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A CONTINUOUS SPECTROPHOTOMETRIC DIRECT ASSAY FOR *PEPTIDYL PROLYL Cis-Trans* ISOMERASES

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Summary. *m*-Nitrotyrosine incorporated into proline peptides of the general sequence -Xxx-Pro-Tyr(*m*-NO_2)- responds to *cis-trans* Xxx-Pro conformational transition by changes in the pK_a of its side-chain hydroxyl (Garel and Siffert, 1979). We exploited this effect to develop a continuous direct (uncoupled) assay for peptidyl prolyl *cis-trans* isomerases. Prior to the enzyme assay, the *cis-trans* equilibrium is perturbed in favor of the *cis* isomer by dissolving the substrate H-Ala-Ala-Pro-Tyr(*m*-NO_2)-Ala-NH_2 in a 470 mM solution of LiCl in trifluoroethanol. Upon addition of substrate to the biological buffer, the conformational equilibrium characteristic for the aqueous medium is restored, and the Ala-Pro isomerization is monitored spectrophotometrically.

Cyclophilin and FK-506 binding protein (FKBP) have been the focus of numerous studies on the role of these enzymes in signal transduction pathways (1), and on the structural effects in the binding of immunosuppressive ligands (2,3). Although inhibition of the peptidyl prolyl *cis-trans* isomerase activity of cyclophilin or FKBP is not sufficient to cause immunosuppression (1,4,5), it is important to understand the catalytic mechanism of the enzymatic isomerization. Peptidyl prolyl *cis-trans* isomerases (6) usually are assayed by employing the chymotrypsin-coupled spectrophotometric method of Fischer (7). This assay, based on the high *trans* Xxx-Pro amide selectivity of chymotrypsin towards substrates of the type Xxx-Pro-Phe-pNA, cannot be used to study the reactivity of peptides extended beyond the *C*-terminal region of the above sequence. This limitation, along with the potential degradation of PPIases by the coupling enzyme, prompted us to consider alternatives to Fischer's classic assay.

Recently, we have shown that the catalytic activity of PPIases is conveniently monitored by using a direct fluorometric assay (8) based on intramolecular quenching through collision. This

Abbreviations: [(Boc)Dab]^8-CsA, [N$^{\varepsilon}$-*tert*-butyloxycarbonyl-diaminobutyryl]^8-CsA; CsA, cyclosporin A; Fmoc, 9-fluorenylmethyloxycarbonyl; HPLC, high pressure liquid chromatography; HR-FABMS, high-resolution fast atom bombardment mass spectrometry; hrFKBP12, human recombinant FK-binding protein 12; MBHA, 4-methylbenzhydrylamine (resin); Nle, norleucine; NMR, nuclear magnetic resonance; PAL, tris(alkoxy)benzylamide linker; PIPES, piperazine-*N,N'*-bis(2-ethane sulfonic acid); pNA, *para*-nitroanilide; PPIases, peptidyl prolyl *cis-trans* isomerases; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; t_R, retention time. Amino acid symbols denote the L-configuration.

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uncoupled assay has been used to characterize the substrate specificity in the *C*-terminal region of cyclophilin and hrFKBP12 (9). Herein, we present a new uncoupled, direct spectrophotometric assay for PPIases, which exploits the change in the ionization state of the *m*-nitrotyrosyl residue upon *cis-trans* isomerization of the adjacent Xxx-Pro amide bond. As in previous reports (8-11), we used the lithium chloride perturbation of the *cis-trans* conformational equilibrium to enhance the population of the *cis* isomer. When the substrate H-Ala-Ala-Pro-Tyr(*m*-NO_2)-Ala-NH_2 is initially dissolved in a 470 mM solution of LiCl in TFE and subsequently added to the assay buffer, the population of the *cis* Xxx-Pro conformer decreases to an equilibrium value, and the isomerization of the proline amide bond is followed spectrophotometrically by recording the change in the absorbance of the *m*-nitrotyrosyl residue.

Materials and Methods

Enzymes were obtained from Dr. Thomas Holzman (Abbott Research Laboratories, cyclophilin) and Dr. Mark Levy (SmithKline Beecham, hrFKBP12). The active-site concentration was determined by titration (10) with [(Boc)Dab]^8-CsA (cyclophilin) and rapamycin (hrFKBP12).

Peptide synthesis. The substrate H-Ala-Ala-Pro-Tyr(*m*-NO_2)-Ala-NH_2 was prepared by manual solid-phase peptide synthesis, starting with an Fmoc-PAL-Nle-MBHA-polystyrene-resin (0.6 g, 0.35 mmol/g; MilliGen/Bioresearch) (12) and using N^{α}-Fmoc-amino acids. Cycles for incorporation of Fmoc-amino acids comprised deprotection with piperidine-*N,N*-dimethylformamide (3:7, v/v; 1 x 2 min + 1 x 8 min) and single coupling (3.0 fold; 90 min) mediated by 1,3-diisopropylcarbodiimide in *N,N*-dimethylformamide; all couplings were ninhydrin (13) or chloranil (14) negative within two hours. Cleavage of a portion of this peptide-resin (256 mg) was achieved with TFA:H_2O (95:5, v/v) (1 mL used per 100 mg peptide-resin), r.t., 2 h, following which resin was removed by filtration through a disposable pipette with a glass wool plug, and washed with 2 mL of TFA. Ice-cold diethyl ether (50 mL) was added to the combined filtrates to induce cloudiness. The suspension was extracted with 10 % acetic acid (4 x 25 mL), and lyophilized. The crude material was purified by reverse-phase high pressure liquid chromatography on a Vydac C_{18} column (22 x 250 mm, 10 μm, 300 Å). The final product (49 μmol) was analyzed by HPLC on a Vydac C-18 column (4.6 x 250 mm), linear gradient over 20 min of CH_3CN-0.036 % TFA and H_2O-0.045 % TFA from 1:19 to 4:1, flow rate 1.2 mL/min, detection at 254 nm; t_R= 12 min. The integrity of the purified peptide was determined by HR-FABMS, and the observed molecular mass was found to agree with the calculated value (calcd. for $C_{23}H_{34}N_7O_8$ 536.2469 [M+H$^+$], found 536.2472).

Enzymatic assays. The substrate TFA· H-Ala-Ala-Pro-Tyr(*m*-NO_2)-Ala-NH_2 was dried over P_2O_5 in vacuo and dissolved in a 470 mM solution of LiCl in TFE (14.6 mM stock solution), which increased the population of the *cis* isomer to 50 % as determined by NMR spectroscopy. Assays were performed at -0.4 ± 0.1 °C to minimize the uncatalyzed thermal isomerization. Cyclophilin (final concentration 360 nM) and hrFKBP12 (final concentration 375 nM) were equilibrated in 990 μl of buffer (0.1 M PIPES, pH 6.75, 100 mM NaCl). The peptide substrate (10 μl of stock solution) was added and the solution was mixed in a spectrophotometric cuvette. Final concentration of LiCl in the assay buffer was 4.7 mM; TFE was present at a concentration of 1 % (v/v). Absorbance readings at 428 nm were collected on a CARY-14 spectrophotometer interfaced to an IBM-compatible computer *via* commercial data acquisition software (OLIS, On Line Instrument Systems, Jefferson, GA).

Results

Figure 1 shows representative spectrophotometric progress curves for cyclophilin- or hrFKBP12-catalyzed Ala-Pro isomerization of the oligopeptide substrate H-Ala-Ala-Pro-Tyr(*m*-NO_2)-Ala-NH_2. Isomerization was followed by monitoring the decrease in absorbance at 428 nm. The peptide was dissolved in a 470 mM solution of LiCl in TFE to increase the population of

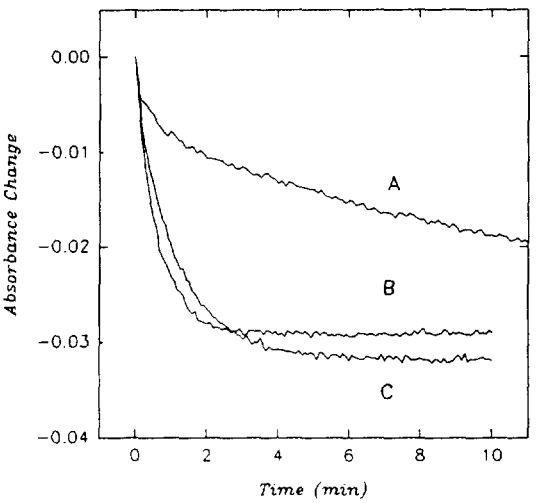

Figure 1.
Spectrophotometric assay of Ala-Pro *cis-trans* isomerization. Experimental conditions: 146 μM H-Ala-Ala-Pro-Tyr(*m*-NO_2)-Ala-NH_2, -0.4 °C, 0.1 M PIPES, pH 6.75, 100 mM NaCl, observation wavelength 428 nm. Curve A - negative control; curve B - human recombinant cyclophilin (360 nM); curve C - hrFKBP12 (375 nM).

the *cis* isomer to 50 % as determined by NMR spectroscopy in d_3-TFE. The equilibrium population of the *cis* isomer in D_2O is 10 %. Assays were conducted at low temperature to minimize the uncatalyzed thermal isomerization. The rate constant k_Δ for uncatalyzed *cis* to *trans* isomerization at -0.4°C ± 0.2 was 2.3 x 10^{-3} s^{-1}, and the corresponding free energy of activation was 19.5 ± 2.9 Kcal/mol. These results are similar to the values reported in the literature for other PPIase substrates (10). The concentration of LiCl (4.7 mM) and TFE (1 %, v/v) has a negligible effect on the activity of cyclophilin and FKBP (10).

Discussion

Peptidyl prolyl *cis-trans* isomerases, including cyclophilin and FKBP, catalyze the *cis-trans* isomerization of the proline amide bond in peptides and proteins (15). The PPIase activity of both enzymes is usually monitored in a chymotrypsin-coupled assay. A significant drawback of the indirect assay is that it only employs substrates that have a *C*-terminal *p*-nitrophenyl group, which precludes structure-reactivity studies in the *C*-terminal region. In addition, the use of a coupling enzyme restricts the assay to PPIases that are not hydrolyzed by the coupling enzyme, and to proteases that are not inhibited by PPIase substrates (16). Uncoupled assays based on NMR techniques, such as saturation transfer (17), 2D nuclear Overhauser enhanced spectroscopy (18), or dynamic NMR spectroscopy (19), have been proposed as alternatives. A disadvantage of these methods is that they require high concentrations of both the substrate and the enzyme, and are time consuming.

In a previous communication (10), we used the lithium chloride/trifluoroethanol solvent system to increase significantly the population of the *cis* Xxx-Pro conformer. This approach was

then applied to develop a continuous PPIase assay which does not require a coupling enzyme and is based on intramolecular quenching through collision (8,9). The fluorogenic substrates may be used only at relatively low concentrations (in the micromolar range), because of the inner filtration effect (20). In order to overcome this instrumental barrier, we took advantage of the *cis-trans* LiCl perturbation effect to developed a continuous UV/VIS spectrophotometric assay.

From solvent-induced isomerization studies of a proline-containing hexapeptide, Garel & Siffert (21) suggested that the isomerization of proline residues in polypeptide chains could be followed spectrophotometrically. This was accomplished by incorporating in the peptide sequence a *m*-nitrotyrosine residue, which attains different ionization states in the *cis* and *trans* conformers of a preceding Xxx-Pro bond. Based on this precedent, we synthesized by solid-phase the substrate H-Ala-Ala-Pro-Tyr(*m*-NO_2)-Ala-NH_2. When PPIase assays are conducted in a strongly buffered solution at pH 6.75, close to the pK_a of an isolated 3-nitrotyrosyl residue (pK_a 6.5), an absorbance change is observed upon *cis-trans* isomerization of the Ala-Pro amide bond with a maximum response at 428 nm. A comparison between the uncatalyzed and the catalyzed Ala-Pro isomerization of the nitrated peptide is shown in Figure 1. Cyclophilin and hrFKBP12 accelerate the Xxx-Pro isomerization significantly.

We developed a mathematical model for the time-course of enzymatic reactions of type $S \rightleftharpoons P$, which are accompanied by a parallel, uncatalyzed, reversible step (arbitrarily, S denotes the *cis* and P the *trans* isomer). Absorbance changes occur in dependence on the current population of *cis* substrate, $x = [S]/[S]_0$, and on the difference molar absorption coefficient $\Delta\varepsilon$, according to the formula $A = A_0 + \Delta\varepsilon\ [S]_0\ (1 - x)$. A_0 is the absorbance at time zero, and $[S]_0$ is the total substrate concentration. The conversion degree x at time t is evaluated by solving the transcendental algebraic equation [1], in which K_{eq} is the *cis-trans* equilibrium constant, x_{eq} the equilibrium concentration of S, lower index zero represents values at initial time, k_Δ is the first-order rate constant for thermal *cis*→*trans* isomerization, k_{cat} and K_m are the corresponding enzyme kinetic parameters, and K_m' is the Michaelis constant in the reverse direction (*trans*→*cis*). A detailed account of applying model equation [1] to determine PPIase kinetic constants from experimental data will be reported separately.

$$\left(\frac{\beta + \alpha(x+1)}{\beta + \alpha(x_0+1)}\right)^{\beta/\alpha}\left(\frac{x - x_{eq}}{x_0 - x_{eq}}\right)^{x_{eq}\gamma+1} - \exp(-\delta k_{cat} t) = 0 \qquad [1]$$

$$\alpha = k_\Delta / k_{cat} \quad ; \quad \beta = \frac{[E]_0}{K_m(1 + [S]_0/K_m')} \quad ; \quad \gamma = \frac{1/K_m + 1/K_m'}{1/[S]_0 - 1/K_m'}$$

$$\delta = (1 + 1/K_{eq})\left[\beta + \alpha(x_{eq}\gamma + 1)\right]$$

In summary, this direct, continuous spectrophotometric assay provides a new method for monitoring enzyme-catalyzed isomerization of Xxx-Pro amide bonds. As in the case of the direct

fluorogenic assay reported earlier (8), it avoids the use of a coupling enzyme, but extends the concentration region beyond that in which spectrofluorometry is applicable. These characteristics provide an alternative method to detect new PPIases (22), and to study the kinetics of PPIase inhibitors.

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