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The *cis/trans* interconversion of the calcium regulating hormone calcitonin is catalyzed by cyclophilin

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The cytosolic peptidyl-prolyl *cis/trans* isomerase cyclophilin from pig kidney can accelerate catalytically the *cis/trans* isomerization of prolyl peptide bonds. One- and two-dimensional ^{1}H NMR spectroscopy was used to prove that the polypeptide hormone calcitonin is a substrate for cyclophilin. Isomerization of only one of the two prolyl peptide bonds is catalyzed significantly. The efficiency of catalysis was calculated by lineshape analysis and NOESY spectroscopy. Cyclosporin A completely blocks the effect of the enzyme on the conformational dynamics of the polypeptide.

Cyclophilin; Peptidyl-prolyl *cis/trans* isomerase; Calcitonin; Cyclosporin A; ^{1}H NMR lineshape analysis; NOESY spectroscopy

1. INTRODUCTION

Peptidyl-prolyl *cis/trans* isomerases (PPIases) catalyse the *cis/trans* isomerization of prolyl peptide bonds in short peptides [1] and accelerate proline limited folding steps of proteins [2–4]. Furthermore, it has been discovered that cyclophilin and FK506BP, the specific binding proteins for the immunosuppressive drugs cyclosporin A (CsA) and FK506, are PPIases [5–8]. Binding of the drugs results in a strong inhibition of the isomerase activity. Recent investigations point to links between the immunosuppressive drugs, their binding proteins and calcium metabolism. It has been shown that the complexes cyclophilin-CsA and FK506BP-FK506 bind to and inhibit allosterically the Ca^{2+}-dependent phosphatase calcineurin [9–11]. However the question of the biological function of the uncomplexed PPIases still remains to be answered since the microbial substances FK506 and CsA are not consituents of mammalian cells. Therefore we search for natural substrates of these enzymes. Human calcitonin is a 32-residue peptide hormone of which the sequence is shown in the previous column.

It inhibits osteoclast activity and promotes Ca^{2+}-uptake of bones [12]. It is widely used as an anti-osteoporosis drug, but its mechanism of action still awaits a satisfactory explanation. As this polypeptide hormone plays a central role in Ca^{2+}-metabolism and contains two proline residues in its sequence we tested the effect of cyclophilin on the *cis/trans* isomerization of the prolyl peptide bonds in human calcitonin by ^{1}H NMR spectroscopy. Our experiments prove that calcitonin in its extended conformation is a substrate for cyclophilin showing a catalytic discrimination between the two prolyl peptide bonds.

1 5 10 15
Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-Gly-Thr-Tyr-Thr-Gln-Asp
└S——————S—┘
20 25
Phe-Asn-Lys-Phe-His-Thr-Phe-**Pro**-Gln-Thr-Ala-Iil-Gly-Val-

30
Gly-Ala-**Pro**-NH_2

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2. MATERIALS AND METHODS

Human calcitonin was a gift from E. Wünsch (MPI, Martinsried). It was dissolved in 10 mM potassium phosphate at pH 6.0 to a concentration of 0.86 mM. 10% D_2O was used for the lock.

The purification of cyclophilin is described in [5] and it was prepared for the NMR experiments according to the procedure given in [13] Aliquots of a stock solution of 190 μM were added to the calcitonin sample to a final concentration of 25 μM. The PPIase-activity was tested with the isomer-specific chymotrypsin test [5] with *cis*-Suc-AFPF-4-nitroanilide as a substrate before and after the NMR experiments.

CsA from Sandoz, Switzerland, was added as powder in excess to the calcitonin/cyclophilin-sample and incubated for 8 hours. Remain-

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ing undissolved CsA was removed by centrifugation. 100% inhibition of the isomerase activity was assayed as described above [5].

All ^{1}H NMR measurements were carried out on a GE Omega 500 MHz NMR spectrometer and transformed using the Omega software. Some data manipulation was done with the inhouse software MAGNE, written by P. Drakenberg. The NOESY data were acquired in the hypercomplex mode with the following parameters: spectral width 6410 Hz in both dimensions, 64 transients, 256 time increments with 2048 complex points and a mixing time of 300 ms. Shifted sine bell squared weight functions were applied in both dimensions and the data were zero filled to a final size of 2k × 1k. Determination of the *cis/trans* interconversion rate by 1D NMR spectroscopy was done by lineshape analysis using a program based on the Bloch equations modified to take exchange into account [14]. Calculation of this rate by 2D chemical exchange spectroscopy was done as described by Baine [15].

3. RESULTS

The sequence specific assignments of the ^{1}H NMR spectrum of human calcitonin have been determined by two-dimensional ^{1}H, ^{15}N and ^{13}C nuclear magnetic resonance spectroscopy (2D NMR). Nuclear Overhauser enhancement spectroscopy (NOESY) connectivities and circular dichroism measurements (data not shown) indicate an extended conformation of calcitonin in water with high flexibility (manuscript in preparation). Due to the two prolyl residues Pro^{23} and Pro^{32} in human calcitonin a maximum of four isomers is expected. Analysis of the ^{1}H NMR spectra reveals the existence of at least three different isomers of the hormone with a ratio of 67 : 25 : 8. Based on the typical NOE-connectivities for *cis*- and *trans*- Xaa–Pro peptide bonds [16b], the major isomer was found to be the all-*trans* conformer (67%), whereas in the two minor isomers the peptide bonds Phe^{22}–Pro^{23} (25%) or Ala^{31}–Pro^{32} (8%) are in the *cis* conformation. The amount of the isomer with both prolyl peptide bonds in the *cis* conformation is too low to be detectable.

Well resolved signals for the isomers (Fig. 1A) indicate that the rate of their interconversion is slow on the NMR time-scale at room temperature ($k < 0.2$ s^{-1}). In the selected region of the ^{1}H NMR spectrum only the δ-protons of the two prolyl residues are located with well separated signals for the isomers and therefore useful to prove an acceleration of their interconversion.

The NOESY spectrum was used for the assignments of the δ-protons of Pro^{23} and Pro^{32} (Fig. 1B). The cross peaks connect those protons which are in close proximity [16a]. The fact that we observe both negative and positive cross peaks in a NOESY spectrum proves the existence of flexible and more rigid regions in this polypeptide. The negative signal at 3.78/3.64 ppm and its mirror image are assigned to the NOESY cross peaks between the two δ-protons from the more flexible C-terminal Pro^{32}-*trans*. The positive signal at 3.78/3.58 ppm is accordingly assigned to the NOESY cross peak between the two δ-protons of Pro^{23}-*trans*. The signal at 3.48/3.35 ppm belongs to the two δ-protons of Pro^{23}-*cis*.

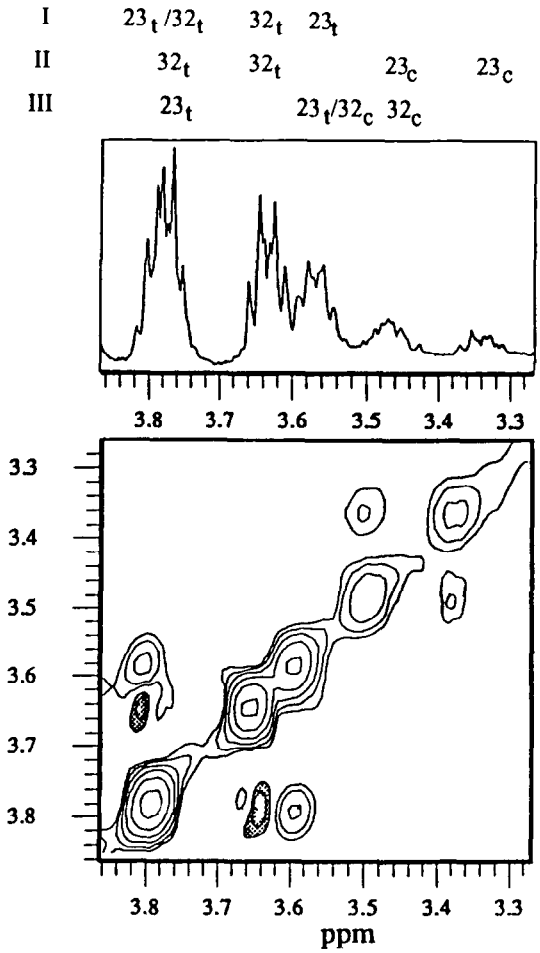

Fig. 1. Expansions of one- and two-dimensional ^{1}H NMR spectra of human calcitonin displaying the δ-proton region for Pro^{23} and Pro^{32}. Panel A shows a 1D spectrum with assignments. The all-*trans* isomer is labeled with I, the Pro^{23}-*cis* Pro^{32}-*trans* with II and the Pro^{23}-*trans* Pro^{32}-*cis* with III, the subscripts t and c indicate the conformation of the specific bond. Panel B depicts the corresponding expansion of a 2D NOESY spectrum with negative peaks shaded.

No cross peak between the δ-protons of Pro^{32}-*cis* was detectable, most likely due to unfavorable correlation times. The cross peak was, however, observed in a TOCSY spectrum allowing the assignment (data not shown).

In addition NOESY spectroscopy can be used to detect isomerization within a molecule, such as catalyzed *cis/trans* isomerization of a prolyl peptide bond. In that case cross peaks between signals from the *cis* and *trans* isomers are observed [14,17].

In order to prove a catalysis of the *cis/trans* isomerization of prolyl peptide bonds in native calcitonin by cyclophilin, we recorded ^{1}H NOESY spectra in the absence (Fig. 2A), in the presence of catalytical amounts of cyclophilin (Fig. 2B) and after inhibition of cyclophilin by CsA (Fig. 2C). Fig. 2B shows four additional cross peaks as a result of magnetisation transfer between the δ-protons of the *cis* and *trans* isomers of Pro^{23}

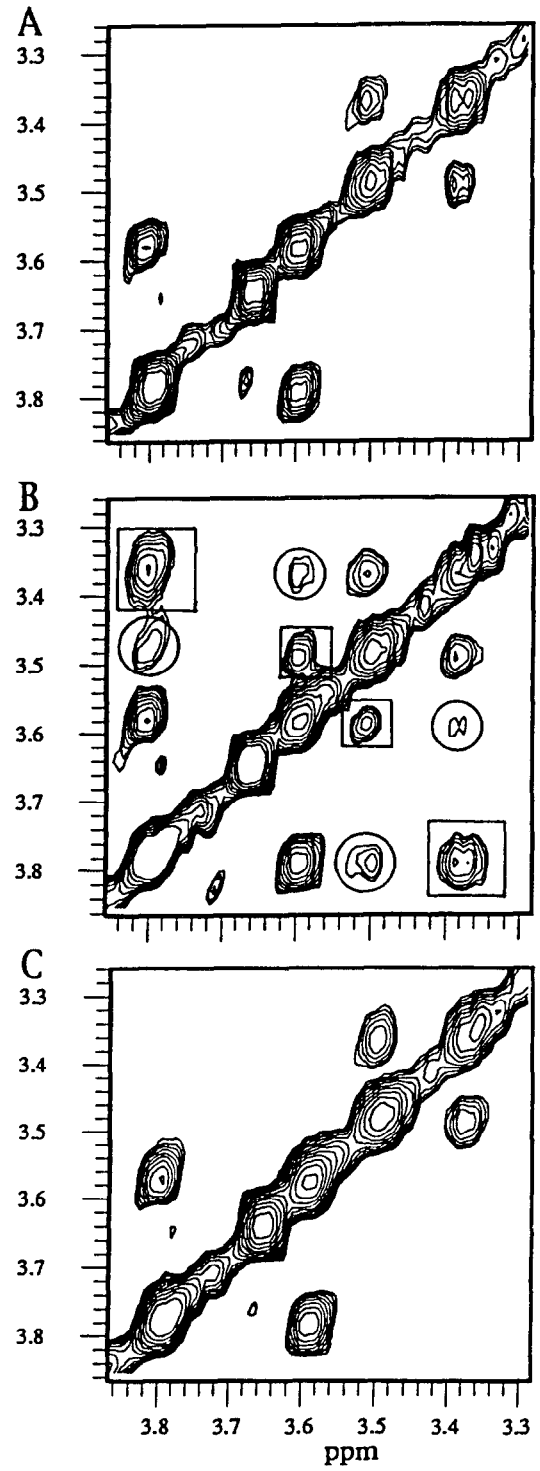

Fig. 2. NOESY spectra showing the catalytic effect of cyclophilin on calcitonin and the inhibition by CsA. The expansion is the same as in Fig. 1B showing positive cross peaks only. Sample and conditions as described in Fig. 1. (A) without cyclophilin, (B) in the presence of 25 μM cyclophilin, (C) sample (B) but incubated with excess of CsA for 8 h. The effects of cyclophilin are marked: boxes identify exchange cross peaks and circles mark conformational exchange-mediated NOESY cross peaks [23] between the δ-protons of the Pro^{23}-*cis* and the Pro^{23}-*trans* isomers.

in the presence of 25 μM cyclophilin. This proves that the interconversion between the *cis* and *trans* isomers of the Phe^{22}–Pro^{23} peptide bond is accelerated. Such exchange cross peaks are observed for several signals as a consequence of differences in chemical shift between the two isomers. Omitting those signals, the remaining spectrum is that of free calcitonin. This indicates that cyclophilin specifically catalyses the *cis/trans* isomerization of the Pro^{23} peptide bond of calcitonin. Additional support for the enzymatic catalysis results from experiments with the tight binding cyclophilin inhibitor cyclosporin A (CsA), which completely inhibits the isomerase activity of cyclophilin (Fig. 2C).

Determination of the efficiency of cyclophilin to catalyze the *cis/trans* isomerization of calcitonin requires knowledge of both the uncatalyzed rate without cyclophilin and the catalyzed rate in the presence of cyclophilin. The uncatalyzed *cis* → *trans* isomerization rate (k_0) of the Pro^{23} peptide bond in free human calcitonin was measured by 1D NMR spectroscopy using line shape analysis [14]. The protons bound to C_2 in His20 in the *cis* and *trans* isomers show well resolved singlets (Fig. 3A) and were therefore used to determine k_0 and the free energy of activation ($\Delta G^{\#}$) for the *cis/trans* isomerization. Fig. 3A–C shows the temperature dependence of the lineshapes and the corresponding calculated spectra with the used *cis* → *trans* isomerization rates. According to the Eyring Equation (1), a plot of $\ln(k_{c\rightarrow t}/T)$ versus $1/T$ for six temperatures between 338K and 363K can be linearly fitted with $y = 23.4 - 9702\times$ and a correlation coefficient of 0.9966. Extrapolation of the linear fit to 300K gives a $k_0 = 0.03\ s^{-1}$. The $\Delta G^{\#}$ at this temperature was calculated to 83 kJ/mol.

$$\ln(k_{c\rightarrow t}/T) = \Delta S^{\#}/R + \ln(R/N\times h) - \Delta H^{\#}/R\times 1/T \quad (1)$$

The same His20 signals were used to obtain the rate constant in presence of 25 μM cyclophilin (k_{obs}). The signal of the *cis* isomer in the spectrum shows a line broadening in the presence of cyclophilin (Fig. 3D) compared to the *cis* signal in the absence of this enzyme (Fig. 3A). This effect is due to the accelerated rate of interconversion which was calculated as $k_{obs} = 1.6\ s^{-1}$ by line shape analysis [14]. This k_{obs} was compared to the value obtained by 2D NMR exchange spectroscopy for the same sample. From the ratio of the diagonal peak to the exchange cross peak [15] a $k_{obs} = 1.3\ s^{-1}$ was obtained showing a good agreement with the results of the line shape analysis.

Interestingly, no catalysis of the Pro^{32} peptide bond was detectable under these conditions, even though the uncatalyzed interconversion rate was the same ($k_0 = 0.03\ s^{-1}$) as that of Pro^{23}. The value for Pro^{32} could be determined with the same method as employed for Pro^{23} but using the *cis* and *trans* signals of the β-protons of Ala^{31}. In the presence of 25 μM cyclophilin, no line

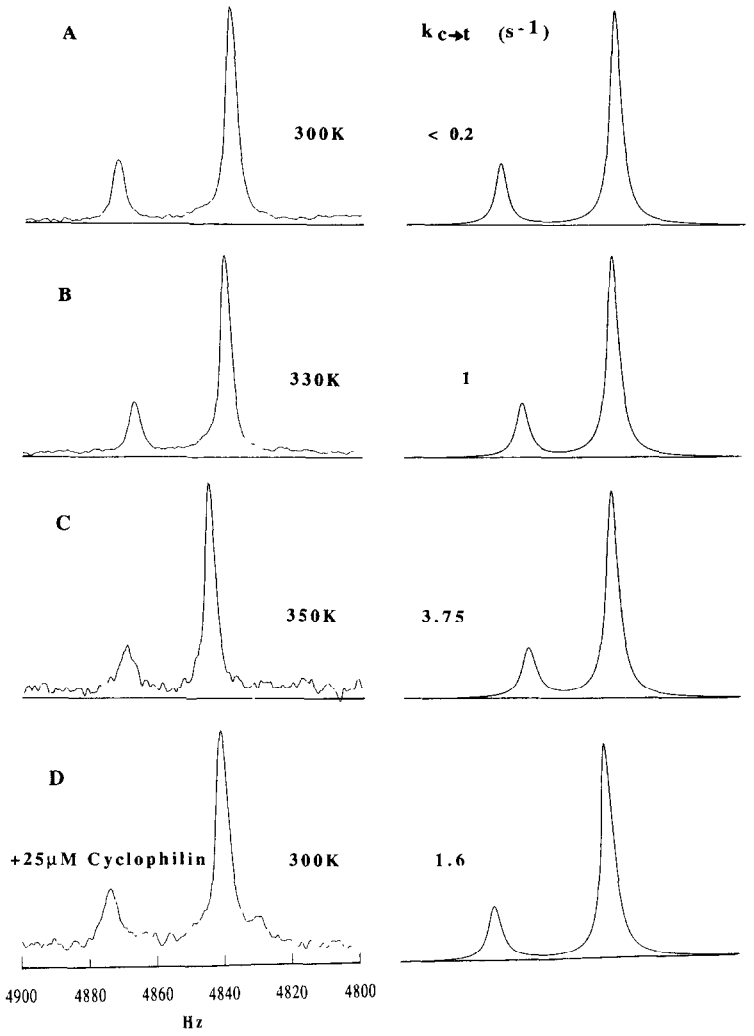

Fig. 3. Left column: Expansion of the 1D ^{1}H NMR spectrum of human calcitonin showing the *cis* and *trans* signals of the C2-protons of His20 at the indicated temperatures (A–C) and in the presence (D) and absence (A) of cyclophilin at the same temperature. Right column: Corresponding calculated spectra using line shape analysis [14]; the used *cis* → *trans* isomeration rates $k_{c\to t}$ are shown respectively.

broadening in the 1D spectrum and no exchange crosspeaks in the 2D spectrum were observed for signals characterizing the *cis* and *trans* isomer of the Ala^{31}–Pro^{32} peptide bond. Therefore the isomerisation rate is smaller than 0.1 s^{-1}, which is the lower limit detectable with these methods. Thus our results clearly show a sequence specificity of cyclophilin for Pro^{23}. This is somewhat surprising in view of the observation by Hsu et al. [18] that cyclophilin will catalyze the *cis/trans* isomerization of small peptides in which Pro is the C-terminal amino acid. However the substrate investigated by Hsu was the free acid whereas calcitonin is amidated at its C-terminal proline.

4. DISCUSSION

Our results demonstrate for the first time that cyclophilin catalyzes *cis/trans* interconversion of a fairly large polypeptide. Up to now only short peptides or refolding proteins have been shown to be substrates for cyclophilin [2–5,18,19]. Previous efforts to catalyze *cis/trans* isomerization in the protein calbindin in its native state [20] by cyclophilin were not successful. We note, however, that even though the prolyl residue is in a flexible loop, the protein has a globular and compact structure that may result in a steric hindrance of interaction between the protein and cyclophilin.

The fact that only the *cis/trans* isomerization of the peptide bond Phe^{22}–Pro^{23} and not Ala^{31}–Pro^{32} is accelerated significantly by cyclophilin clearly shows a sequence specifity within one substrate.

Recent discoveries support evidence for a connection between cyclophilin and the important intracellular regulatory systems that involve calcium-ions and protein phosphorylation [9,10]. Our experiments prove that calcitonin, a hormone important for the regulation of

Ca^{2+}-uptake and Ca^{2+}-homeostasis, is a naturally occurring substrate for cyclophilin. In the presence of CsA, cyclophilin can not catalyse the *cis/trans* isomerization of calcitonin. At times of calcium stress calcitonin protects the skeleton by inhibiting osteoclast activity [12]. CsA, however, shows the inverse effect producing high bone remodeling with resorption exceeding formation causing a significant bone loss [21,22]. Further studies will show the biological relevance of our findings.

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