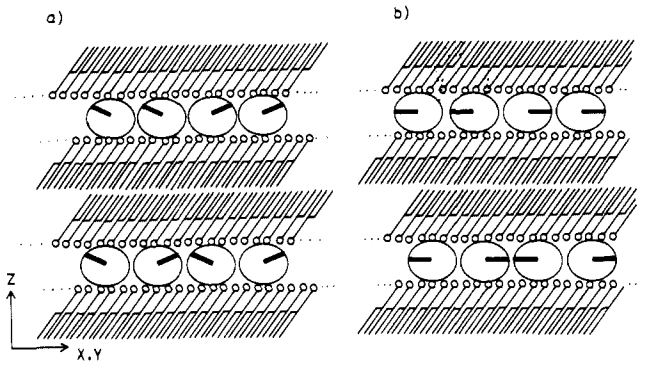

Figure 2. Schematic illustration of met-Mb molecules immobilized in cast multibilayer films: (a) cast from Tris·HCl buffer; (b) cast from phosphate buffer. The protein molecules show at least one-dimensional ordering along the Z axis. The heme plane is expressed by filled rectangles.

perature ([met-Mb] = 0.06–0.5 mM, μ = 0.02). Multilayer films were obtained by spreading the dispersion on Fluoropore membranes (Sumitomo Electric) and allowing it to stand at 20–25 °C for a few days. Low-temperature ESR spectra were measured with a JEOL JES-RE2X X-band spectrometer equipped with a liquid helium cryostat with 100-kHz magnetic field modulation.

The cast film containing met-Mb was self-supporting and showed a gel-to-liquid crystal phase-transition behavior very similar to that of the bilayer alone. This confirms that the bilayer structure is maintained even in the presence of immobilized met-Mb. An ESR spectrum of met-Mb powder is characteristic of high-spin iron(III) porphyrin (Figure 1a)[13] and is composed of two components, $g_\perp$ = 5.9 and $g_\parallel$ = 2.0, where $g_\parallel$ (g_{ZZ}) represents the principal value of the g tensor that is parallel to the normal of the heme plane (i.e., perpendicular to the heme plane), and $g_\perp$ ($g_{XX} = g_{YY}$) represents the principal value of the g tensor that is perpendicular to the normal of the heme plane (i.e., parallel to the heme plane). It is interesting that ESR spectra of the cast film show strong anisotropy depending on the angle (θ) between the normal of the film plane (Z axis) and the applied magnetic field H_0: Figure 1b–d. When the film plane is parallel to the magnetic field (θ = 90°), a strong signal of the $g_\perp$ component appears at 1000 G but the $g_\parallel$ signal is weak. As angle θ decreases from 90° to 0°, the $g_\parallel$ component is intensified, assumes a maximum at θ = 15–20°, and is then slightly decreased. The $g_\perp$ signal shows complementary changes. The observed angular dependence clearly indicates that the heme plane of met-Mb is oriented at an angle of 15–20° against the bilayer surface.[14]

This specific orientation is maintained even when the ratio of met-Mb over amphiphile ([met-Mb]/[**1**]) is varied from 1/40 to 1/330. The overall dimension of myoglobin is about 45 × 35 × 25 Å, and it is presumed that 30 basic amino acid residues and 20 acidic amino acid residues are distributed on the protein surface, forming a few charged domains.[17] The molecular cross section of **1** is ca. 50 Å^2 as estimated from CPK model building and from the data of its surface monolayer.[18] The ratio of [met-Mb]/[**1**] = 1/40 corresponds to a situation where met-Mb molecules cover almost completely the polar bilayer surface in the multibilayer film.

Denaturation apparently does not occur with met-Mb included in the cast film. Its ESR data are in good agreement with those of native met-Mb: see above. Absorption maxima of met-Mb in the cast film (Soret band, 409 nm; Q band, 500, 540, and 630 nm) coincide with those of met-Mb itself dissolved in Tris buffer. The active site of the protein is not disturbed seriously by immobilization under the current conditions.

The orientation of met-Mb is dependent on buffer and the ionic strength of the casting solution. When phosphate buffer (pH 7.5, 10 mM) was used instead of Tris·HCl buffer, the ESR spectra of the resulting cast film showed the strongest $g_\parallel$ signal at θ = 0°. This anisotropy corresponds to the orientation of the heme plane parallel to the plane of the cast film. On the other hand, an increased ionic strength of the casting solution (μ = 0.2 instead of the original 0.02) caused some loss in the orientational specificity. The electrostatic interaction of the polar groups at the protein surface and the phosphate head group of the bilayer membrane appears to determine the protein orientation.

In conclusion, myoglobin molecules were incorporated into multilamellar films of a phosphate bilayer membrane without detectable denaturation. The myoglobin molecule can fill the polar interbilayer region and maintains at least the one-dimensional order due to electrostatic interactions with the bilayer surface. This is schematically illustrated in Figure 2. The present approach is distinguished from the previous examples in that two-dimensional placement of proteins is attained by simple solvent casting. Improved anisotropy was found relative to those of membrane-bound proteins in a matrix of biolipids.[7] This indicates an advantage of synthetic bilayer membranes as matrices. We envisage a wide range of biochemical and biotechnological applications.

Acknowledgment. We are grateful to Dr. Y. Ishikawa for ESR simulation.

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Peptidyl-Prolyl Cis–Trans Isomerase Activity of Cyclophilin Studied by One-Dimensional ^{1}H Nuclear Magnetic Resonance Spectroscopy

Victor L. Hsu,[†] Robert E. Handschumacher,[‡] and Ian M. Armitage*[,†,§]

Departments of Molecular Biophysics and Biochemistry, Pharmacology, and Diagnostic Radiology Yale University, P.O. Box 3333 333 Cedar Street, New Haven, Connecticut 06510

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Recent advances in multidimensional NMR[1] spectroscopy have made it possible to study and determine the structures of many biologically interesting molecules at atomic resolution.[2] Additionally, lest one forget, NMR is also uniquely useful in studying the dynamic and kinetic properties of these biomacromolecules. In fact, certain functional properties of molecules whose large size is not amenable to detailed structure elucidation by even three-

* Address correspondence to this author at the Department of Molecular Biophysics and Biochemistry.
† Department of Molecular Biophysics and Biochemistry.
‡ Department of Pharmacology.
§ Department of Diagnostic Radiology.

(1) Abbreviations: BzFAP, benzoyl-Phe-Ala-Pro; CsA, cyclosporin A; CyP, cyclophilin; NMR, nuclear magnetic resonance; PPIase, peptidyl-prolyl cis–trans isomerase; Succ-AAPF-pNA, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide.
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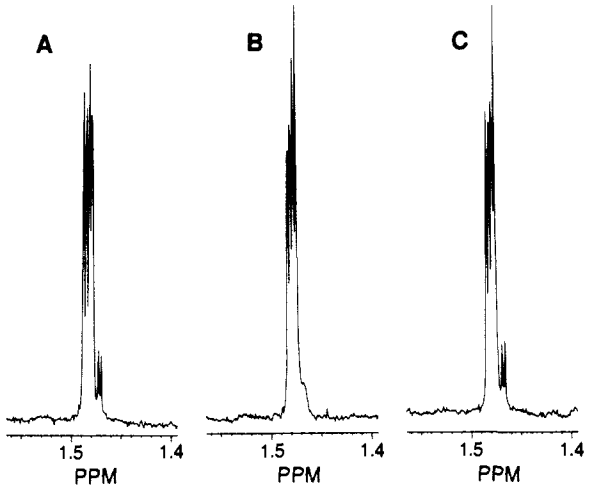

Figure 1. Effect of CyP and CsA on the alanine methyl spectral region of Succ-AAPF-pNA. All spectra were collected at 500 MHz, 25 °C, 25 mM potassium phosphate, 400 mM NaCl, pH 6.8 (except for pH jump experiments, see text). Dithiothreitol or β-mercaptoethanol was added as an antioxidant to samples containing CyP. (A) Free Succ-AAPF-pNA (300 μM peptide). The small doublet slightly upfield of the larger multiplet is due to the minor isomer. A total of 512 scans were collected. (B) The addition of CyP increases the isomerization of the Ala-Pro bond (same sample as in A plus 1 μM CyP). (C) Inhibition of the isomerase activity of CyP by addition of CsA (5 μM) to the sample in B.

dimensional NMR[3,4] can often be characterized by one-dimensional spectroscopy.

A protein of considerable current interest, cyclophilin, has not yet had a complete resonance assignment (a necessary first step for structure determination) primarily because of its large size (163 amino acids, M_w 17 737 daltons).[5-7] First isolated in 1984,[8] cyclophilin (CyP) was found to be a high-affinity binding protein for the immunosuppressive drug cyclosporin A (CsA) in both lymphoid and nonlymphoid mammalian cells.[9,10] However, when the enzyme peptidyl-prolyl cis-trans isomerase (PPIase) from hog kidney was sequenced early last year, it was found to be identical with CyP.[11,12] This added a new dimension to the role of CyP and suggests that protein folding or conformational changes may constitute a new mechanism of signal transduction or its control. It has been shown that isomerization about the proline imide bond is the rate-determining step in the folding of several proteins.[13,14] The connection between protein folding and the immune response was further strengthened recently when it was discovered that a specific binding protein for another highly potent immunosuppressive drug, FK506,[15] was also a proline isomerase.[16,17]

Table I. Rate Constants (k) for the Cis-Trans Isomerization of Benzoyl-Phe-Ala-Pro

	% cis[a]	method	rate,[b] s^{-1}
uncatalyzed	14.3	pH jump	$3.3 \times 10^{-3} \pm 0.9 \times 10^{-3}$
CyP catalyzed[c]	13.8	saturation transfer	0.44 ± 0.04

[a] BzFAP (2.62 mM), 25 mM potassium phosphate, 400 mM NaCl, pH 6.8, 25 °C. [b] Uncertainties calculated at 95% confidence intervals. [c] BzFAP (2.62 mM), 16 μM CyP.

The isomerase activity of CyP has been previously studied spectrophotometrically to extract quantitative kinetic data by using a coupled assay in which the nitroanilide group of the model substrate, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Succ-AAPF-pNA), is cleaved by chymotrypsin only when the Ala-Pro bond is trans.[12] Unfortunately, this is an indirect assay which is dependent on having a chromophore attached to the carboxy terminus and assumes that the substrate, products, or inhibitors of the isomerase do not affect the activity of the protease. These problems can be overcome, however, by the direct NMR measurement of the isomerization rates of peptides and proteins.

Although the solubility of Succ-AAPF-pNA is limited in aqueous solvents, we have used this peptide substrate to qualitatively demonstrate the change in proline isomerization rate upon the addition of CyP. The one-dimensional ^1H NMR spectrum of this tetrapeptide exhibits minor peaks arising from the cis isomer, in particular the methyl group of Ala2 and the nitroanilide group. The observed chemical shift difference between two exchanging resonances is proportional to environment shielding differences and inversely proportional to the kinetic exchange rates, and although these rates cannot be quantitatively determined from these shift differences by the NMR methods presented here, an upper limit can be approximated. Measuring line widths of exchanging resonances has also been used as a method of determining exchange rates.[18] As can be seen in the alanine methyl region of the spectrum (Figure 1A), the addition of CyP causes exchange broadening and a decrease in the chemical shift difference between the exchanging methyl resonances (Figure 1B), indicating an increased isomerization rate. The inhibition of CyP isomerization activity by the addition of CsA to this same sample restores the chemical shift difference (Figure 1C) to that seen in the uncatalyzed sample.

To quantitate the isomerase activity of CyP by NMR, other model proline-containing peptides with greater chemical shift dispersion of the exchanging resonances have been studied. One example is the tripeptide benzoyl-Phe-Ala-Pro (BzFAP), a substrate for the angiotensin-converting enzyme. The rate for the uncatalyzed isomerization of its proline was determined by measuring the percent cis content in a series of one-dimensional spectra acquired as a function of time after a rapid change in pH from 1.5 to 8.0:[19]

$$\frac{[\text{cis}]_t - [\text{cis}]_0}{[\text{cis}]_\infty - [\text{cis}]_0} = \exp\left(-\frac{k}{[\text{trans}]_\infty}t\right) \quad (1)$$

The measured rate (Table I) is similar to those reported by Grathwohl and Wüthrich[19] for other short proline-containing peptides as well as the values reported for Succ-AAPF-pNA.[12,20]

For rates faster than ~0.1 s^{-1}, saturation transfer techniques[19,21-24] can be used to measure the chemical exchange rate.

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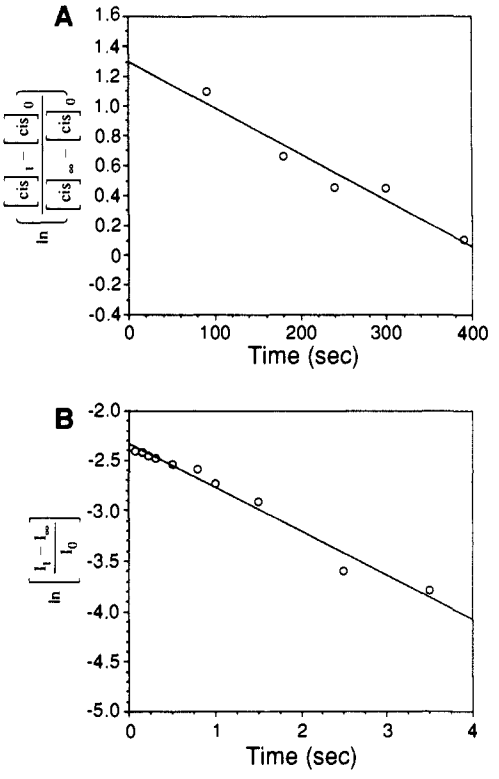

Figure 2. Plots from which the rate constants for the uncatalyzed and CyP-catalyzed isomerization of BzFAP were determined. For a description of the ordinate axes, see text. (A) pH jump experiment with the uncatalyzed BzFAP sample (2.62 mM BzFAP). The data have been multiplied by $[trans]_\infty$. (B) Saturation transfer experiment of the catalyzed BzFAP sample (same sample as in A plus 16 μM CyP). Note that the time range (abscissa) for the uncatalyzed experiment is 2 orders of magnitude greater.

This technique was used to determine the CyP-catalyzed BzFAP isomerization rate. The relationship between isomerization rate and saturation transfer is given by[22]

$$\frac{I_t - I_\infty}{I_0} = \exp(-kt) \qquad (2)$$

where I_t is the intensity of the unsaturated resonance when an exchanging resonance is saturated for a given length of time. The saturation period was varied between 0.05 and 5.0 s. As Figure 2 demonstrates, CyP catalyzes the isomerization rate by a factor of 135 (Table I). The CyP-catalyzed rate is similar to the catalyzed rate determined spectrophotometrically for Succ-Ala-Ala-Pro-Phe-pNA by using similar substrate:enzyme ratios.[12] As expected, the equilibrium distribution of the two proline conformers remained the same in the presence of CyP. The increased rate observed in the presence of CyP can be attributed to a lowering of the activation energy of isomerization, which can be quantified by comparing Arrhenius plots of the rate constants for both the uncatalyzed and CyP-catalyzed reactions.

A major advantage of NMR spectroscopy as a means of quantifying isomerase activity is that it is a direct measurement of the substrate being studied. Additionally, due to the high substrate:enzyme ratios normally encountered, only those resonances arising from the substrate are observed, and the broad overlapped resonances associated with large proteins are not seen (Figure 3). Thus, with enzyme concentrations low enough to render enzyme resonances spectroscopically "invisible", the spectra of the enzyme–substrate system are "enzyme edited". Furthermore, there is no need for isotopically labeled (^2H, ^{13}C, ^{15}N, ^{19}F, etc.) substrates. In those cases where lower substrate:enzyme ratios are required to determine turnover numbers, K_m, etc., the use of heteronuclear NMR experiments with NMR-labeled substrates would be advantageous, and these studies are in progress.[25]

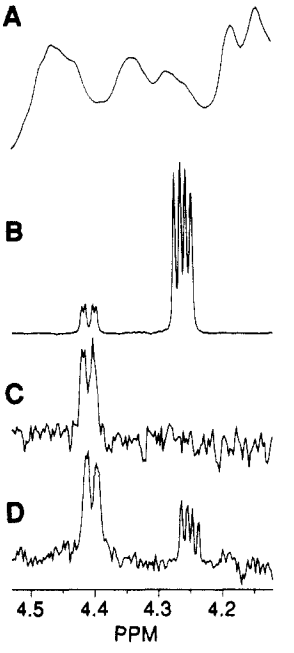

Figure 3. Proline H_α region of the BzFAP–CyP system. (A) Spectrum of free cyclophilin (1.2 mM CyP). (B) Spectrum of BzFAP–CyP (2.62 mM BzFAP, 16 μM CyP) showing the trans (4.25 ppm) and cis (4.40 ppm) resonances. (C) Difference spectrum in the absence of CyP from on- and off-resonance saturation (800 ms) of the minor cis resonance (2.62 mM BzFAP). (D) Difference spectrum in the presence of CyP from on- and off-resonance saturation (800 ms) of the minor cis resonance demonstrating increased chemical exchange (2.62 mM BzFAP, 16 μM CyP).

Previous studies of enzyme mechanisms by NMR spectroscopy have been primarily structural investigations of enzyme–substrate complexes[26] or have relied upon the availability of isotopically labeled substrates and/or time-consuming two-dimensional NMR experiments.[27] In the present report we have demonstrated that kinetic aspects of substrate–enzyme interactions in an equilibrium system can be monitored by one-dimensional ^1H NMR. We feel that these simple one-dimensional ^1H NMR techniques[28] will prove extremely useful in characterizing other substrates without resorting to labeled analogues. Such studies should make valuable contributions to establishing the molecular mechanism of action of cyclophilin and other isomerases in the immune response.

Note Added in Proof. We thank a reviewer for commenting on the enhanced sensitivity of this approach compared to the poor signal-to-noise ratio associated with the coupled chymotrypsin assay.

Acknowledgment. We thank Chris Proto for preparing and purifying the cyclophilin samples and Dr. N. Gillis for the BzFAP samples. This work was supported by grants from the National Institutes of Health (GM 40660 and DK 18778) and the American Cancer Society (CH 67). NMR instrumentation and computational facilities were provided by grants from the NIH (RR 03475), NSF (DMB 8610557), and ACS (RD 259).

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