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Kinetic β-deuterium isotope effects suggest a covalent mechanism for the protein folding enzyme peptidylprolyl *cis/trans*-isomerase

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The *cis/trans* interconversion of Glt-Ala-Ala-Pro-Phe-4-nitroanilide and Glt-Ala-Gly-Pro-Phe-4-nitroanilide was studied both enzymatically and nonenzymatically by measuring kinetic β-deuterium isotope effects. The hydrogen atom at the α-carbon atom of the Xaa residue within the Xaa-Pro moiety was substituted by deuterium. In the nonenzymatic case the transition state of rotation is reflected by $k_H/k_D > 1$. When catalysed by 17 kDa PPIase the same bond rotation is characterized by $k_H/k_D < 1$. This suggests a covalent mechanism of catalysis which involves an approximately tetravalent carbon of the prolyl imidic bond for the transition state of reaction.

cis/trans interconversion; Prolyl imidic bond; Deuterium isotope effect; Prolyl *cis/trans*-isomerase; Cyclophilin; Cyclosporin

1. INTRODUCTION

PPIases have been discovered recently in pig kidney cortex by an assay which is based on isomer specific proteolysis using α-chymotrypsin. This protease exhibits a distinct conformational specificity for substrates with proline in P_2-position [1,2]. Several PPIases have been found in kidney but the 17 kDa enzyme was most abundant. Its amino acid sequence is identical with the sequence of bovine cyclophilin and nearly identical with human splenocyte cyclophilin recognized as the putative cytosolic receptor of the immunosuppressant undecapeptide CsA [3,4]. The 17 kDa enzyme has the unique property of accelerating in vitro the formation of the native structure in unfolded proteins [5-7]. The reason why several unfolded proteins loose their slow kinetic phases during the refolding reaction in the presence of PPIase is quite obvious. The rate limiting step of some slow phases is the *trans* → *cis* interconversion of -Xaa-Pro- bonds and consequently the rate of folding is increased specifically by the 17 kDa enzyme. A single sulphydryl group appears to be essential for the enzymatic activity [3]. However, the catalytic mechanism is unknown at present. Here we have measured both the rates of *cis* → *trans* interconversions and the conformational equilibra of specifically ^{2}H-labelled substrates of the 17 kDa PPIase. The purpose is to compare the data with the protium analog of the substrates. The paper reports evidence concerning the transition states of imidic peptide bond rotation. These are utilized to provide a description for the enzymatic catalysis that includes a tetrahedral intermediate. This unstable compound is formed by addition of an enzymatic nucleophile to the carbonyl group of the prolyl peptide bond.

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Abbreviations: PPIase, peptidylprolyl *cis/trans*-isomerase; β-^{2}H KSIE, kinetic β-deuterium isotope effect; CsA, cyclosporin A; Glt, glutaryl

2. EXPERIMENTAL

2.1. *Materials*

17 kDa PPIase was obtained from pig kidney cortex as

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described elsewhere [1,5]. Preparations used here generally showed a specific activity of 140 U/mg protein. The enzyme migrates as a single band in the reducing SDS-PAGE. It is a mixture of two closely related PPIases differing in their isoelectric points [4]. Chymotrypsin (350 U/mg, from bovine pancreas) was purchased from Merck, Darmstadt.

Glt-Ala-Ala-Pro-Phe-4-nitroanilide (m.p. 114–117°C) and Glt-Ala-Gly-Pro-Phe-4-nitroanilide (m.p. 142–144°C) as well as their ^{2}H-labelled congeners were prepared by the stepwise method described previously [8]. Ala-2-$^2H_1$ and Gly-2-$^2H_2$ were synthesized by exchange of ^{2}H from ^2H_2O/acetic anhydride using Ac-Xaa-OH as the starting material. After dissolving of the acylated amino acid in boiling acetic anhydride, ^2H_2O (99.7%, Isocommerz, Berlin) was added in portions. The mixture was held 3 h at 30°C and then evaporated in vacuo. This procecure was repeated five times. After the final evaporation the residue was taken up in EtOAc and crystallized. The racemic Ac-Ala-2-$^2H_1$ was dissolved in 60 ml ^2H_2O and subjected to deacylation with acylase I (from porcine kidney, Serva, Heidelberg) at pH 7.5, held under pH-stat conditions for 8 h at 37°C. After the pH had been adjusted to 5.5, treatment with charcoal and subsequent evaporation of the solvent occurred. Ala-2-$^2H_1$ could be crystallized from EtOH.

The ^{2}H content of the amino acid was calculated from ^{1}H-NMR spectra. The yields were 80% ^{2}H in Ala-2-$^2H_1$ and 98% ^{2}H in Gly-2-$^2H_2$.

2.2. *Determination of β-^{2}H KSIEs*

The general method of isomer-specific proteolysis performed using α-chymotrypsin was described elsewhere [1,8]. The reaction was conducted in silica cells (d = 1 cm) containing 1.45 ml reaction solution maintained at 1.4°C in the primary sample compartment of a Perkin-Elmer 356 UV-VIS spectrophotometer. The reactions were started by injection of 10 μl peptide substrate (in EtOH) into the buffer solution containing PPIase (stock solution ~ 1 mg protein/ml) and α-chymotrypsin (20–60 μM). The final substrate concentration for the determination of isotope effects was in the range of 31.4–50.1 μM. Mixing of the reaction solution was performed manually.

The substrates exhibited the following catalytic constants for chymotryptic hydrolysis.

Glt-Ala-Ala-Pro-Phe-4-nitroanilide: k_{cat} 37.1 s^{-1}, K_m 73 μM, k_{cat}/K_m 5.1 × $10^5 M^{-1} s^{-1}$.

Glt-Ala-Gly-Pro-Phe-4-nitroanilide: k_{cat} 10.4 s^{-1}, K_m 120 μM, k_{cat}/K_m 8.7 × $10^4 M^{-1} s^{-1}$.

The conditions for the determination of catalytic constants were 0.035 M Hepes buffer at pH 7.8 containing 0.2 M NaCl and 0.01 M Ca^{2+} at 25°C. K_m values are corrected for the percentage of unreactive *cis*-isomer in solution.

Rate constants for the *cis* → *trans*-interconversion were evaluated by fitting the data to the integrated first-order rate equation by nonlinear least-square analysis using 20 appropriately spaced data points. The standard deviations were less than 3% of a single measurement.

Initial rates were calculated from the first-order rate constants. No deviation occurs in the first-order kinetics even at the first data point measured after the dead time of mixing (~ 15 s). Coincidentally, even at the highest substrate concentration used here in the PPIase catalysed reaction (1.09 mM (*cis* + *trans*) Glt-Ala-Ala-Pro-Phe-4-nitroanilide) no deviation from first-order kinetics was obtained during the entire kinetic trace.

The products of the reaction (*cis* + *trans*) Glt-Ala-Xaa-Pro-Phe-O$^-$ and 4-nitroaniline show negligible influence on the kinetics of the PPIase catalysed interconversion.

3. RESULTS AND DISCUSSION

It is generally accepted that β-^{2}H KSIEs of carbonyl reactions are predominantly hyperconjugative in origin [9]. Thus, gain of hyperconjugation in going from reactant to transition state of the nonenzymatic *cis* → *trans* interconversion is

Table 1

First-order rate constants k and equilibrium constants K (K = [cis]/[trans]) for the uncatalyzed *cis* → *trans* interconversion of Glt-Ala-Xaa-Pro-Phe-4-nitroanilide in 0.035 mol · l^{-1} Hepes buffer, pH 7.8, at 1.4°C[a]

Xaa	k (× 10^{-3} s^{-1})	K	k_H/k_D	K_H/K_D
-Ala-	2.12 ± 0.05	0.1082 ± 0.0004		
-Ala-2-$^2H_1$-	2.03 ± 0.04	0.1094 ± 0.0009	1.053 ± 0.03[b]	0.989 ± 0.009[b]
-Gly-	1.79 ± 0.04	0.1223 ± 0.0008		
-Gly-2-$^2H_2$-	1.70 ± 0.03	0.1250 ± 0.0006	1.053 ± 0.03	0.981 ± 0.008

[a] From least square fitting of 12 replicate pairwise determinations (^{1}H-substrate and ^{2}H-substrate in alternation) of first-order rate constants

[b] Corrected for 20% undeuterated substrate

Comparison of matched pairs of isotopic substrates instead of calculating the mean values of the entire body of data yields identical isotope effects but smaller limits of error than indicated in the table

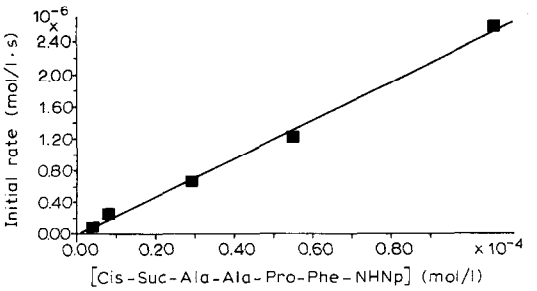


Fig.1. Plot of initial rate against substrate concentration of the 17 kDa PPIase (3.6 nM) catalyzed *cis* → *trans* interconversion in 0.035 M Hepes buffer (pH 7.8) at 2°C.

documented by $k_H/k_D > 1$ (table 1). This situation is expected to result from a major loss of resonance stability of the peptide bond in the transition state. In the same way the isotope effects k_H/k_D and K_H/K_D which are indicative of the *trans* → *cis* interconversion suggest a nearly symmetric transition state with the proline ring perpendicular to the plane of the peptide bond. The magnitude of the β-^{2}H KSIE is independent of the number of ^{2}H. This is consistent with the restricted conformational mobility of the -Xaa-Pro- bond (dihedral angle ψ) found by others [10], which is observed even if the proline containing sequences are truncated.

For the interpretation of the β-^{2}H KSIEs in the presence of the 17 kDa PPIase it is necessary to evaluate the enzyme kinetics of the irreversible *cis* → *trans* interconversion under the specific conditions of the assay. As shown in fig.1, the rate can be expressed as k_{cat}/K_m within the range of substrate concentrations (31.4–50.1 μM; sum of [cis] and [trans]) used in the determination of β-^{2}H KSIEs.

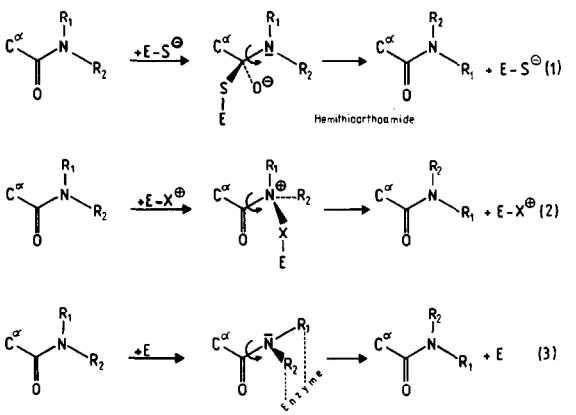
Table 2 reports the β-^{2}H KSIEs of two substrates at various concentrations of PPIase. No significant dependence on the enzyme concentration of the isotope effect was observed. However, in contrast to their nonenzymatic counterpart the enzymatic β-^{2}H KSIEs are all inverse in their nature. We interpret this to originate from the loss of hyperconjugation in the transition state of the reaction of the free enzyme with the *cis*-substrate. This implies that the sp^2 hybridisation of the carbonyl group disappears during reaction [11–13]. The magnitude of the KSIE depends on several factors [14–16], among which the remaining carbonium ion character of the transition state is the most important. In the light of the relatively large k_H/k_D obtained for the PPIase reaction, a nearly tetrahedral transition state (or intermediate) of bond rotation may be assumed. It appears further that the peptide conformation favours hyperconjugative orbital overlap. Three possible catalytic mechanisms for PPIase are proposed in scheme 1. Only the nucleophilic pathway (scheme 1, pathway 1) is in agreement with the β-^{2}H KSIEs observed here. The tetrahedral state whose formation is connected with β-^{2}H KSIEs < 1 allows free rotation of the C-

Table 2

First-order rate constants k for the 17 kDa PPIase catalyzed *cis* → *trans* interconversion of Glt-Ala-Xaa-Pro-Phe-4-nitroanilide at various enzyme concentrations[a]

PPIase (μg/ml)	k_H ($\times 10^{-2}$ s^{-1})	k_D ($\times 10^{-2}$ s^{-1})	k_H/k_D
Xaa = -Ala- and -Ala-$^2H_1$-			
0.306	1.59 ± 0.09	1.72 ± 0.1	0.909 ± 0.08[b]
0.441	2.24 ± 0.07	2.42 ± 0.07	0.911 ± 0.04[b]
0.772	3.66 ± 0.07	3.97 ± 0.07	0.906 ± 0.03[b]
Xaa = -Gly- and -Gly-$^2H_2$-			
0.482	1.16 ± 0.05	1.28 ± 0.06	0.906 ± 0.06
0.811	1.78 ± 0.04	1.93 ± 0.04	0.922 ± 0.03
1.20	2.51 ± 0.07	2.74 ± 0.09	0.916 ± 0.04
1.44	3.31 ± 0.08	3.63 ± 0.09	0.912 ± 0.04

[a] From least square fitting of 7 replicates pairwise determinations
[b] Corrected from 20% undeuterated substrate
For the conditions see table 1



Scheme 1. Hypothetical mechanisms for PPIase catalyzed reactions. The nonenzymatic peptide bond rotation resembles pathway 3, but necessarily lacking the transition state stabilization by the protein.

N bond. The finding of a single essential thiol group for catalytic activity [3] points to a possible candidate for the enzymatic nucleophile. Phase transfer of the substrate into the putative nonpolar active site of 17 kDa PPIase, thought to be responsible for the rate enhancement of the enzymatic *cis* → *trans* interconversion [17] (scheme 1, pathway 3) should produce a small β-^{2}H KSIE >1 [18]. Therefore this mechanism is ruled out by the observed data. The nucleophilic mechanism of catalysis of the 17 kDa PPIase suggested here could explain the tight binding of this protein (cyclophilin) to CsA. This cyclic peptide contains a single *cis*-peptide bond in the solid state and in apolar solvents located N-terminally to MeLeu 10 [19]. Within cells, CsA is mainly bound to 17 kDa PPIase. In vivo investigations show that unlike MeLeu 4, MeLeu 6 and MeLeu 9, the MeLeu 10 residue is well protected from demethylation and oxidation by metabolic enzymes [19]. It follows that the MeLeu 10 residue should not be freely accessible within the CsA/cyclophilin complex, It could therefore be reasoned that this amino acid residue is buried in the active center of 17 Kda PPIase and may be involved in the strong binding (K_i = 2.6 nM [3]) of CsA by PPIase. Thus, it is not unreasonable to assume that a covalent bond in the CsA/cyclophilin complex contributes to the tightness of the binding reaction. This bond is indicated as hemithioorthoamide in scheme 1, pathway 1.

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