a mediator of protein-protein interactions [13,16,17].

Previous studies have shown that CyPA binds to the capsid domain of the *gag* polyprotein (and subsequently to the capsid protein) through a proline-rich region in the capsid domain, $Pro(Xxx)_4$ $Pro222(Xxx)_2$ $Pro(Xxx)_5Pro$ (Fig. 1B) [7,8,10,18,19]. CyPA packaging into the virion is essential for viral infectivity as mutations in *gag* or reagents such as CsA which disrupt the *gag*-CyPA interaction inhibit viral replication [8,20–25]. Possible roles of CyPA in the life cycle of HIV-1 have been suggested based on the structural studies of Gitti et al. [26] and Gamble et al. [16]. Gitti and coworkers determined the solution structure of the amino-terminal two-thirds of the capsid protein by NMR. Based on this structure, it was suggested that CyPA catalyzes the *trans-cis* interconversion about Pro^{90} of the capsid protein (*Pro222* of intact *gag* polyprotein), a possible molecular switch in virion disassembly. Gamble and coworkers determined the X-ray structure of a complex between the truncated capsid protein and CyPA. CyPA was found to interact with Pro^{90} and it was suggested that CyPA may function to weaken the association of the capsid protomers so as to function in virion disassembly. The premise behind the current study is that CyPA, in addition to other possible functions, may catalyze isomerization about the Xxx-Pro peptide bonds [9] in the *gag* polyprotein to the conformer cleaved by HIV protease. Noteworthy is the fact that of the eight cleavage sites in the *gag* and *gag-pol* polyproteins, three sites involve an Xxx-Pro scissile peptide bond (Fig. 1A) [4,27,28].

In this study, a potential mechanistic role for CyPA in the HIV-1 replicative cycle was probed using nuclear magnetic resonance (NMR) methods and the model Pro containing heptapeptide of the HIV-1 *gag* polyprotein. Saturation transfer and nuclear Overhauser effect spectroscopy (NOESY) techniques were utilized to quantitate the peptidyl-prolyl *trans-cis* isomerization kinetics of the heptapeptide in the presence of CyPA.

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2. Materials and methods

2.1. Sample preparation

The heptapeptide model substrate (Ser-Gln-Asn-Tyr-Pro-Ile-Val) was obtained from Sigma Chemical Company and used without further purification. Samples were prepared by dissolving the heptapeptide in H_2O, ^2H_2O, or a potassium phosphate (KPi)/^2H_2O buffer (0.05 M KPi, 0.15 M NaCl, and 0.02% NaN_3). When needed, CyPA from a stock of recombinant CyPA (1 mg/ml) originating from a human Jurkat T-cell lymphoma line [29] and CsA (kindly supplied by J. Borel of Sandoz, Inc. of Switzerland) were added. NaO^2H and ^2HCl were used to alter the pH of the sample as needed.

2.2. NMR techniques

^{1}H-NMR spectra were recorded on a Bruker AMX500 NMR spectrometer. The data was processed with the Felix software (Biosym Technologies, San Diego, CA) running on a SGI computer.

The *trans-cis* ratios at pH 9.15, 7.0, 5.73, and 1.56 in ^2H_2O were determined from 1-dimensional spectra at 25°C using 0.6 ml of a 2 mM heptapeptide sample in a 5-mm NMR tube. The measurements were made using the following parameters: data points, 8K; scans, 64; pulsewidth, 77°. The same method was used to determine the *trans-cis* ratio of the heptapeptide in a KPi/^2H_2O buffer.

One-dimensional NOE difference experiments on the heptapeptide (1.6 mM) in the absence and presence of CyPA (25 μM) with and without CsA (25 μM) were conducted in a KPi/^2H_2O buffer at pH 6.8. The measurements were made using 0.67 ml of each sample in a 5-mm NMR tube at 25°C. Three hundred and twenty transients were collected in multiples of eight alternating between on and off-resonance saturation of the proline δH resonance in the *cis* conformation at 3.37 ppm. The parameters were as follows: 16K data points; on-resonance pulse, 3.32 ppm; and off-resonance pulse, 10.08 ppm. Exponential multiplication of the time domain signal by 5.0 Hz was used before Fourier transformation. To reduce the possibility of perturbation of the nearby downfield *cis* multiplet, the selective saturation pulse was intentionally placed a few hundredths of a ppm to higher field from the center of the *cis* multiplet.

Saturation transfer methods were used to measure exchange rates as previously reported [30] on the substrate (2.0 mM) in the absence and presence of CyPA (45 μM). The relationship between isomerization rate and saturation transfer is given by equation 1 [30,31]:

$$(I_t - I_\infty)/I_0 = \exp(-k_{t\rightarrow c}t) \quad (1)$$

where I_t is the intensity of the *trans* multiplet at 3.74 ppm after irradiation of its corresponding *cis* multiplet at 3.37 ppm for a time equals t; I_∞ is the intensity of the *trans* multiplet at 3.74 ppm after irradiation at 3.37 ppm for an infinite time; and I_0 is the equilibrium intensity of the *trans* multiplet without irradiation of its corresponding *cis* multiplet at 3.37 ppm. The rate constant, $k_{t\rightarrow c}$, for *trans-cis* interconversion, is given by the slope of the best fit line through the data on a plot of $\ln[(I_t - I_\infty)/I_0]$ vs. time t. The following NMR acquisition parameters were used: 128 scans, 8K data points, 77° pulse, and 25°C. The on-resonance pulse was applied at 3.32 ppm, the off-resonance at 5.60 ppm, and the sweep width was 12 ppm. The saturation period of the *cis* multiplet was varied from 20 ms to 2.0 s. For the substrate in the presence of CyPA, this straight forward procedure was sufficient to determine the catalyzed *trans-cis* interconversion rate, $k_{t\rightarrow c}$, of the substrate at 25°C. On the other hand, the interconversion rate of the free substrate at 25°C was too slow to be measured by this technique which requires that the rates be within one order of magnitude of the T_1. Higher temperatures (60–80°C) were attempted with the hope that an Arrhenius plot of the rates vs. temperature could be used to extrapolate back to the uncatalyzed *tran-cis* interconversion rate at 25°C.

Two-dimensional NOESY experiments were performed on substrate (1.6 mM), substrate (1.6 mM):CyPA(25 μM), and substrate(1.6 mM):CyPA(25 μM):CsA(25 μM) samples in KPi/^2H_2O at pH 6.8. These measurements were made on 0.67 ml of sample in a 5-mm NMR tube with the following parameters: temperature 25°C, 512 increments in t_1, 2K complex data points in t_2, 48 scans, 600 ms mixing time, and time-proportional phase incrementation, TPPI.

3. Results and discussion

Sequence-specific resonance assignments obtained from DQF-COSY spectra revealed separate resonances for the *cis* and *trans* conformers of the Tyr-Pro peptide bond in the heptapeptide substrate (Ser-Gln-Asn-Tyr-Pro-Ile-Val). The aromatic region of the heptapeptide (Fig. 2A) exhibits splitting of both the 2,6 (δ_{trans} = 7.09 ppm) and the 3,5 (δ_{trans} = 6.76 ppm) proton resonances of Tyr. This splitting indicates a slow conformational exchange about the Tyr-Pro peptide bond. Further support for the slow *trans-cis* interconversion is evidenced in a parallel splitting of the δH prolyl resonances (Fig. 2B). Integration of either the aromatic resonances or the δH prolyl resonances establishes a *trans-cis* ratio of 70:30 ± 5% for the heptapeptide in either ^2H_2O or KPi/^2H_2O which is pH-insensitive throughout the pH range of 1 to 9.

Fig. 3 shows the expansion of the aromatic region of the spectrum of the peptide alone and also with and without

A)

Site	P5	P4	P3	P2	P1		P1'	P2'	P3'	P4'
1	Val	Ser	Gln	Asn	Tyr	*	Pro	Ile	Val	Gln
2	Lys	Ala	Arg	Val	Leu	*	Ala	Glu	Ala	Met
3	Ser	Ala	Thr	Ile	Met	*	Met	Gln	Arg	Gly
4	Arg	Pro	Gly	Asn	Phe	*	Leu	Gln	Ser	Arg
5	Val	Ser	Phe	Asn	Phe	*	Pro	Gln	Ile	Thr
6	Cys	Thr	Leu	Asn	Phe	*	Pro	Ile	Ser	Pro
7	Gly	Ala	Glu	Thr	Phe	*	Tyr	Val	Asp	Gly
8	Ile	Arg	Lys	Val	Leu	*	Phe	Leu	Asp	Gly

B)

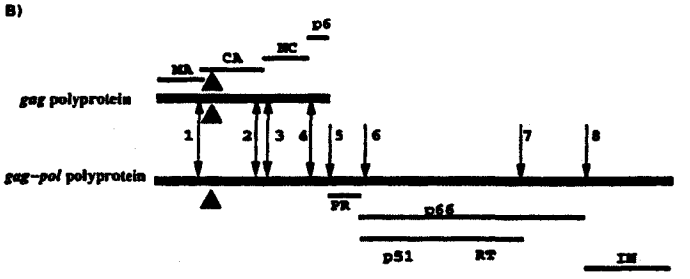

Fig. 1. HIV-1 protease cleavage sites in the *gag* and *gag-pol* precursor polyproteins of HIV-1. (A) The amino acid sequences of the eight documented cleavage sites in the *gag* and *gag-pol* polyproteins. The asterisks indicate the scissile bonds. (B) A schematic depiction of the cleavage sites on the *gag* and *gag-pol* polyproteins. Numbered arrows indicate sites of cleavage and the mature proteins formed upon cleavage are indicated as labeled fragments above and below the polyproteins. The CyPA binding site and its proline-rich sequence is indicated by an arrow to the precursor polyproteins and the capsid protein.

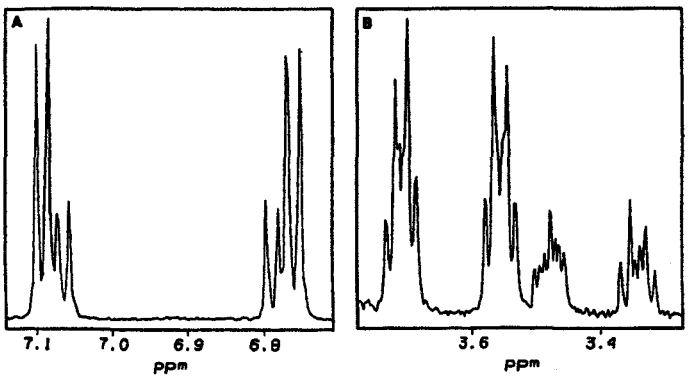

Fig. 2. Tyrosyl aromatic (A) and proline δH (B) resonances of the heptapeptide substrate in ^2H_2O (Ser-Gln-Asn-Tyr-Pro-Ile-Val, 2 mM peptide, and pH 7.0).

CyPA and CsA. Comparison of the spectra of the free peptide (Fig. 3A) with the peptide in the presence of CyPA (Fig. 3B) indicates that CyPA enhances the rate of *trans-cis* isomerization as seen by selective broadening/shifting of the smaller *cis* peaks in the tyrosyl region upon addition of CyPA. The broadening was not the result of increased viscosity with the addition of CyPA as the line-broadening effect reversed upon CsA addition (Fig. 3C).

To quantitate the catalytic effect of CyPA, saturation transfer experiments were conducted on the heptapeptide. The high field δH prolyl *cis* resonance (3.37 ppm) was saturated as indicated by an 'X' in Fig. 4A which shows the enlargement of the proline δH region of the heptapeptide substrate. Fig. 4B–D show the NOE difference spectra of the heptapeptide, the heptapeptide with CyPA, and the heptapeptide with CyPA plus CsA, respectively. With saturation of the highest field *cis* resonance (X) in the heptapeptide alone, no saturation was transferred to its corresponding *trans* peak (X′) (Fig. 4B). It should be noted that the B_2 saturation pulse was not completely selective for the highest field *cis* resonance (3.37 ppm); a small amount of saturation at the adjacent low field *cis* resonance (3.50 ppm, labeled Z) was observed. This lack of absolute specificity, however, does not compromise the conclusions drawn from further analysis. In contrast to the free substrate (Fig. 4B), saturation transfer was observed from the highest field *cis* resonance at 3.37 ppm (X) to the lowest field *trans* resonance at 3.74 ppm (X′) of the substrate in the presence of CyPA (Fig. 4C). Again, the less than perfect selectivity of the B_2 saturation field is seen in that the partial saturation of the *cis* resonance at 3.50 ppm (Z) which, now in the presence of CyPA, is transferred to its corresponding *trans* resonance at 3.58 ppm (Z′). Addition of a molar equivalent of CsA blocked the transfer of saturation observed for the substrate in the presence of CyPA, a result consistent with CsA inhibition of CyPA catalysis.

Quantitative measurement of the *trans-cis* isomerization rate was conducted utilizing saturation transfer methodology. The *cis* resonance at 3.37 ppm was irradiated for a variable time period and the intensity of the corresponding *trans* resonance at 3.74 ppm was observed (see methods for details). In the presence of CyPA, the interconversion rate for the Tyr-Pro peptide bond in the model heptapeptide at 25°C was found to be 2.5 s^{-1} (Fig. 5). In the absence of CyPA, the *trans-cis* interconversion rate was too slow at 25°C for measurement by this method. Therefore, saturation transfer experiments were conducted at elevated temperatures (60–80°C) with the hope to extrapolate the uncatalyzed rate at 25°C from an Arrhenius plot. However, temperature dependent resonance shifts in the δH Pro region of the spectrum resulted in the degeneracy of resonances compromising a quantitative analysis by saturation transfer.

Enhancement of the *trans-cis* isomerization rate was also evidenced in NOESY spectra. Fig. 6 shows an enlargement

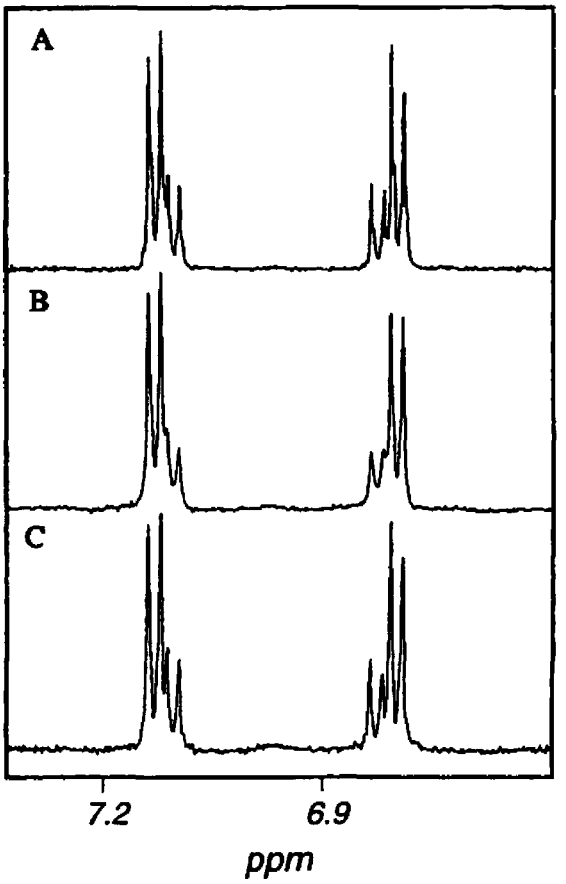

Fig. 3. Effect of CyPA and CsA on the tyrosyl spectral region of Ser-Gln-Asn-Tyr-Pro-Ile-Val at pH 6.8. (A) Heptapeptide substrate (1.6 mM peptide). (B) Substrate (1.6 mM) with CyPA (25 μM). (C) Substrate (1.6 mM), CyPA (25 μM), and CsA (25 μM).

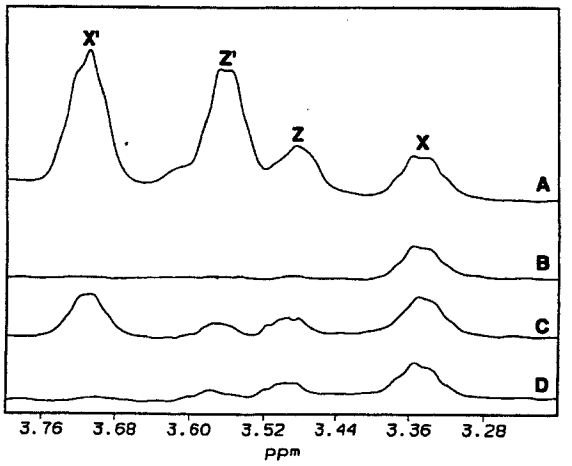

Fig. 4. Observation of saturation transfer from *cis* resonance at 3.37 ppm (X) to the *trans* resonance at 3.74 ppm (X'). (A) δH proline region. (B) NOE difference spectra of free substrate (1.6 mM peptide). (C) NOE difference spectra of substrate (1.6 mM) with CyPA (25 μM). (D) NOE difference spectra of substrate (1.6 mM), CyPA (25 μM), and CsA (25 μM).

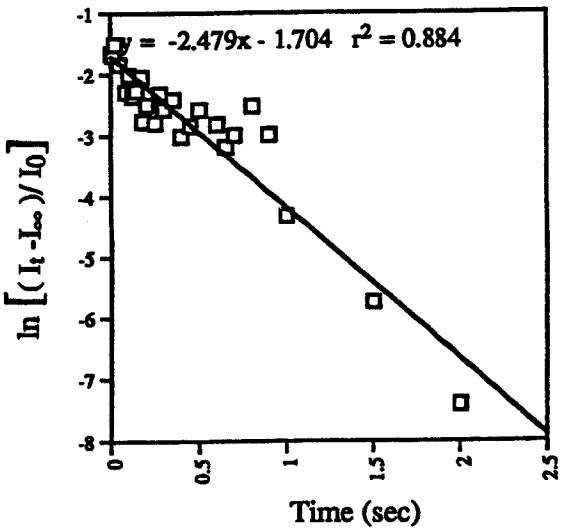

Fig. 5. 1-D ^{1}H-NMR saturation transfer experiment of the CyPA catalyzed Ser-Gln-Asn-Tyr-Pro-Ile-Val substrate at pH 6.8. The sample contains heptapeptide (2.0 mM), CypA (45 μM), and 50 mM KPi.

of the 1–3.0 ppm region of the spectrum for heptapeptide, heptapeptide:CyPA, and heptapeptide:CyPA:CsA. Comparison of the spectra of free heptapeptide (Fig. 6A) to the spectra of heptapeptide plus CyPA (Fig. 6B), reveals the appearance of exchange cross peaks (marked with arrows) between the $\gamma_c \leftrightarrow \gamma_T$ and $\beta_c \leftrightarrow \beta_T$ protons of the Pro residue. These cross peaks are eliminated upon addition of CsA (Fig. 6C).

Our studies indicate that CyPA catalyzes the *trans-cis* isomerization about the Tyr-Pro peptide bond in a model substrate, Ser-Gln-Asn-Tyr-Pro-Ile-Val, for a HIV-1 protease cleavage site in the *gag* polyprotein. These findings raise the interesting possibility that CyPA may be functioning in the HIV-1 virion to catalyze the generation of the conformers of *gag* and *gag-pol* precursor polyproteins necessary for cleavage by HIV protease.

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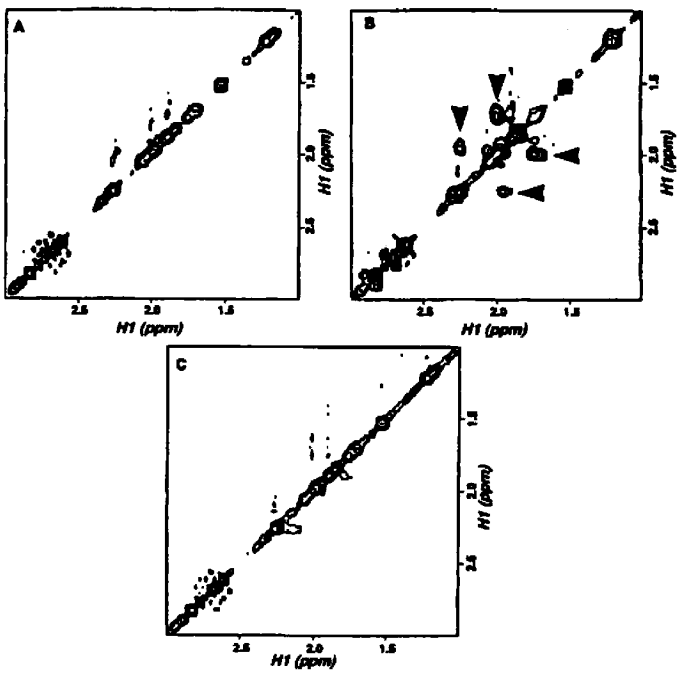

Fig. 6. Effect of CyPA and CsA on Ser-Gln-Asn-Tyr-Pro-Ile-Val at pH 6.8 using 2-D NOESY experiments. (A) Heptapeptide substrate (1.6 mM peptide). (B) Substrate (1.6 mM) with CyPA (25 μM). (C) Substrate (1.6 mM), CyPA (25 μM), and CsA (25 μM). Exchange cross peaks between the $\gamma_c \leftrightarrow \gamma_T$ and $\beta_c \leftrightarrow \beta_T$ protons of the Pro residue are indicated by the arrows.

References

[1] Peterlin, B.M. and Luciw, P.A. (1988) AIDS 2, (Suppl) S29–S40.
[2] Kohl, N.E., Emini, E.A., Schleif, W.A., Davis, L.J., Heimbach, J.C., Dixon, R.A.F., Scolnick, E.M. and Sigal, I.S. (1988) Proc. Natl. Acad. Sci. USA 85, 4686–4690.
[3] Darke, P.L., Leu, C.-T., Davis, L.J., Heimbach, J.C., Diehl, R.E., Hill, W.S., Dixon, R.A.F. and Sigal, I.S. (1989) J. Biol. Chem. 264, 2307–2312.
[4] Oroszlan, S. and Tözsér, J. (1990) Virology 1, 369–378.
[5] Luban, J., Bossolt, K.L., Franke, E.K., Kalpana, G.V. and Goff, S.P. (1993) Cell 73, 1067–1078.
[6] Klasse, P.J., Schulz, T.F. and Willison, K.R. (1993) Nature 365, 395–396.
[7] Franke, E.K., Yuan, H.E. and Luban, J. (1994) Nature 372, 359–362.
[8] Thali, M., Bukovsky, A., Kondo, E., Rosenwirth, B., Walsh, C.T., Sodroski, J. and Göttlinger, H.G. (1994) Nature 372, 363–365.
[9] Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T. and Schmid, F.X. (1989) Nature 337, 476–478.
[10] Handschumacher, R.E., Harding, M.W., Rice, J., Drugge, R.J. and Speicher, D.W. (1984) Science 226, 544–547.
[11] McKeon, F. (1991) Cell 66, 823–826.
[12] Schreiber, S.L. (1992) Cell 70, 365–368.
[13] Schreiber, S.L. and Crabtree, G.R. (1992) Immunol. Today 13, 136–142.
[14] Heitman, J., Movva, N.R. and Hall, M.N. (1992) New Biolog. 4, 448–460.
[15] Gething, M.J. and Sambrook, J. (1992) Nature 355, 33–45.
[16] Gamble, T.R., Vajdos, F.F., Yoo, S., Worthylake, D.K., Houseweart, M., Sundquist, W.I. and Hill, C.P. (1996) Cell 87, 1285–1294.
[17] Luban, J. (1996) Cell 87, 1157–1159.
[18] Cullen, B.R. and Heitman, J. (1994) Nature 372, 319–320.
[19] Schutkowski, M., Drewello, M., Wöllner, S., Jakob, M., Reimer, U., Scherer, G., Schierhorn, A. and Fischer, G. (1996) FEBS Lett. 394, 289–294.
[20] Karpas, A., Lowdell, M., Jacobson, S.K. and Hill, F. (1992) Proc. Natl. Acad. Sci. USA 89, 8351–8355.
[21] Rosenwirth, B., Billich, A., Datema, R., Donatsch, P., Hammerschmid, F., Harrison, R., Heistand, P., Jaksche, H., Mayer, P., Peichl, P., Quesniaux, V., Schatz, F., Schuurman, H.-J., Traber, R., Wenger, R., Wolff, B., Zenke, G. and Zurini, M. (1994) Antimicrob. Agents Chemother. 38, 1763–1772.
[22] Braaten, D., Franke, E.K. and Luban, J. (1996) J. Virol. 70, 3551–3560.
[23] Bartz, S.R., Hohenwalter, E., Hu, M.-K., Rich, D.H. and Malkovsky, M. (1995) Proc. Natl. Acad. Sci. USA 92, 5381–5385.
[24] Steinkasserer, A., Harrison, R., Billich, A., Hammerschmid, F., Werner, G., Wolff, B., Peichl, P., Palfi, G., Schnitzel, W., Mlynar, E. and Rosenwirth, B. (1995) J. Virol. 69, 814–824.
[25] Billich, A., Hammerschmid, F., Peichl, P., Wenger, R., Zenke, G., Quesniaux, V. and Rosenwirth, B. (1995) J. Virol. 69, 2451–2461.
[26] Gitti, R.K., Lee, B.M., Walker, J., Summers, M.F., Yoo, S. and Sundquist, W.I. (1996) Science 273, 231–235.
[27] Griffiths, J.T., Phylip, L.H., Konvalinka, J., Strop, P., Gustchina, A., Wlodawer, A., Davenport, R.J., Briggs, R., Dunn, B.M. and Kay, J. (1992) Biochemistry 31, 5193–5200.
[28] Martin, J.A. (1992) Antiviral Res. 17, 265–278.
[29] Liu, J., Albers, M.W., Chen, C.-M., Schreiber, S.L. and Walsh, C.T. (1990) Proc. Natl. Acad. Sci. USA 87, 2304–2308.
[30] Hsu, V.L., Handschumacher, R.E. and Armitage, I.M. (1990) J. Am. Chem. Soc. 112, 6745–6747.
[31] Campbell, I.D., Dobson, C.M., Ratcliffe, R.G. and Williams, R.J.P. (1978) J. Magn. Reson. 29, 397–417.