

- Shames, S. L., Fairlamb, A. H., Cerami, A., & Walsh, C. T. (1986) *Biochemistry* 25, 3519-3526.
- Shames, S. L., Kimmel, B. E., Peoples, O. P., Agabian, N., & Walsh, C. T. (1988) *Biochemistry* 27, 5014-5019.
- Sullivan, F. X., & Walsh, C. T. (1991) *Mol. Biochem. Parasitol.* 44, 145-147.
- Sullivan, F. X., Shames, S. L., & Walsh, C. T. (1989) *Biochemistry* 28, 4986-4992.
- Sullivan, F. X., Sobolov, S. B., Bradley, M., & Walsh, C. T. (1991) *Biochemistry* 30, 2761-2767.
- Taylor, J. W., Ott, J., & Eckstein, F. (1985a) *Nucleic Acids Res.* 13, 8749-8764.
- Taylor, J. W., Ott, J., & Eckstein, F. (1985b) *Nucleic Acids Res.* 13, 8764-8785.
- Tutic, M., Lu, X., Schirmer, R. H., & Werner, D. (1990) *Eur. J. Biochem.* 188, 523-528.
- Vanoni, M. A., Wong, K. K., Ballou, D. P., & Blanchard, J. S. (1990) *Biochemistry* 29, 5790-5796.
- Wong, K. K., & Blanchard, J. S. (1989) *Biochemistry* 28, 3586-3590.

Determination of Kinetic Constants for Peptidyl Prolyl Cis-Trans Isomerases by an Improved Spectrophotometric Assay[†]

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ABSTRACT: The kinetic properties and substrate specificity of two well-characterized peptidyl prolyl cis-trans isomerases (PPIases), cyclophilin and the FK-506 binding protein (FKBP), have been previously examined [Fischer, G., Bang, H., Berger, E., & Schellenberger, A. (1984) *Biochim. Biophys. Acta* 791, 87-97; Harrison, R. K., & Stein, R. L. (1990) *Biochemistry* 29, 1684-1689; Albers, M. W., Walsh, C. T., & Schreiber, S. L. (1990) *J. Org. Chem.* 55, 4984-4986]. The chymotrypsin-coupled enzymatic assay employed in these studies suffers from two serious shortcomings. Due to the low equilibrium population of the X-cis-Pro-Phe-pNA isomer (the PPIase substrate), in conjunction with the low solubility of *p*-nitroaniline generated by chymotrypsin hydrolysis, substrate concentrations in the saturating region are not experimentally attainable. Secondly, the uncatalyzed cis-trans isomerization obscures the interpretation of the initial velocity. As a result of these limitations, the steady-state kinetic parameters (K_m , k_{cat}) have not been determined. Here we introduce an improved version of the spectrophotometric assay and report for the first time the Michaelis constants and turnover numbers for both PPIases with established substrates. The improvements in the experimental conditions originate in a medium-induced increase in the equilibrium population of the cis X-Pro conformer and in conducting the assay at 0 °C to suppress the uncatalyzed thermal isomerization. In addition, we present a rigorous mathematical model of the spectrophotometric progress curves that accounts for the contributions of the residual background rate. For Suc-Ala-Ala-cis-Pro-Phe-pNA with bovine cyclophilin, $K_m = 0.98 \pm 0.14$ mM and $k_{cat} = 13200 \pm 880$ s⁻¹; for recombinant human cyclophilin, $K_m = 0.87 \pm 0.084$ mM and $k_{cat} = 12700 \pm 550$ s⁻¹. The kinetic parameters for Suc-Ala-Leu-cis-Pro-Phe-pNA with FKBP are $K_m = 0.520 \pm 0.08$ mM and $k_{cat} = 344 \pm 26$ s⁻¹. We also demonstrate that [(Boc)Dab]⁸-CsA, a cyclosporin A analogue, is a tight-binding, slow-binding inhibitor of cyclophilin and that another cyclosporin A analogue, [Me⁵Bth]¹-CsA, is a competitive inhibitor of the same enzyme.

Peptidyl prolyl cis-trans isomerase¹ (PPIase,² EC 5.2.1.8), discovered by Fischer and co-workers in 1984 (Fischer et al., 1984a), catalyzes the cis-trans isomerization of X-Pro peptide bonds. This class of enzymes facilitates the refolding of some denatured proteins in vitro (Lang et al., 1987; Lang & Schmid, 1988; Lin et al., 1988; Schönbrunner et al., 1991) and is believed to have an important role in the folding of newly synthesized proteins in vivo, where proline isomerization has been proposed as a potential rate-limiting step (Brandts et al., 1975). The discovery of PPIase coincided with the discovery of cyclophilin, a protein receptor for the immunosuppressive

drug cyclosporin A (Handschumacher et al., 1984). The two proteins were subsequently shown to be identical (Takahashi et al., 1989; Fischer et al., 1989a). Intriguingly, the FK-506 binding protein, a receptor for the powerful immunosuppressant FK-506, also has PPIase activity (Siekierka et al., 1989; Harding et al., 1989). The fact that both PPIases bind

¹ Enzymes that catalyze an intramolecular cis-trans isomerization are categorized under the EC 5.2 class, cis-trans isomerases. This enzyme has also been referred to as a "rotamase".

² Abbreviations: PPIase, peptidyl prolyl cis-trans isomerase, (CyP, cyclophilin); TFE, trifluoroethanol; Suc, succinyl; pNA, *p*-nitroanilide; AU, absorbance units; PMSF, phenylmethanesulfonyl fluoride; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CsA, cyclosporin A; [(Boc)Dab]⁸-CsA, [*N*-(*t*-butoxycarbonyl diaminobutyl)]⁸-CsA; [Me⁵Bth]¹-CsA, [5-methyl-3-hydroxy-2-(methylamino)-6-octenoic acid]¹-CsA.

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immunosuppressive drugs suggests a link between protein folding and immunosuppression, but the connection of PPIases to the regulation of immune response is tentative at best (Bierer et al., 1990a; Sigal et al., 1990, 1991). Regardless of the uncertainty concerning their physiological role, PPIases represent a novel class of enzymes, and there is a distinct possibility that an effective PPIase inhibitor may have an important biological activity. As a basis for the rational design of PPIase inhibitors, there is a need to clearly understand the catalytic mechanism of these enzymes; a logical starting point is the determination of the steady-state kinetic parameters.

Peptidyl prolyl *cis-trans* isomerases are normally assayed by employing the spectrophotometric method of Fischer (Fischer et al., 1984a), which exploits the high conformational selectivity of chymotrypsin toward chromogenic substrates of the type X-Pro-Phe-pNA. The hydrolysis of the C-terminal *p*-nitroanilide bond occurs selectively only in the *trans* X-Pro conformer (Neil et al., 1966; Fischer et al., 1984b). The coupled assay has proven very valuable for detecting PPIase activity of proteins such as cyclophilin, FK-506 binding protein, and others (Fischer et al., 1984a; Siekierka et al., 1989; Harding et al., 1989)³ and was used to probe the catalytic mechanism of this class of enzymes (Harrison & Stein, 1990a; Harrison et al., 1990; Liu et al., 1990; Albers et al., 1990). Studies of PPIases have been hampered by rather severe limitations due to the physical properties of both the chromogenic substrates and products (*p*-nitroaniline), as well as the kinetic complexity of the coupled assay. The PPIase-catalyzed *cis-trans* isomerization is accompanied by two competing processes: (1) the parallel, reversible, thermal *cis-trans* isomerization and (2) the sequential, irreversible, chymotrypsin-catalyzed hydrolysis of the *trans* isomer. To a limited degree, the relative importance of the three reactions can be controlled by adjusting the concentrations of both enzymes. For example, some substrates are cleaved very rapidly at high concentrations of chymotrypsin, so that the excess *trans* isomer present at the beginning of the assay is consumed in a "burst phase" during the mixing time. The major obstacle is in the restricted range of substrate concentrations. In an aqueous solution, the equilibrium population of the *cis* X-Pro conformer is approximately 10%, and thus 90% of the total absorbance change arises from the burst phase. This leads to an unfavorable signal-to-noise ratio, despite the very high spectroscopic sensitivity ($\Delta\epsilon$ *p*-nitroaniline = 13300 M⁻¹ cm⁻¹ at 390 nm, pH 8.0, 0 °C). Moreover, the substrate concentration cannot be arbitrarily increased because of the low solubility of *p*-nitroaniline. Due to these inherent limitations, the total substrate concentration has customarily been limited to 20–250 μ M; this equates to an upper limit of 25 μ M for the *cis* isomer. To accurately determine the steady-state kinetic parameters, velocities need to be measured at concentrations at least approaching the Michaelis constant, which was previously estimated to be greater than 20 mM for the cyclophilin substrate Suc-AA-*cis*-PF-pNA (Harrison & Stein, 1990a)—a value 3 orders of magnitude higher than the maximum concentration attained in the original assay.

We report herein an improved method for monitoring the peptidyl prolyl isomerase activity. The new assay incorporates three improvements that enable us to carry out the enzyme kinetics under saturating conditions: (1) a novel solvent system that increases the equilibrium population of the *cis* X-Pro isomer from 10% to approximately 50% (70% in some cases)

for a variety of peptide substrates; (2) the use of low temperature and detection at varied wavelengths; and (3) a rigorous mathematical analysis of the spectrophotometric progress curves. This improved assay has provided, for the first time, the means for accurate determination of steady-state kinetic parameters for PPIase substrates (k_{cat} , K_m) as well as inhibition patterns for PPIase inhibitors. In addition, we show that a cyclosporin A analogue is a linear competitive inhibitor of bovine cyclophilin and that another analogue displays time-dependent binding ("slow-binding") to cyclophilin.

MATERIALS AND METHODS

Enzymes. CyP was isolated from calf thymus by the method of Holzman et al. (1991) with the following modifications. The tissue was homogenized in buffer (20 mM Tris/HCl, 100 mM NaCl, 1 mM PMSF, pH 7.0) followed by a 30-min stirring on ice. The pH precipitation was performed at pH 5.0 instead of pH 5.5. The dissolved, dialyzed 40–60% ammonium sulfate material was applied to a DEAE-Sepharose column (2.6 × 30 cm) preequilibrated in 20 mM Hepes/NaOH, pH 8.0. The column was eluted isocratically, and the cyclophilin eluted with the *second* peak. This protein was concentrated for the size-exclusion step (Sephacryl S-100 HR, 2.6 × 90 cm, 20 mM Hepes/NaOH, pH 8.0, 100 mL/h). Fractions with PPIase activity were pooled, dialyzed versus 5 mM Hepes/NaOH, pH 8.0, and applied to a CM-Sepharose column (2.6 × 45 cm) preequilibrated in the same buffer. A 500-mL linear gradient of NaCl (0–75 mM) in buffer was used to elute the protein.

Recombinant human CyP (neutral pI) was a generous gift from Dr. T. Holzman, Abbott Research Laboratories.

FKBP was partially purified from calf thymus. The thymus was homogenized in buffer (25 mM Tris/HCl, 100 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM PMSF, pH 7.5). After centrifugation to remove the cellular debris, the supernatant was heated to 60 °C for 20 min. This mixture was centrifuged, and protein from the supernatant was precipitated by the addition of ammonium sulfate (80% saturation). The collected protein was dissolved in a minimum of the cell lysis buffer and applied to a Sephacryl S-100HR column (2.6 × 90 cm) preequilibrated in this buffer. The PPIase activity of fractions was assayed by employing a modification of the procedure of Fischer and co-workers (1984a) using both Suc-AAPF-pNA and Suc-ALPF-pNA as substrates. Fractions that contained a high PPIase activity when Suc-ALPF-pNA was used as the substrate were pooled; SDS-PAGE confirmed the presence of a 12-kDa protein. The PPIase activity of this pooled protein was inhibited by both FK-506 and rapamycin.

Solvents and Substrates. Trifluoroethanol (TFE) and tetrahydrofuran (THF) were purchased from Aldrich Chemical Co. and distilled over sodium before use; LiCl (Aldrich) was dried in vacuo at 150 °C for 24 h. The peptide substrates, Suc-AAPF-pNA (Sigma) and Suc-ALPF-pNA (prepared by V.K.), were dried in vacuo over P₂O₅ at room temperature.

Substrates were dissolved in TFE or THF that contained LiCl (235–470 mM). These solutions (5–250 mM) were prepared in vials fitted with septa under an atmosphere of argon to exclude water vapor. The percentage of the *cis* isomer in the sample was determined in the following manner. A cuvette containing the assay buffer and chymotrypsin (6 mg/mL), but no PPIase, was zeroed on the spectrophotometer at 390 nm. The peptide substrate was added with mixing to give a final concentration of approximately 75 μ M; the *trans* isomer of the substrate was consumed by chymotrypsin hydrolysis during the mixing time. The absorbance of the sample was immediately recorded; this corresponds to the amount of the *trans* isomer present in the original mixture. After 30 s,

³ We have also partially purified at least one protein that possesses PPIase activity but that is not inhibited by CsA, FK-506, or rapamycin (J.L.K., unpublished observation).

cyclophilin (approximately 50 nM) was added to increase the rate of cis-to-trans isomerization, and the absorbance was measured after the chymotrypsin-catalyzed hydrolysis was complete (1 min). The amount of the cis isomer was calculated from the difference between the initial and the final absorbance. The difference molar absorption coefficient $\Delta\epsilon$ was calculated from the total absorbance change; the results are given as $\Delta\epsilon$ ($M^{-1} cm^{-1}$) followed by the corresponding wavelength in parentheses (in nanometers): 13300 (390), 1250 (445), 995 (450), 460 (455), 267 (460). In the kinetic experiments, higher wavelengths were used at higher total substrate concentrations, so that the optical density was kept below 2.0 absorbance units.

The cis-trans equilibrium for the peptide substrates was also determined independently, by observing the ^{13}C NMR peaks of the C^β and C^γ proline resonances (cis, $\delta = 35$ and 24, respectively; trans, $\delta = 33$ and 27, respectively). The results obtained by use of the NMR method agreed with the chymotrypsin method to within 2%.

Assay for Peptidyl Prolyl Isomerization. The assay buffer (865 μL of 50 mM Hepes, 100 mM NaCl, pH 8.0 at 0 °C; final concentration 43 mM Hepes, 86 mM NaCl) and PPIase (10 μL of a 300–700 nM stock solution; final concentration 3–7 nM) were preequilibrated in the spectrometer until the temperature reached 0.0 °C. Immediately before the assay was started, 100 μL of chymotrypsin solution (60 mg/mL in 0.001 M HCl; final concentration 6 mg/mL) was added. The peptide substrate, dissolved in LiCl/TFE, was added to the cuvette; additional cosolvent was added to bring the total of substrate and cosolvent to 25 μL , and the solution was mixed. The final volume in a masked, semimicro 1-cm path length cell was 1.0 mL. After a delay from the onset of mixing (usually 10 s for Suc-AAPF-pNA, 30 s for Suc-ALPF-pNA), the absorbance of *p*-nitroaniline was followed at an appropriate wavelength until the reaction was complete (2–10 min). Final concentrations of LiCl in the assay were 6–12 mM; TFE was present at a concentration of 2.5% (v/v).

Absorbance readings were collected on a CARY 14 spectrophotometer interfaced to a IBM computer, by use of commercial data acquisition software (OLIS; On Line Instrument Systems, Jefferson, GA). The progress curves were analyzed by nonlinear least-squares fit to the integrated rate equation (eq 2) (see Results).

Determination of the Steady-State Kinetic Parameters for PPIase Substrates. PPIases were assayed at varied substrate concentrations as indicated above. Data were collected at varied wavelength (445, 455, and 460 nm for bovine CyP; 445, 450, 455, and 460 nm for recombinant human cyclophilin and bovine FK-506 binding protein). Other conditions of the assays are outlined in Table I.

Inhibition Assays and Active Site Titration. The inhibition assays were performed in the same manner as indicated for the determination of substrate kinetic parameters, with the following exception. A 10- μL aliquot of the inhibitor in DMSO was added to the PPIase solution in the assay buffer; after preincubation for an appropriate period of time at 0 °C (10–150 min, depending on the inhibitor), the assay was started by the addition of chymotrypsin and the substrate. At higher concentrations of inhibitor, the total assay time was increased from 2–5 min to 10–20 min; the extent of conversion at the end of the assay was typically higher than 80%. The concentration of cyclophilins was established by active site titration with [(Boc)Dab]⁸-CsA ($K_i = 2.4$ nM); the enzyme concentration was approximately 3-fold higher than the K_i of the inhibitor. The concentration of FKBP (approximately 10 nM)

Table I: Determination of the Michaelis Constants (K_m) and Turnover Numbers (k_{cat}) for Peptidyl Prolyl Cis-Trans Isomerases at 0 °C

| | human CyP | bovine CyP | bovine FKBP |
|--|------------------|------------------|-------------------|
| conditions | | | |
| substrate | Suc-AAPF-pNA | Suc-AAPF-pNA | Suc-ALPF-pNA |
| [LiCl] in TFE (mM) | 470 | 235 | 470 |
| [S] stock 1 (mM) ^a | 160 | 250 | 120 |
| percentage cis | 60 | 35 | 48 |
| [S] stock 2 (mM) ^a | 32 | 55 | 30 |
| percentage cis | 67 | 55 | 48 |
| [S] _{cis} range (μM) | 100–2250 | 100–2400 | 70–2400 |
| [E] _p (nM) | 3.7 ^b | 3.4 ^b | 13.0 ^c |
| results | | | |
| k_1 (uncatalyzed, s ⁻¹) | 0.0020 | 0.0020 | 0.0016 |
| K_m (μM , for cis) | 870 \pm 84 | 980 \pm 140 | 520 \pm 85 |
| k_{cat} (s ⁻¹ , for cis) | 12700 \pm 550 | 13200 \pm 880 | 344 \pm 26 |
| k_{cat}/K_m ($10^6 M^{-1} s^{-1}$) | 14.6 \pm 1.5 | 13.4 \pm 2.1 | 0.66 \pm 0.12 |

^a Stock solution of the indicated peptide dissolved in the LiCl/TFE mixture. ^b Active site titration with [(Boc)Dab]⁸-CsA (K_i 2.4 nM). ^c Active site titration with rapamycin (K_i 0.25 nM).

was determined by titration with the inhibitor rapamycin ($K_i = 0.25$ nM).

Solvent Deuterium Isotope Effect. Recombinant human cyclophilin (3.7 nM) was assayed with Suc-AA-cis-PF-pNA (0.02–2.0 mM). The assays were performed under identical conditions as indicated above for the determination of steady-state kinetic parameters, with the exceptions that buffer and chymotrypsin solutions were prepared in deuterium oxide instead of water for D₂O assays and that all assays were performed at 4 °C. Deuterium content was determined by infrared absorption spectroscopy at 1668 nm (Northrop et al., 1991); all samples contained 98.1 \pm 0.4% D₂O. The difference molar absorption coefficients for *p*-nitroaniline in the D₂O samples were determined as indicated previously.

RESULTS

Assay of Peptidyl Prolyl Cis-Trans Isomerization. The PPIase assay first described by Fischer and co-workers (1984a) is based on the observation that chymotrypsin cleaves the C-terminal amide bond only in the trans X-Pro conformer of the chromogenic substrate, X-Pro-Phe-pNA. The rapid hydrolysis perturbs the cis-trans conformational equilibrium, which allows one to monitor the PPIase-catalyzed cis-to-trans isomerization. In the original assay, the substrate Suc-AAPF-pNA was used at a concentration of 75 μM , of which 7.5 μM corresponds to the active cis isomer. The substrate was preincubated with the PPIase in an assay buffer; after reaching thermal equilibrium, chymotrypsin was added to initiate the reaction. Under these conditions, the burst phase due to the cleavage of the excess trans isomer is completed during the mixing time, and the remaining absorbance change is due to the cis-trans isomerization. A representative spectrophotometric progress curve is shown in Figure 1, curve A.

In the improved assay described in this paper, the substrate is dissolved in LiCl/TFE, which increases the population of the cis isomer from 10–12% to 40–60%, and in some cases to 70%; the exact percentage of the cis isomer seems to depend on traces of moisture. Addition of small amounts of water ($\leq 1\%$ v/v) lowers the percentage of the cis isomer to an intermediate value (20–30%), and higher amounts decrease the cis population to the value characteristic for aqueous solutions. In the absence of lithium chloride, the equilibrium population of the cis isomer in trifluoroethanol is 10–15%. A representative progress curve for the new assay conditions is shown in Figure 1, curve B. By combining the medium-induced shift

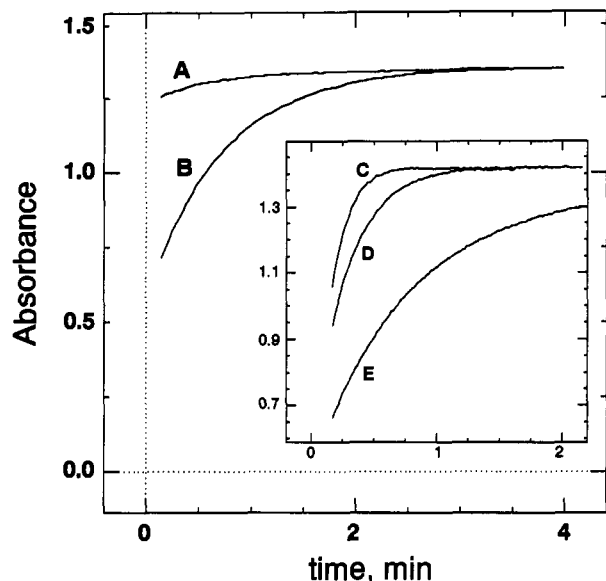


FIGURE 1: Representative progress curves obtained in assays of bovine cyclophilin (3.4 nM) with Suc-AAPF-pNA (100 μ M; chymotrypsin 6 mg/mL; 0 $^{\circ}$ C; pH 8.0). (Curve A) Substrate dissolved in DMSO (10% cis). (Curve B) Substrate dissolved in 470 mM LiCl/TFE (55% cis). (Inset) Time-dependent binding of [(Boc)Dab]⁸-CsA (10.5 nM) to recombinant human cyclophilin (7.4 nM) assayed with Suc-AAPF-pNA (64 μ M cis; 105 μ M total peptide). (Curve C) No preincubation. (Curve D) Enzyme and inhibitor preincubated at 0 $^{\circ}$ C for 1 min. (Curve E) Enzyme and inhibitor preincubated for 150 min. The horizontal axis is in units of time (minutes); the vertical axis is in units of absorbance (390 nm).

of the cis-trans equilibrium with the variation in the monitoring wavelength, the initial cis substrate concentration can be varied from 2 μ M to 3 mM. The concentrations of LiCl (6–12 mM) and TFE (2.5% v/v) had little effect on the activity of the PPIases, as determined with control experiments that used DMSO and ethanol as the solvents for the peptide substrates.

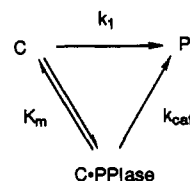
Numerical Analysis of the Progress Curves To Obtain Initial Velocities. An important aspect of the PPIase assay is that the uncatalyzed thermal isomerization complicates the kinetic analysis. For substrate concentrations that are much smaller than the Michaelis constant ($[C]_0 \ll K_m$), the concentration of PPIase is usually chosen so that the uncatalyzed reaction contributes less than 10% to the initial velocity; this yields first-order progress curves, which can be fit to an exponential (Fischer et al., 1989b; Harrison & Stein, 1990a). At intermediate substrate concentrations ($[C]_0 \approx K_m$) the first-order analysis is no longer applicable. Customarily, the determination of the initial velocity would include graphical interpolation of the initial part of the progress curve. However, as substrate concentration is raised, the observed initial velocity (after the burst) contains an ever-increasing contribution from the uncatalyzed background isomerization. Graphical analysis necessarily overestimates the initial velocity; instead, progress curves must be analyzed by an equation that accounts for both the enzyme-catalyzed and background reactions.

Progress curves for substrates that are cleaved by chymotrypsin much more rapidly than the rate of the cis-to-trans isomerization, e.g., Suc-AAPF-pNA or Suc-ALPF-pNA, can be described by the differential rate equation (eq 1)

$$\frac{d[C]}{dt} = -k_1[C] - k_{cat}[E]_p \frac{[C]}{[C] + K_m} \quad (1)$$

derived for Scheme I, where C represents the cis isomer of the substrate and $[E]_p$ is the concentration of PPIase. In eq 1,

Scheme I: Kinetic Mechanism for the PPIase-Chymotrypsin Coupled Assay



K_m and k_{cat} are the Michaelis constant and the turnover number for the PPIase-catalyzed cis-to-trans isomerization and k_1 is the first-order rate constant for the corresponding uncatalyzed process.

Equation 1 can be analytically integrated to provide eq 2

$$E' \ln \left(1 - \frac{[P]}{[C]_0 + K_m + E'} \right) + K_m \ln \left(1 - \frac{[P]}{[C]_0} \right) + k_1 t (K_m + E') = 0 \quad (2)$$

as the theoretical model of the time vs absorbance data. E' stands for $[E]_p k_{cat}/k_1$, and $[P]$ is the concentration of *p*-nitroaniline, formally defined as $[C]_0 - [C]$, where $[C]_0$ is the initial concentration of the cis substrate and $[C]$ is the concentration at time t . The first-order rate constant for thermal isomerization (k_1) was determined independently in identical experiments conducted in the absence of the enzyme, and the data were analyzed by exponential fit.

The experimental absorbance data from each progress curve were analyzed by nonlinear least-squares optimization of K_m and k_{cat} in eq 2 (Marquardt, 1963). At any arbitrary time t , the concentration of the product $[P]$ (and thus absorbance) was computed from the transcendental rate equation (eq 2) by using the Newton-Raphson method. From the optimized values of K_m and k_{cat} the initial velocities v_0 were calculated by applying eq 3. Standard errors ΔK_m and Δk_{cat} were ob-

$$v_0 = k_{cat}[E]_p \frac{[C]_0}{[C]_0 + K_m} \quad (3)$$

tained within the Marquardt algorithm from the final curvature matrix for the fit to eq 2 (Bevington, 1969). This provided the basis for the estimate of errors for v_0 (Δv_0); these were estimated as in eq 4, which follows from eq 3 according

$$\Delta v_0 = \left[\left(\frac{k_{cat}[E]_p[C]_0}{([C]_0 + K_m)^2} \Delta K_m \right)^2 + \left(\frac{[E]_p[C]_0}{[C]_0 + K_m} \Delta k_{cat} \right)^2 \right]^{1/2} \quad (4)$$

to the theory of propagation of experimental errors.

Steady-State Kinetic Parameters of PPIase Substrates. Initial velocities corresponding to the PPIase-catalyzed cis-to-trans isomerization of Suc-AAPF-pNA or Suc-ALPF-pNA were determined as indicated in the preceding paragraph, at the initial cis isomer concentration in the range 0.07–2.50 mM. The results were analyzed by nonlinear least-squares optimization of K_m and V_{max} in the Michaelis-Menten equation $v_0 = V_{max}[C]_0/([C]_0 + K_m)$. The value of the Michaelis constant was subsequently used as a fixed parameter in the active site titration (see below); the enzyme concentration thus obtained was used to calculate turnover numbers k_{cat} from V_{max} . The steady-state kinetic parameters of all three PPIases examined here are summarized in Table I. A representative plot of velocity versus substrate concentration is shown in Figure 2 (solid line); the double-reciprocal plot is shown as an inset.

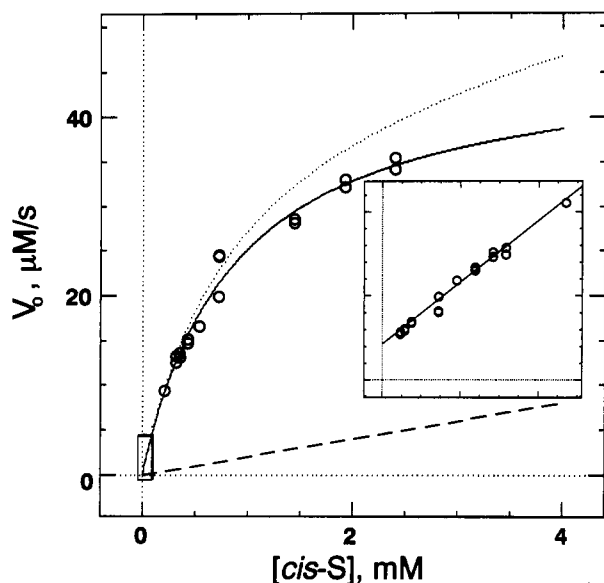


FIGURE 2: Initial velocity of recombinant human cyclophilin-catalyzed *cis*-*trans* isomerization of Suc-AA-*cis*-PF-pNA (O). The solid line represents the best fit to the Michaelis-Menten equation, as indicated in the text ($k_{\text{cat}} = 13200 \pm 880 \text{ s}^{-1}$; $K_m = 0.98 \pm 0.14 \text{ mM}$); the inset shows the corresponding double-reciprocal plot. The *total* initial velocity (---) includes the contribution from the *thermal* uncatalyzed isomerization (---). The box near the origin indicates the concentrations of *cis* substrate attainable prior to the modifications described in this paper.

Active Site Titration of PPIases. To determine the enzyme concentration, the initial velocities were analyzed by nonlinear least-squares optimization of K_i' and $[E]_p$ in eq 5 derived by

$$v_0 = (v_{00}/2[E]_p) \times \left([E]_p - [I]_0 - K_i' + \sqrt{([E]_p - [I]_0 - K_i')^2 + 4[E]_0 K_i'} \right) \quad (5)$$

$$K_i' = K_i \left(1 + \frac{[C]_0}{K_m} \right)$$

Morrison (1969) for competitive tight-binding inhibition. The velocity v_{00} (eq 6) corresponds to zero inhibitor concentration.

$$v_{00} = k_{\text{cat}}[E]_p \frac{[C]_0}{[C]_0 + K_m} \quad (6)$$

In order to avoid the problem of the mutual correlation of v_{00} and $[E]_p$, the value of v_{00} was determined in multiple (typically five) experiments.

Inhibition of Bovine Cyclophilin by $[\text{Me}^5\text{Bth}]^1\text{-CsA}$. The initial velocity for the PPIase-catalyzed *cis*-to-*trans* isomerization of Suc-AAPF-pNA was measured at *cis* substrate concentrations ranging from 0.5 to 2.1 mM. Three sets of data were collected at various concentrations of the inhibitor $[\text{Me}^5\text{Bth}]^1\text{-CsA}$: 0, 70, and 100 nM. The concentration of the bovine cyclophilin was 6.8 nM, as determined in an active site titration experiment. The results from all three series of experiments were pooled and analyzed by nonlinear least-squares optimization of the inhibition constants (K_{ii} , K_{is}) in eq 7. Model discrimination analysis, assuming pure com-

$$v_0 = k_{\text{cat}}[E]_p \frac{[C]_0/K_m}{1 + [C]_0/K_m + [I]_0/K_{is} + [C]_0[I]_0/K_m K_{ii}} \quad (7)$$

petitive inhibition or noncompetitive mixed-type inhibition, was

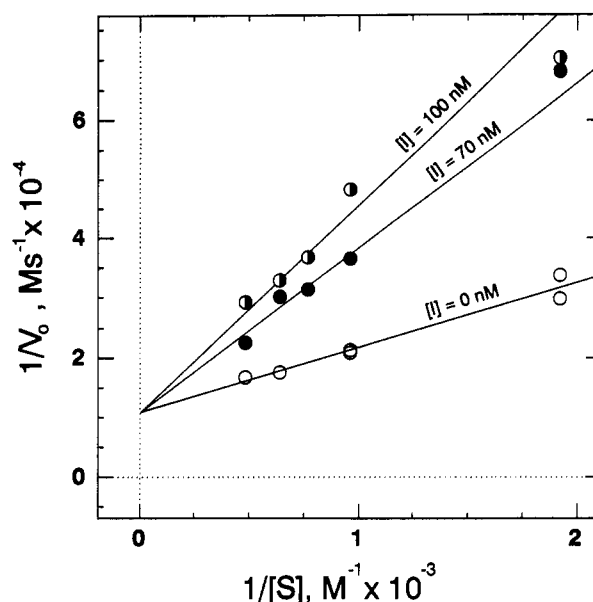


FIGURE 3: Double-reciprocal plot for the competitive inhibition of bovine cyclophilin (6.8 nM) by $[\text{Me}^5\text{Bth}]^1\text{-CsA}$ (0 nM, O; 70 nM, ●; 100 nM, ●). The substrate concentration ranged from 0.5 to 2.1 mM Suc-AA-*cis*-PF-pNA. The inhibition constant K_{ii} is $45 \pm 3 \text{ nM}$.

performed according to the rules formulated by Mannervik (1982). The following criteria were used to discriminate between pure competitive and mixed-type inhibition: (a) successful convergence of parameters K_{ii} and K_{is} in the regression analysis and (b) meaningful values of parameters and low standard deviations. The value of K_{ii} , the uncompetitive component of the overall inhibition, was lacking in convergence; any value of K_{ii} higher than 10 μM satisfied the mixed-type model, but the estimated standard error of K_{ii} was greater than 100%. On the other hand, the competitive inhibition constant quickly converged to the sample value regardless of the model (competitive/mixed-type), and the estimated standard deviation was small ($K_{is} = 45 \pm 3 \text{ nM}$). These results indicate that $[\text{Me}^5\text{Bth}]^1\text{-CsA}$ acts as a competitive inhibitor of human cyclophilin. The diagnostic double-reciprocal plot is shown in Figure 3.

Time-Dependent Inhibition of Human Cyclophilin by $[(\text{Boc})\text{Dab}]^8\text{-CsA}$. Interestingly, the inhibitor $[(\text{Boc})\text{Dab}]^8\text{-CsA}$ exhibits slow onset of inhibition of human cyclophilin. In Figure 1, curve C represents a PPIase assay conducted in the absence of the inhibitor ($[\text{cyclophilin}] = 7.4 \text{ nM}$). Curve D corresponds to an assay in which cyclophilin and the inhibitor (10.5 nM) were preincubated for 1 min prior to the addition of the substrate, while in the case of curve E, the preincubation period was 150 min. The difference between curves D and E illustrates that upon preincubation the initial velocity decreases due to the slow formation of a tightly bound enzyme-inhibitor complex.

DISCUSSION

A primary objective at the start of this work was to overcome the limitations of the chymotrypsin-coupled PPIase assay by shifting the conformational equilibrium toward the *cis* X-Pro isomer. The effects of temperature, organic cosolvents, and ionic strength were investigated by using Suc-AAPF-pNA as the prototypical PPIase substrate, but none of these factors alone produced a significant effect. An alternative approach stems from the elegant work of Seebach and co-workers, who established that the addition of lithium chloride dramatically increases the solubility of peptides in tetrahydrofuran (Seebach et al., 1989). This work prompted us to investigate the effect

of the LiCl/THF system on the cis-trans conformational equilibrium; this salt/solvent system shifts the equilibrium from 12% to approximately 35–40% cis X-Pro isomer in Suc-AA-PF-pNA. A systematic survey of other organic solvents in combination with lithium chloride led to the discovery of LiCl/TFE, which increases the population of the cis isomer to 70%.⁴ The novel effect of lithium salts dissolved in organic solvents on the conformational equilibrium appears to be general for proline-containing peptides. Although the exact percentage of the cis isomer depends on the particular peptide sequence, the yield is higher than 40% cis in all cases tested. In the PPIase assays, this translates into a 5–7-fold absolute increase (10-fold relative increase) in the spectrophotometric signal. It is interesting that lithium chloride in tetrahydrofuran, a cation-solvating solvent, shifts the cis-trans equilibrium in the same direction as in trifluoroethanol, an anion solvator. For the peptides examined here, the absolute magnitude of the effect in THF is lower than in TFE; however, other experimental considerations may arise that will favor the use of THF.

In order to further increase the useful substrate concentration in the assay, the formation of *p*-nitroaniline was monitored at varied wavelengths, away from the absorption maximum (λ_{max} 390 nm). No deviations from the Lambert-Beer law were observed at wavelengths in the range 390–460 nm, up to 4.0 mM total substrate concentration. Close to the solubility limit of *p*-nitroaniline (approximately 5 mM at 0 °C), the spectroscopic response becomes nonlinear, possibly due to light scattering. The corresponding substrate concentration thus represents an upper limit for the PPIase-chymotrypsin coupled assay. In summary, by using the TFE/LiCl solvent system and by varying the monitoring wavelength, it is possible to extend the concentration of Suc-AA-*cis*-PF-pNA in cyclophilin assays from approximately 100 μ M (Fischer et al., 1989b) to approximately 3 mM—a 30-fold gain. This is illustrated in Figure 2, in which the box at the origin indicates the useful cis substrate concentration range prior to the modifications described herein. Similarly, in the assays of the FK-506 binding protein, the concentration of Suc-AL-*cis*-PF-pNA could be increased to about 2.5 mM; the latter substrate was used for FKBP assays because of the 12-fold higher specificity number (k_{cat}/K_m) in comparison to Suc-AA-*cis*-PF-pNA (Harrison & Stein, 1990b).

Harrison and Stein (1990a) demonstrated that at low substrate concentration the contribution of the uncatalyzed, thermal isomerization can be neglected when the concentration of the enzyme is properly adjusted. However, at high substrate concentration, in the vicinity of the Michaelis constant, this approach can no longer be applied because the required amount of PPIase would bring the initial velocity above the limit measurable with a conventional spectrometer. In our modified procedure, the thermal isomerization is partially suppressed by conducting the assays at 0 °C. The residual contribution of the uncatalyzed process is accounted for by fitting the spectroscopic progress curves to the integrated rate equation (eq 2); the dependence of the thermal background

on the substrate concentration is shown in Figure 2 (broken line).

The saturating substrate concentrations that can be achieved with the modified assay enabled us to determine steady-state kinetic parameters for several PPIases. In the case of both human and bovine cyclophilin, previous indirect estimates for the Michaelis constant (>20 mM) and the turnover number ($>40\,000$ s⁻¹) (Harrison & Stein, 1990a) are significantly higher than the values shown in Table I; the values for the specificity number (k_{cat}/K_m) are similar to those reported (Harrison & Stein, 1990a; Liu et al., 1990). K_m and k_{cat} for bovine FKBP are reported here for the first time; k_{cat}/K_m agrees with the published data (Harrison & Stein, 1990b).

The ability to use relatively high cis substrate concentrations in the kinetic studies makes it possible to determine the inhibition patterns for PPIase inhibitors. Two examples illustrate the utility of the assay. The synthetic cyclosporin analogue, [Me⁵Bth]¹-CsA, is a competitive inhibitor of bovine cyclophilin (Figure 3; $K_i = 45$ nM), which suggests that CsA analogues are binding to the active site of the enzyme. It was also found that the tight-binding PPIase inhibitor, [(Boc)Dab]⁸-CsA, displays a slow onset of inhibition; this is the first demonstration of slow-binding inhibition for a CsA analogue. We propose that the slow onset of inhibition may be caused by cis-to-trans isomerization of amide bonds in the inhibitor; NMR experiments have shown that upon binding of cyclosporin A to cyclophilin, the conformation of the MeLeu⁹-MeLeu¹⁰ amide bond changes from cis to trans (Fesik et al., 1990). Detailed kinetic analysis of the time-dependent inhibition will be reported elsewhere. Rapamycin was found to be a tight-binding inhibitor of FKBP, and the observed inhibition constant (0.25 nM) is in good agreement with a previous report (Bierer et al., 1990b). In this case there was no evidence of time-dependent inhibition—an observation that may be related to the absence of slow binding of FK-506 to the same enzyme (Harrison & Stein, 1990b).

The catalytic efficiency of PPIases has previously been evaluated on the basis of estimated kinetic constants (Harrison & Stein, 1990b; Albers et al., 1990), and it is of interest to reexamine the estimates in light of the results indicated in Table I. The catalytic efficiency of PPIases is rigorously defined as a ratio of two first-order rate constants, one that describes the cis-to-trans isomerization of the enzyme-substrate complex, and the other that characterizes the corresponding uncatalyzed process. The lowest estimate for the enzymatic rate constant is represented by the turnover number (k_{cat}), so that the catalytic efficiency can be expressed as the ratio k_{cat}/k_1 . According to this definition, the catalytic efficiency of cyclophilin at 0 °C is approximately 5×10^6 ; taking into account the literature data concerning the temperature effects on the enzymatic and the thermal isomerization, the estimated catalytic efficiency of cyclophilin is 2×10^5 at 37 °C. For the FK-506 binding protein at 0 °C, the catalytic efficiency is 2.2×10^5 , in sharp contrast to a previous report of 9 (Albers et al., 1990).⁵ It is clear that both PPIases greatly accelerate the isomerization; the acceleration factor indicates that PPIases lower the activation barrier for cis-to-trans isomerization by approximately 16 kcal/mol at 0 °C; this is a remarkable effect considering that the energy barrier for the uncatalyzed isomerization itself is close to 19–20 kcal/mol. This is in conflict with thermodynamic data previously published (Harrison & Stein, 1990a) in which the enzyme apparently lowers the

⁴ Solvents that had little or no effect on the cis-trans equilibrium of proline-containing peptides when used in conjunction with LiCl were ethanol, dimethyl sulfoxide, dimethylformamide, and hexafluoro-2-propanol. The substitution of LiClO₄ for LiCl with TFE as the solvent also shifted the equilibrium to 50–70% cis isomer. The substitution of NaCl for LiCl in the TFE/LiCl and tetrahydrofuran/LiCl had no effect (i.e., 10% cis) on this equilibrium. The effects of the LiCl/TFE system on poly(amino acids) have been examined previously (Lotan, 1974); however, the effect on the cis-trans equilibrium of proline-containing peptides could not be anticipated from this report.

⁵ In this paper the authors calculate $k_{\text{enz}}/k_{\text{unc}}$. Apparently k_{enz} is the rate for the enzyme-catalyzed reaction, and k_{unc} is the first-order rate constant for the uncatalyzed reaction; k_{cat} was not determined.

energy of activation by 3.1 kcal/mol at 0 °C.⁶ It is also noteworthy that within an order of magnitude, the apparent second-order rate constant for the enzyme–substrate association (k_{cat}/K_m ; Table I) approaches the estimated diffusion limit of $10^8 \text{ M}^{-1} \text{ s}^{-1}$ —another indication of high catalytic efficiency.

The mechanism by which PPIases catalyze the cis–trans isomerization of X-Pro peptide bonds is not well understood but appears to deviate from catalytic mechanisms utilized by other protein-processing enzymes. Secondary β -deuterium isotope effects (Harrison & Stein, 1990a; Harrison et al., 1990), site-directed mutagenesis, and metal chelation experiments (Liu et al., 1990) have shown that thiol groups or metal ions are not required for catalysis. On the basis of the insensitivity of k_{cat}/K_m to pH (5.5–9.0) and to solvent deuterium, general acid–base catalysis was also ruled out (Harrison & Stein, 1990a). Solvent isotope effects and pH effects on k_{cat} had not been evaluated previously because it was not possible to measure k_{cat} with the original assay. With our modified assay we measured the effect of solvent deuterium (D_2O vs H_2O) on human recombinant cyclophilin assayed with Suc-AA-cis-PF-pNA. Within the limits of the experimental error, the steady-state kinetic parameters (k_{cat} and K_m) were unaffected.

These data suggest that the catalytic mechanism does not involve making or breaking of bonds to hydrogen in rate-limiting steps. Indeed, to account for the apparent noninvolvement of functional catalytic groups, Harrison and Stein (1990a) presented a novel catalytic mechanism, in which the driving force arises exclusively from the noncovalent enzyme–substrate binding interaction to stabilize a distorted, twisted C–N amide bond; the carbonyl group was proposed to remain trigonal. This process was designated “catalysis by distortion”. Subsequently, the same catalytic mechanism was proposed for FKBP by inspection of the structure of FK-506 (Rosen et al., 1990) and from thermodynamic and kinetic arguments (Albers et al., 1990). While the idea that binding energy derived from noncovalent protein–ligand interactions can be utilized in subsequent chemical steps is widely accepted (Jencks, 1975) and has also been invoked for protein processing enzymes, e.g., proteases (Fruton, 1976), the proposed catalysis by distortion is unusual in that no catalytic groups are postulated to accelerate the isomerization of cis-to-trans amide bonds.

The lack of involvement of catalytic groups is surprising because a plausible catalytic functionality would be a proton or a hydrogen-bond donor. Molecular orbital calculations suggest that protonation on the amide nitrogen dramatically lowers the barrier to rotation between cis and trans forms, whereas protonation on the carbonyl oxygen, which is favored, raises the barrier to isomerization (Armbruster & Pullman, 1974). Thus, an alternative catalytic mechanism consistent with the available experimental data is one that stabilizes the transition state for isomerization by protonation on, or hydrogen bonding to, the tetrahedral nitrogen lone pair formed as the amide is rotated. In view of the pH titration data, the proton donor would either have a high pK_a ($pK_a > 9.0$) or be shielded from bulk solvent (Rose et al., 1990). Alternatively, a catalytic group could stabilize the transition state by hydrogen bonding to the tetrahedral nitrogen lone pair, a process that would not give a measurable solvent isotope effect. En-

zymic donor groups that satisfy these criteria include side-chain hydroxyl groups such as found in serine, threonine, or tyrosine. Recently, Wiederrecht et al. (1991) suggested that two tyrosines found in FK-506 binding proteins and several cyclophilin-related proteins are conserved residues; it is possible that one of the phenolic hydroxyls might serve as a catalytic group. Other potential contributors to catalysis have not yet been fully evaluated. For example, hydration has been shown to stabilize the planar form of amide bonds, and conversely, desolvation increases their flexibility (Scheiner & Kern, 1977); consequently, desolvation of the amide bond in a hydrophobic active site of PPIase may be a contributing factor that facilitates the isomerization process.

The use of the lithium chloride/trifluoroethanol solvent system creates an opportunity to develop novel PPIase assays that do not require a coupling enzyme. In this case the shift from the conformational equilibrium characteristic for the aqueous phase is achieved by the mixture of a lithium salt in an organic solvent, rather than by continuous removal of the trans isomer by hydrolysis. A possible loss of spectroscopic sensitivity in alternative detection methods (e.g., fluorescence of labeled peptide substrates, intrinsic CD signals), compared to the convenient monitoring of the intensely chromophoric *p*-nitroaniline, is outweighed by other advantages. A direct assay for PPIase activity would allow systematic exploration of substrate specificity, by using peptide sequences that extend beyond the C-terminal side of proline. Although assays based on NMR methods, e.g., saturation transfer (Hsu et al., 1990) and 2D nuclear Overhauser enhanced spectroscopy (Justice et al., 1990), have the same potential for studying diverse peptide sequences without the use of a coupling enzyme, these techniques are not as convenient as spectrophotometric or fluorometric assays, and the substrates may not be always be soluble at the high concentrations needed for NMR experiments. The LiCl/TFE (or LiCl/THF) solvent system may also be useful in the conformational studies of peptides and in protein folding. We are currently investigating alternative methods for the detection of the peptidyl prolyl cis–trans isomerization, with the aim to examine the structure–activity relationship for PPIase substrates and to characterize the molecular events associated with the time-dependent inhibition.

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REFERENCES

- Albers, M. W., Walsh, C. T., & Schreiber, S. L. (1990) *J. Org. Chem.* 55, 4984–4986.
- Armbruster, A. M., & Pullman, A. (1974) *FEBS Lett.* 49, 18–21.
- Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, p 242, McGraw-Hill, New York.
- Bierer, B. E., Somers, P. K., Wandless, T. J., Burakoff, S. J., & Schreiber, S. L. (1990a) *Science* 250, 556–559.
- Bierer, B. A., Mattila, P. S., Standaert, R. F., Herzenberg, L. A., Burakoff, S. J., Crabtree, G., & Schreiber, S. L. (1990b) *Proc. Natl. Acad. Sci. U.S.A.* 87, 9231–9235.
- Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry* 14, 4953–4963.
- Fesik, S. W., Gampe, R. T., Holtzman, T. F., Egan, D. A., Edalji, R., Lully, J. R., Simmer, R., Helfrich, R., Kishore, V., & Rich, D. H. (1990) *Science* 250, 1406–1409.

⁶ Rigorous determination of the thermodynamic activation parameters for an enzyme-catalyzed reaction would use $k = k_{\text{cat}}$ in the Eyring analysis. Because this value was not available to them, Harrison and Stein (1990a) used an arbitrarily defined $k = (k_{\text{cat}}/K_m)[\text{PPIase}]_{\text{standard state}}$. This treatment gives a correct value for ΔH^\ddagger but incorrect values for ΔS^\ddagger and ΔG^\ddagger ; without access to k_{cat} , the above thermodynamic analysis cannot be successfully attempted.

- Fischer, G., Bang, H., Berger, E., & Schellenberger, A. (1984a) *Biochim. Biophys. Acta* 791, 87-97.
- Fischer, G., Bang, H., & Mech, C. (1984b) *Biomed. Biochem. Acta* 43, 1101-1111.
- Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., & Schmid, F. X. (1989a) *Nature* 337, 476-478.
- Fischer, G., Berger, E., & Bang, H. (1989b) *FEBS Lett.* 250, 267-270.
- Fruton, J. S. (1976) *Adv. Enzymol. Relat. Areas Mol. Biol.* 44, 1-36.
- Handschumacher, R. E., Harding, M. W., Rice, J., Drugge, R. J., & Speicher, D. W. (1984) *Science* 226, 544-547.
- Harding, M. W., Galat, A., Uehling, D. E., & Schreiber, S. L. (1989) *Nature* 341, 758.
- Harrison, R. K., & Stein, R. L. (1990a) *Biochemistry* 29, 1684-1689.
- Harrison, R. K., & Stein, R. L. (1990b) *Biochemistry* 29, 3813-3816.
- Harrison, R. K., Caldwell, C. G., Rosegay, A., Melillo, D., & Stein, R. L. (1990) *J. Am. Chem. Soc.* 112, 7063-7064.
- Holzman, T. F., Egan, D. A., Edalji, R., Simmer, R. L., Helfrich, R., Taylor, A., & Burres, N. S. (1991) *J. Biol. Chem.* 266, 2474-2479.
- Hsu, V. L., Handschumacher, R. E., & Armitage, I. A. (1990) *J. Am. Chem. Soc.* 112, 6745-6747.
- Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol.* 43, 219-410.
- Justice, R. M., Jr., Kline, A. D., Sluka, J. P., Roeder, W. D., Rodgers, G. H., Roehm, N., & Mynderse, J. S. (1990) *Biochem. Biophys. Res. Commun.* 171, 445-450.
- Lang, K., & Schmid, F. X. (1988) *Nature* 331, 453-455.
- Lang, K., Schmid, F. X., & Fischer, G. (1987) *Nature* 329, 268-270.
- Lin, L.-N., Hasumi, H., & Brandts, J. F. (1988) *Biochim. Biophys. Acta* 956, 256-266.
- Liu, J., Albers, M. W., Chen, C. M., Schreiber, S. L., & Walsh, C. T. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87, 2304.
- Lotan, N. (1974) in *Peptides, Polypeptides and Proteins, Proceedings of the 2nd Rehovot Symposium* (Blout, E. R., Bovey, F. A., Goodman, M., & Lotan, N., Eds.) pp 157-165, Wiley, New York.
- Mannervik, B. (1982) *Methods Enzymol.* 87, 370-390.
- Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math.* 11, 431-441.
- Morrison, J. F. (1969) *Biochim. Biophys. Acta* 185, 269-286.
- Neil, G. L., Niemann, C., & Hein, G. E. (1966) *Nature* 210, 903-907.
- Northrop, D. B., Rebholz, K. L., & Bencke Marti, K. M. (1991) in *Enzyme Mechanisms from Isotope Effects* (Cook, P., Ed.) pp 331-338, CRC Press, Ann Arbor, MI (in press).
- Rose, I. A., Fung, W.-J., & Warms, J. V. B. (1990) *Biochemistry* 29, 4312-4317.
- Rosen, M. K., Standaert, R. F., Galat, A., Nakatuska, M., Schreiber, S. L. (1990) *Science* 248, 863-866.
- Seebach, D., Thaler, A., & Beck, A. K. (1989) *Helv. Chim. Acta* 72, 857-867.
- Scheiner, S., & Kern, C. W. (1977) *J. Am. Chem. Soc.* 99, 7042-7050.
- Schönbrunner, E. R., Mayer, S., Tropschug, M., Fischer, G., Takahashi, N., & Schmid, F. X. (1991) *J. Biol. Chem.* 266, 3630-3635.
- Siekierka, J. J., Hung, H. Y., Poe, M., Lin, C. S., & Sigal, N. S. (1989) *Nature* 341, 755.
- Sigal, N. H., Dumont, F., Durette, P., Siekierka, J. J., Peterson, L., Rich, D. H., Dunlap, B. E., Staruch, M. J., Melino, M. R., Koprak, S. L., Williams, D., Witzel, B., & Pisano, J. M. (1991) *J. Exp. Med.* 173, 619-628.
- Wiederrecht, G., Brizuela, L., Elliston, K., Sigal, N. H., & Siekierka, J. J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 1029-1033.