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THE DETECTION OF PROLINE ISOMERASE ACTIVITY IN FK506-BINDING PROTEIN BY TWO-DIMENSIONAL ^{1}H NMR EXCHANGE SPECTROSCOPY

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Summary: ^{1}H NMR assignments of the trans and cis isomers of succinyl-Ala-Ala-Pro-Phe-p-nitroanilide were accomplished by two-dimensional NMR techniques. Conformational exchange between the cis and trans isomers was not detected in the two-dimensional exchange spectra (NOESY) until catalytic amounts of FK506-binding protein (FKbp) were added. The addition of FK506 to the enzyme-substrate solution inhibited the enzyme and removed the substrate exchange peaks.

Cyclosporin A (CsA) and FK506, immunosuppressive agents that find utility in organ transplantation, have been found to bind tightly to proteins that also display cis/trans peptidyl prolyl isomerase (PPI) activity (1-4). Both of these compounds inhibit activation of T-cells, presumably by blocking the production of early T-cell activation genes, for example interleukin-2 (5). The relation between the inhibition of T-cell activation and the binding of CsA and FK506 to their respective isomerases remains obscure, although it has been suggested that these compounds interfere either with the function of T-cell specific transcription factors or with a T-cell specific signal transmission system (6).

The measurement of PPI activity relies on the biphasic cleavage by chymotrypsin on the substrate succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Suc-AAPF-pNA) (7). The biphasic nature of this proteolysis is caused by the enzyme's inability to cleave the substrate when the Ala-Pro amide bond has the cis conformation (8). The cis portion of the substrate then undergoes thermal isomerization to the trans form, which can then be cleaved by chymotrypsin. The PPI activity is measured by examining the increase in the final-phase cleavage.

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A more direct method for measuring PPI activity would be to follow the substrate cis/trans exchange via ^1H NOESY spectra. The feasibility of this technique to observe the activity of FKbp is demonstrated.

Methods

The substrate Suc-AAPF-pNA, purchased from Sigma Chemical Co., was determined to be pure by high pressure liquid chromatography.

FKbp was expressed using a cDNA clone encoding human FKbp obtained from G. Verdine and S. Schreiber (Harvard Univ.). The insert was subcloned into a lambda cI857 regulated lambda PL-expression plasmid such that the FKbp cDNA insert encoded the second cistron of a two cistron expression scheme (9). E. coli cells bearing this plasmid were grown at 32° and expression was induced with a shift to 42°. The FKbp was purified by a protocol adapted from one supplied by Verdine and Schreiber. After 4 hr. cells were harvested, then suspended in 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 2 mM $CaCl_2$, 10 mM $MgCl_2$, 5% glycerol, and lysed with sonic bursts. The lysate was centrifuged and the supernatant applied to DEAE-cellulose equilibrated with 50 mM Tris-Cl, pH 7.5, 2 mM 2-mercaptoethanol. Protein fractions not absorbing to the column were incubated at 56° for 20 min. and then centrifuged. Ammonium sulfate was added to the supernatant till the concentration was 1.8 M and then applied to a phenyl-Sepharose column. FKbp was eluted with a decreasing ammonium sulfate gradient in 100 mM phosphate, pH 7.0. Protein concentrations were estimated by the Bradford protocol.

FK506 was isolated from *Streptomyces tsukabaensis* obtained from the Fermentation Research Institute, Tsukuba, Ibaraki, Japan. The organism was grown in submerged culture in stirred fermenters. The procedures employed to purify FK506 were similar to those described previously (10). Acetone extract of the separated mycelium was combined with the broth filtrate. Active material was recovered by Diaion HP-20 resin adsorption and elution, solvent partitioning, gravity silica gel chromatography, and preparative HPLC on Rainin Dynamax 60Å 8 μm silica columns using a heptane/tetrahydrofuran mobile phase.

NMR samples were prepared by dissolving 3 mG of substrate in 50 μL of perdeuterated dimethylsulfoxide, then adding to 450 μL of a 20 mM phosphate (pH reading 7.8) deuterium oxide. Sodium 3-trimethylsilylpropionate was added as a reference subsequent to the 2D data collection. FKbp (2.4 mG) was dialyzed with a 5 mM dithiothreitol, 20 mM phosphate (pH reading 7.8) deuterium oxide. The enzyme solution was concentrated to 1.0 mL, and 80 μL were added to the substrate sample. FK506 (0.5 mG) was dissolved in 500 μL perdeuterated dimethylsulfoxide and 20 μL of this solution were added to the sample.

Spectra were collected on a Bruker AM-500 spectrometer with the probe temperature regulated to 27°C. NOESY spectra were collected as previously described (11). Repetition rate for NOESY experiments was 2 s, while for the 1D spectra it was 10 s. Frequency discrimination was accomplished by time proportional phase incrementation (12). Data were

processed using FTNMR (Hare Research). 2D matrices were 1024 points in $\omega 1$ and 2048 points in $\omega 2$. Polynomial baseline corrections were used.

Results and Discussion

The ^1H NMR spectra of Suc-AAPF-pNA showed multiple sets of signals for this substrate in aqueous solution (figure 1). A detailed examination revealed that two sets of signals exist: one representing 89% of the substrate and the other only 11%. Previous studies have verified that the cause of this heterogeneity is the existence of both the cis and trans conformations at the Ala-Pro amide bond (8).

^1H NMR Assignments on this substrate proceeded using the well-established strategies of Wüthrich (13). It is based on the scheme of grouping all protons within amino acid residues by the presence of scalar couplings as determined by standard two-dimensional spectra. This is followed by connecting the unknown residues via nuclear Overhauser enhancement (NOE) between any of the protons within the residue with the amide proton of the following residue.

The assignment of the dominant species of the substrate proceeded without complication (spectra not shown) and allowed the critical juncture at Ala-2 and Pro-3 to be assigned to the trans conformation. This conformation was identified by the presence of sequential NOE's between

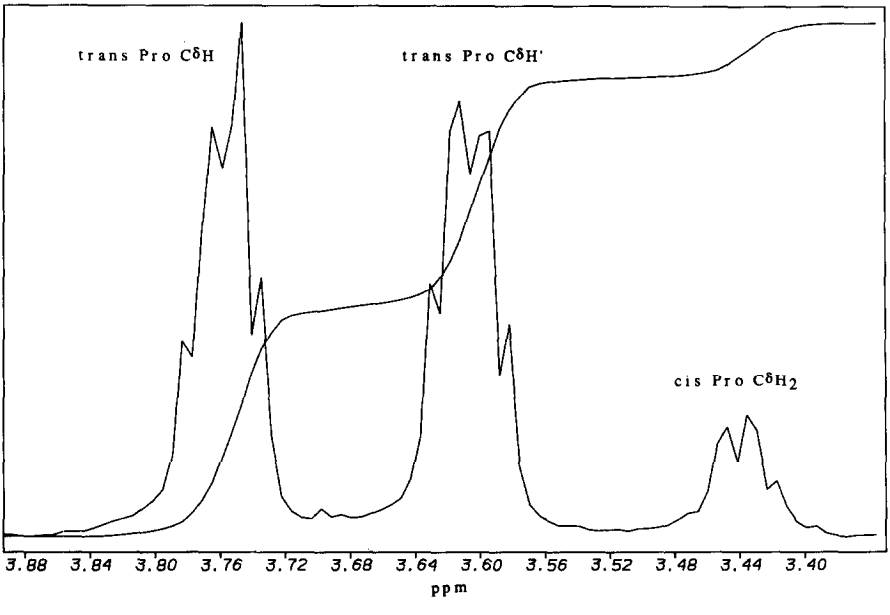

Figure 1: Expanded region (3.36 to 3.90 ppm) of a 1D spectrum taken on Suc-AAPF-pNA displaying the proline delta signals. Peaks are identified according to their isomer; an integral is also displayed.

Table I: Assigned ^1H Chemical Shifts (±0.01 ppm) for Suc-AAPF-pNA at pH 7.8, 27°C in 10% dimethylsulfoxide†

Residue	NH	$C^{\alpha}H$	$C^{\beta}H$	others
Ala-1	8.17	4.28	1.36	
	8.16	4.34	1.33	
Ala-2	8.31	4.56	1.33	
	8.05	4.13	1.28	
Pro-3		4.42	2.26,1.83	$C^{\gamma}H$ 1.99,1.99 $C^{\delta}H$ 3.76,3.61
		4.53	2.24,2.11	1.86,1.57 3.44,3.44
Phe-4	8.43	4.65	3.23,3.15	$C^{\delta}H_2$ 7.30 $C^{\varepsilon}H_2$ 7.36
	8.62	4.70	3.24,3.19	

† Values for the trans conformation are underlined while those for the cis are not.

the alanine residue and the delta signals of the proline. Because of limited signal-to-noise, the assignment of the minor component as the cis form of the substrate, relied on the presence of an additional set of the component amino acids in the 2D spectra. No sequential NOE's were detected between the minor residue components. The chemical shift assignments are listed in Table I according to conformer and sequence position.

An examination of Table I shows that the conformational change has its strongest effect on the chemical shifts of protons closest to the Ala-Pro amide bond. The proline $C^{\delta}H$ signals are displaced by 0.15 and 0.30 ppm by this amide bond rotation. Because these signals are well-isolated from each other and from all other proton signals in the spectrum, this affords an opportunity to study the exchange between the cis and trans conformations by examining the transfer of magnetization between the two sets of signals.

The NOESY experiment is particularly suited for this type of study because it records the transfer of magnetization both by chemical and conformational exchange as well as by NOE (11). Shown in figure 2A is the proline $C^{\delta}H$ region of a NOESY spectrum collected on the substrate sample. The absence of cross peaks between the trans signals and the cis signal indicates that no detectable magnetization is transferred between the two conformers within the 400 milliseconds of mixing time.

However, the addition of FKbp produces clear evidence (figure 2B) of an exchange between the cis and trans forms, consistent with previous

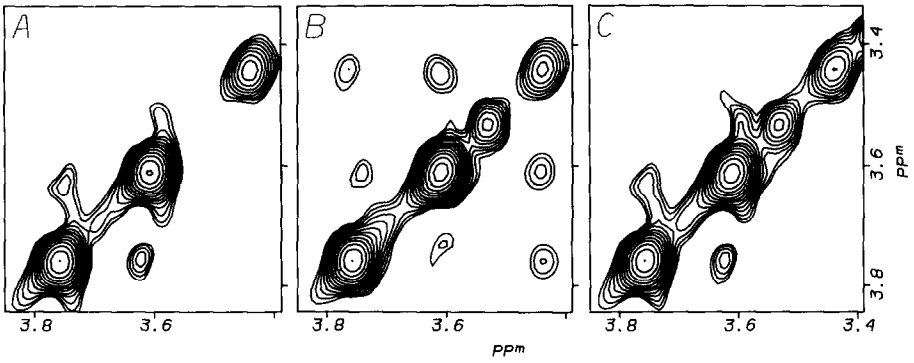

Figure 2: Similar region from three different NOESY spectra showing the three proline delta diagonal peaks and their accompanying cross peak. NOESY spectra were collected in an identical manner using a 0.4 s mixing time. Spectra are processed and plotted in an identical manner. (A) The NMR sample contains 10 mM Suc-AAPF-pNA (solution conditions described in Methods Section). (B) To the sample used in (A), FKbp was added to produce an approximate enzyme concentration of 30 μM. (C) To the sample used in (B), FK506 was added to produce an approximate concentration of 40 μM.

reports (3,4,14). An additional diagonal peak is seen at 3.54 ppm and is assigned to the dithiothreitol within the enzyme solution.

To be certain that the observed cross peaks were in fact due to the direct catalytic effect of the FKbp on the substrate, the inhibitor FK506 (14) was added to the sample. The resulting NOESY spectrum is shown in figure 2C. The presence of the inhibitor has removed the cross peaks from between the cis and the two trans signals. Thus the cross peaks observed in figure 2B are due to the catalytic activity of FKbp on the cis/trans conversion rate of the substrate.

By measuring the cis/trans cross peak volume, it should be possible to measure directly the catalytic rates of FKbp. Future studies will thus concentrate on the feasibility of obtaining accurate Michaelis constants by examining peak volumes as a function of the substrate concentration.

This method of enzymatic analysis should have two distinct advantages over the standard chymotrypsin assay. The first is that the standard assay has potential complications because of both the added chymotrypsin and the large amounts of succinyl-Ala-Ala-Pro-Phe product generated before the rate measurement begins. The NMR method, however, contains only the FKbp and its substrate and represents a simpler experimental condition.

The second advantage relates to the range of potential substrates that could be studied with the NMR assay. While the chymotrypsin proced-

ure requires that a Pro-Phe-p-nitroanilide be present on the C terminus of the substrate, the NMR method carries no such restriction. The possibility exists, therefore, to explore the action of the PPI on substrates that bear defined amino acids C-terminal to the proline residue. Thus, the specificity of the isomerases for residues C-terminal of the proline can, for the first time, be facilely explored.

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