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Peptidyl-prolyl *cis-trans* isomerase activity as studied by dynamic proton NMR spectroscopy

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Recently the identity of the peptidyl-prolyl *cis-trans* isomerase (PPIase), which accelerates the *cis/trans* isomerization of prolyl peptide bonds and cyclophilin, the binding protein for the immunosuppressive drug Cyclosporin A (CsA), was discovered. The PPIase catalysis toward the substrate Suc-Ala-Phe-Pro-Phe-pNA has been studied by ^{1}H NMR spectroscopy. Using the bandshape analysis technique the rate of interconversion between the *cis* and *trans* isomers of the substrate could be measured in the presence of PPIase and under equilibrium conditions. The acceleration is inhibited by equimolar amounts of CsA. The results provide evidence that the PPIase catalysis is more complex than a simple exchange between two states.

Peptidyl-prolyl *cis-trans* isomerase; Cyclophilin; Cyclosporin A; Prolin *cis-trans* isomerism; ^{1}H dynamic NMR; Bandshape analysis

1. INTRODUCTION

The enzyme peptidyl-prolyl *cis-trans* isomerase (PPIase) is the first isolated member of a new family of enzymes, which catalyses conformational interconversion within peptides and proteins. It accelerates the *cis/trans* isomerization of prolyl amide bonds [1] and the rate limiting step in the refolding of several proteins [2–5]. Recently it was discovered that the 17 kDa PPIase from pig kidney is identical to cyclophilin, the binding protein of the immunosuppressive drug cyclosporin A (CsA) [6,7]. The establishment of CsA as a tightly binding inhibitor of prolyl isomerase revealed a new relationship between *cis/trans* peptide bond isomerism, protein folding and signal transduction in the immune system. Furthermore, it has recently been shown that the specific binding protein for another immunosuppressive drug, FK506, is also a prolyl *cis-trans* isomerase [8,9]. The connection between the isomerase activity and the immune response is, however, still unknown. To answer these complex questions a clear understanding of the mechanism of PPIase catalysis will be necessary. In order to obtain more reliable data on the catalytic activity of PPIase we have used dynamic NMR spectroscopy to study the acceleration of the isomerization rate in the substrates Suc-Ala-Xaa-Pro-Phe-pNA (Xaa = Ala, Phe) as a function of PPIase concentration. This technique allows us to study the catalytic activity under equilibrium conditions, contrary to chymotrypsin coupled spectrophotometric method, which acts irreversibly [1]. Furthermore, we are not limited to substrates having the nitro-phenyl group.

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2. MATERIALS AND METHODS

PPIase was purified according to the preparation scheme previously published [6]. Purity was checked by SDS-PAGE showing a single band for PPIase. The protein used in the NMR experiments was diafiltrated into 50 mM phosphate buffer, pH 6, and concentrated by ultrafiltration to a concentration of 570 μM. The molar concentration of PPIase was determined by monitoring A_{280} using an extinction coefficient of 8140 $M^{-1} \cdot cm^{-1}$.

The enzyme activity of PPIase was monitored by using the chymotrypsin test [1].

Substrate peptides were from Bachem, Switzerland, and were dissolved to a concentration of 5 mM in a buffer containing 50 mM sodium phosphate, pH 6.0, and 10% D_2O used as lock in the NMR spectrometer. CsA was a gift from Sandoz, Switzerland, and was dissolved in methanol-d_4 to a concentration of 530 μM. Aliquots from a stock solution of PPIase (10.1 mg/ml) were added directly into the 5 mm NMR tube.

^{1}H NMR measurements were performed at 500.13 MHz on a GE Omega 500 spectrometer and the acquired data were processed using Omega software on a Sun 3/260 work station.

The theoretical spectra used to determine the *cis/trans* interconversion rate were calculated on a Motorola 68000 based computer using a program based on the Block formalism as modified by McConnell [10] to take exchange into account.

3. RESULTS

The ^{1}H NMR bandshape analysis technique was used to study the acceleration of the *cis/trans* isomerization of different peptides due to added PPIase as well as its inhibition by CsA. In this study we initially used the

standard substrate Suc-Ala-Ala-Pro-Phe-pNA (data not shown). Unfortunately for this peptide the *cis/trans* ratio is only 0.1 making it difficult to reliably quantify the isomerase activity by bandshape analysis. Therefore the substrate Suc-Ala-Phe-Pro-Phe-pNA with a *cis* content of 27% has been used throughout this investigation even though the k_{cat}/K_m for PPIase catalysis is lower than that for the standard substrate [11].

The uncatalyzed *cis/trans* interconversion is slow on the NMR time scale at 10°C, resulting in sharp, well resolved, signals from the *trans* and *cis* isomers, in particular for the methyl protons from alanine (Fig. 1, left) and the *ortho*-protons from the nitroanilide ring (Fig. 2). Addition of increasing amounts of PPIase results in a successive broadening of the corresponding signals and eventually a merging of them into one resonance, as can be seen from Figs 1 and 2. This shows that the rate of the *cis/trans* isomerization has been accelerated. The enzyme catalyzed enhancement of the prolyl peptide bond isomerization can be determined by comparing calculated and experimental bandshapes as exemplified in Fig. 1. The transverse relaxation time, T_2, was obtained from a signal not affected by the exchange and the chemical shifts were internally referenced to an impurity signal. The uncatalyzed *cis*⟶*trans* interconversion rate in the free peptide is 4.3×10^{-3} s^{-1} at 10°C, as could be determined by isomer specific proteolysis [1]. Based on the NMR experiments we find that the rate enhancement factor performed by PPIase is 6 per μg enzyme, whereas the value obtained by the chymotrypsin test is 14.

In the calculation of the theoretical spectra a lineshape program valid for exchange between 2 protons spin coupled to another proton was used. As can be seen from Fig. 1d it is impossible to obtain an overall satisfactory agreement between experimental and calculated spectra using a simple model with an exchange between 2 sites only. An extra broadening and change in the chemical shift of the *cis* signal is obvious. Another manifestation of this effect can be seen in Fig. 2 where also the resonance from the *meta*-protons from the nitroanilide ring broadens and shifts as a function of added enzyme even though the chemical shift is the same for the *cis* and *trans* isomers for these protons. We should consider that the maximum of the enzyme/substrate ratio for all experiments is only 0.048. Therefore a simple association to a protein/peptide complex cannot be the explanation to the observed extra broadening.

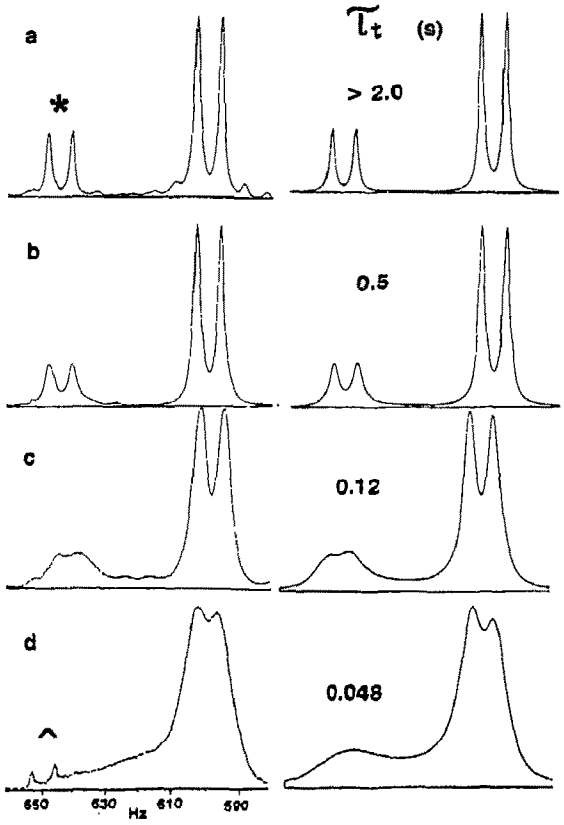

Fig. 1. 500 MHz ^{1}H NMR spectra of Suc-Ala-Phe-Pro-Phe-pNA showing the region with the alanyl methyl group, at 10°C and pH 6.0, [peptide] = 5 mM. The signal marked * is from the *cis* isomer. Signals marked ^ are from impurities in the PPIase solution. *Left column:* experimental spectra. (a) Without PPIase, (b)–(d) with 24 μM, 104 μM and 240 μM PPIase, respectively. *Right column:* calculated bandshapes. τ_t indicates the lifetimes of the *trans* isomer.

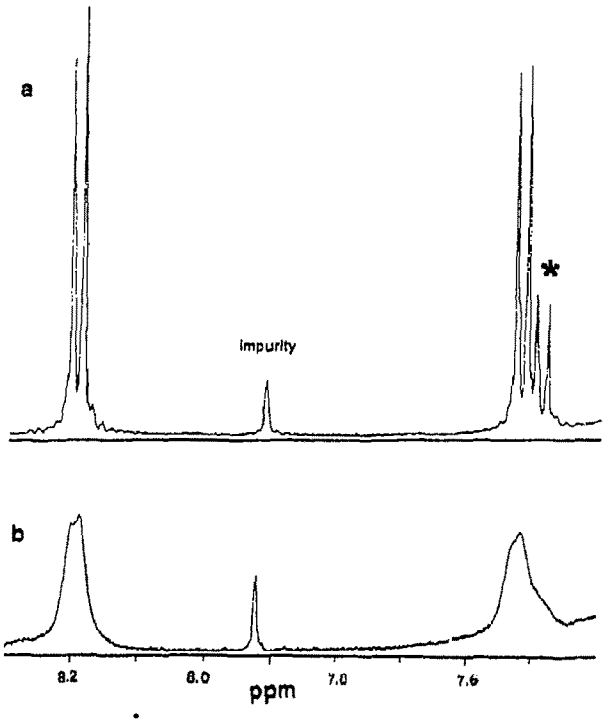

Fig. 2. 500 MHz ^{1}H NMR spectra of Suc-Ala-Phe-Pro-Phe-pNA showing the *meta*- (left) and *ortho*-protons (right) of *p*-nitroanilide. Conditions for (a) and (b) as in Fig. 1a and d, respectively.

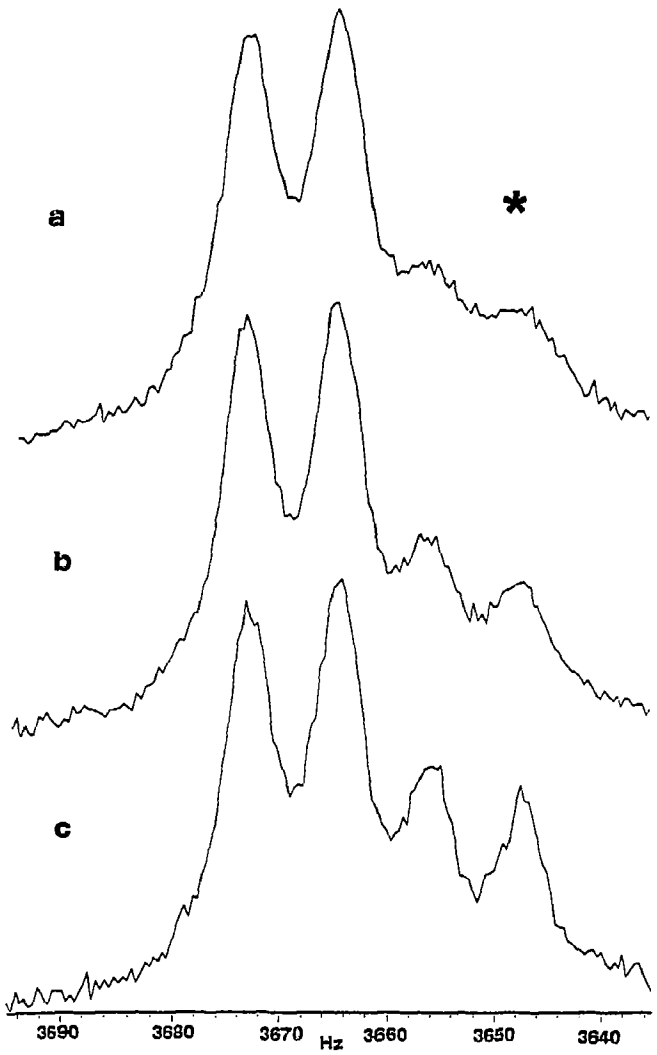

Fig. 3. The *ortho*-proton signals from *p*-nitroanilide of Suc-Ala-Phe-Pro-Phe-pNA. General conditions as in Fig. 1. [E] = 50 μM (a) without CsA, (b) with 35 μM CsA, and (c) with 55 μM CsA.

Fig. 3 shows the effect of addition of CsA. From this figure it is evident that the inhibition of the PPIase activity is complete when approximately one equivalent of CsA has been added.

4. DISCUSSION

Dynamic NMR spectroscopy allows us to study the PPIase catalysis directly and in more detail without changing the *cis/trans* equilibrium, contrary to the indirect and irreversible chymotrypsin coupled test. We could thus show that PPIase accelerates the reversible *cis/trans* interconversion and that the immunosuppressive drug CsA inhibits the isomerase activity, also in the NMR experiment. However, the enzyme activity calculated by means of NMR bandshape analysis is significantly lower than the one obtained from the isomer specific proteolysis. We have to consider that the conditions for these experiments are quite different. In the isomer-specific proteolysis system PPIase catalysis acts on the pure *cis* isomer since all initially present *trans* peptide is cleaved by chymotrypsin during a fast initial process. The Suc-Ala-Phe-Pro-Phe peptide does not inhibit the PPIase reaction, whereas the presence of the uncleaved *trans* peptide in the ^{1}NMR experiment can be looked upon as a product inhibition. Assuming that the binding constants for *cis* and *trans* isomers to the active site are the same and that the peptide concentration is above the K_m, this will result in a 9-fold higher rate in the chymotrypsin test as compared to NMR for Suc-Ala-Ala-Pro-Phe-pNA and about 3-fold for Suc-Ala-Phe-Pro-Phe-pNA. This is also close to what we have observed.

The results from the bandshape analysis provide, furthermore, evidence that the assumed model of a simple exchange between 2 states may be inadequate since there is additional broadening and shift of several signals besides that expected due to the *cis/trans* exchange.

It may also be worth pointing out that this result could be obtained using the oldfashioned 1D NMR experiments but could not be observed using more sophisticated experiments like 2D NOESY [12] or saturation transfer [13].

Another advantage in using NMR instead of the chymotrypsin coupled test is that there is no longer a need for the C-terminal chromogenic residue as has also been pointed out by Hsu et al. [13]. This opens new possibilities to probe substrate specificity of PPIase and other prolyl isomerases and to investigate their effect on biologically active peptides.

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