ANALYTICAL BIOCHEMISTRY **252,** 299–307 (1997)
ARTICLE NO. AB972330

A Protease-Free Assay for Peptidyl Prolyl *cis/trans* Isomerases Using Standard Peptide Substrates

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Received June 11, 1997

Peptidyl prolyl *cis/trans* isomerases (PPIases) are ubiquitous and abundant enzymes catalyzing peptide bond *cis/trans* isomerization adjacent to proline in peptides and proteins. An uncoupled protease-free assay of PPIase activity has been developed using the standard tetrapeptide substrates of the proteolytically coupled test system. Differences in the UV/vis absorption spectra of *cis* and *trans* conformations of Suc-Ala-Xaa-Pro-Phe-(Y-) anilide (Xaa = Ala, Leu, Phe; Y = 4-nitro, 2,4-difluoro) were exploited to monitor the time course of the *cis/trans* isomerization subsequent to a solvent jump from 0.47 M LiCl/trifluoroethanol into aqueous solution. The utility of the assay has been demonstrated by the determination of the Michaelis–Menten constants of cytosolic cyclophilin (Cyp18) and of the proteolytically sensitive FK506-binding protein-like PPIase SlyD from *Escherichia coli.* Furthermore, similar inhibition constants were estimated for the reversible inhibition of human Cyp18 by cyclosporin A (CsA) with both the proteolytically coupled and the novel uncoupled PPIase assay.

Key Words: **peptidyl prolyl *cis/trans* isomerase; kinetics; cyclophilin; cyclosporin A.**

Peptidyl prolyl *cis/trans* isomerases (PPIases[1] EC 5.2.1.8) are ubiquitous and abundant enzymes conserved from procaryotes to eucaryotes (reviewed in 1). PPIases accelerate the slow prolyl isomerization[2] in peptides and unfolded proteins. Enzymes of the cyclophilin and the FK506 binding protein (FKBP) family are the cellular receptors of the immunosuppressive drugs cyclosporin A (CsA) and FK506, respectively, acting in a gain of function model in T cells (1). The enzymatic activity of PPIases is generally assessed by an assay that is based on isomer-specific proteolysis using tetrapeptide derivatives (Suc-Ala-Xaa-Pro-Yaa-(4-) nitroanilides with Xaa, Yaa for any natural amino acid) as standard substrates (2, 3). The major part of the reported kinetic constants of PPIase catalysis has been evaluated with this type of proline peptides. Due to the quasi-irreversibility of the prolyl isomerization under these conditions, the proteolytically coupled test permits the calculation of data for *cis* prolyl bonds[2] only.

Another assay used routinely for the enzymatic characterization of PPIases is based on the acceleration of slow kinetic phases during the refolding of denatured proteins (4, 5). Obviously, the quasi-irreversible refolding of denatured proteins under strong native conditions does not permit the measurement of enzymatic constants at the *cis/trans* equilibrium as well.

It was inferred from the microscopic rate constants of a cyclophilin (Cyp)18-catalyzed prolyl isomerization that this member of the enzyme class may have evolved to accelerate interconversions at or near the *cis/trans* equilibrium *in vivo* (6). Therefore, assaying PPIases under reversible conditions of the substrate isomerization proved useful for approaching the cellular situation more closely.

Another disadvantage of the proteolytically coupled assay arises from the presence of very high concentrations of the helper protease in the reaction mixture. The digestion-prone PPIases and putative proteinaceous ligands may be cleaved during the running time of the assay, which usually results in complete misinterpretation of the kinetic traces. Recently, these limitations were partially circumvented by incorporation of the nonnatural 3-nitrotyrosine into oligopeptides. The *cis/trans* interconversions of such peptides can be followed either by UV/vis spectroscopy (7) or along with

[1] Abbreviations used: Boc, *tert*-butyloxycarbonyl, CsA, cyclosporin A, Cyp, cyclophilin, FKBP, FK506 binding protein, PMSF, phenylmethylsulfonyl fluoride, PPIase, peptidyl prolyl *cis/trans* isomerase, SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Suc, succinyl, TFE, trifluoroethanol.

[2] The term prolyl isomerization is used throughout the paper for the *cis/trans* isomerization of the peptide bond preceding proline. Similarly, the term prolyl bond is synonymous with the peptide bond preceding proline.

0003-2697/97 $25.00

appropriately suited fluorescent groups by analyzing the change in the intramolecular fluorescence quenching (8). So far, no detailed analysis of kinetic parameters for the catalysis of prolyl isomerization by PPIases has been undertaken with the 3-nitrotyrosyl peptide derivatives, preventing direct comparison to the standard assay peptides.

In another approach, the Michaelis–Menten parameters for the prolyl isomerization of Suc-Ala-Phe-Pro-Phe-(4-) nitroanilide catalyzed by Cyp18 were estimated recently by NMR line shape analysis under reversible conditions (6). This technique may be the method of choice for determination of microscopic rate constants and mechanistic investigations. However, dynamic NMR measurements are rather time consuming and thus not suited for kinetic screening experiments and assays involving low concentrations of substrates and enzymes.

Here we present a novel uncoupled assay for PPIase activity using standard substrates that combines advantages of both the spectrophotometric and the NMR-based assays as far as it is protease-free, quick, and facile. This new test exploits the observation that prolyl isomerization can be followed by means of UV/vis spectroscopy due to a minor difference in the absorption coefficients of *cis* and *trans* conformers of tetrapeptide anilides remote from far UV peptide bond absorption.

To demonstrate its reliability, this protease-free assay was used to determine the Michaelis–Menten parameters for the catalysis of the prolyl isomerization of tetrapeptide derivatives of the general structure Suc-Ala-Xaa-Pro-Phe-(4-) nitroanilide by the PPIases Cyp18 and *Escherichia coli* SlyD under reversible conditions. The latter FKBP-like protein was previously found to be inactive in the coupled assay (9). Furthermore, we addressed the question of whether the determination of the inhibition constant K_i for inactivation of Cyp18 by CsA is disturbed under conditions of isomer-specific proteolysis. Considerable differences were found for the dissociation constants for the interaction of Cyp18 and CsA determined by either competition kinetics of the Cyp18 catalyzed prolyl isomerization (10–12) or binding assays acting under reversible conditions (13–16). Thus, the K_i for inhibition of Cyp18 by CsA was determined under protease-free conditions by means of the uncoupled test.

MATERIAL AND METHODS

Chemicals

Bovine α-chymotrypsin, isobutylchloroformate, succinic anhydride, and dimethylformamide were purchased from Merck (Darmstadt, Germany). Buffer salts 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and *N*-tris-(hydroxymethyl)-methylglycine (Tricine) were from Serva (Heidelberg, Germany). Succinyl-Ala-Ala-Pro-Phe-(4-) nitroanilide, succinyl-Ala-Phe-Pro-Phe-(4-) nitroanilide, succinyl-Ala-Leu-Pro-Phe-(4-) nitroanilide, and Boc-Ala-Ala-Pro were obtained from Bachem (Heidelberg, Germany). Trifluoroethanol (TFE), *N*-methylmorpholine, and phenylmethylsulfonyl fluoride (PMSF) were from Aldrich (Steinheim, Germany). Subtilisin and bovine serum albumin were purchased from Sigma (Deisenhofen, Germany). LiCl (analytical grade) was from Fluka (Neu-Ulm, Germany). Recombinant human Cyp18 and authentic *E. coli* SlyD were gifts of J. Rahfeld and S. Hottenrott, respectively.

Synthesis of Tetrapeptide Derivatives

The tetrapeptide anilide derivatives were synthesized using standard procedures. The *tert*-butyloxycarbonyl (Boc) moiety was used for temporary protection of the amino function. We used the mixed anhydride method (isobutylchloroformate/*N*-methylmorpholine) for the generation of amide bonds and succinic anhydride/diisopropylamine in dimethylformamide for the final succinylation of the tetrapeptide anilides.

After synthesis of the Boc-Phe-anilides starting from Boc-Phe and the appropriate substituted aniline, we deprotected these compounds acidolytically using 1.3 N HCl in acetic acid. The resulting Phe-anilide hydrochloride was elongated by condensation with Boc-Ala-Ala-Pro. After deprotection of the resulting tetrapeptide derivatives and final succinylation we purified the products by preparative HPLC using different acetonitrile/water mixtures containing 0.1% trifluoroacetic acid as eluent.

All peptides were characterized by ES-MS and ^{13}C and ^{1}H NMR spectroscopy. The purity of all compounds was checked by TLC and HPLC and for the deprotected substances by capillary electrophoresis.

Determination of Absorption Coefficients

At a given total peptide concentration, the absorption coefficients can be calculated from solvent jump experiments provided the known ratios (f_1) of the *cis*/*trans* isomers in 0.47 M LiCl/TFE and aqueous buffer (f_2) and the concentration of peptide (C_0). The *cis*/*trans* equilibrium was determined as described (17) from the ratio of the amplitudes of the kinetic phases of the isomer-specific proteolysis after injection of the peptide substrates from either 0.47 M LiCl/TFE or aqueous buffer. The total peptide concentration of the stock solution C_0 was calculated from the absorbance measured after completion of the reaction using the absorption coefficients of the respective substituted aniline. Absorption coefficients of 13,454 $M^{-1} \cdot cm^{-1}$ at 390 nm for 4-nitroaniline and of 1452 $M^{-1} \cdot cm^{-1}$ for 2,4-difluoroaniline at 290 nm were used for calculation of the concentration of stock solutions.

According to Lambert–Beer's law, total absorption

coefficients of the peptide anilides can be calculated from the initial and the final absorbance for zero time (ϵ_i) and the final state (ϵ_f) of every solvent jump experiment by division with C_0. Using these coefficients, Eqs. [1] and [2] can be formulated,

$$\epsilon_c = \frac{\epsilon_i - (1 - f_1)\cdot\epsilon_t}{f_1} \quad [1]$$

$$\epsilon_t = \frac{f_1\cdot\epsilon_f - f_2\cdot\epsilon_i}{f_1 - f_2}, \quad [2]$$

where ϵ_c and ϵ_t are the absorption coefficients for the *cis* and the *trans* isomer, respectively.

However, due to the finite mixing time of the reagents when starting the solvent jump measurements, ϵ_i cannot be measured directly. Instead, ϵ_i was calculated by extrapolation to zero time according to first-order kinetics. First-order rate constants were obtained from recorded time-dependent spectra by nonlinear regression (2). The UV/vis spectra of peptide substrates injected from solutions in 0.47 M LiCl/TFE into 35 mM Hepes pH 7.8 at 9.5°C were collected using a Hewlett–Packard diode array spectrophotometer HP8452A. A 1-cm silica cell was used. Spectra were recorded between 230 and 530 nm with a spectral bandwidth of 2 nm for 13 min and a cycle time of 10 s starting along with injection.

Isomer-Specific Proteolysis

Isomer-specific proteolysis was performed in 35 mM Hepes, pH 7.8, at 9.5°C. The substrates of the general structure Suc-Ala-Xaa-Pro-Phe-(4-) nitroanilide (Xaa = Ala, Leu, Phe) were used as 100 mg/ml stock solutions in 0.47 M LiCl/TFE. Cyp18 was used from a 470 nM solution in 35 mM Hepes, pH 7.8, obtained by 500-fold dilution from a 235 μM stock solution in 2 mM Tricine, pH 8.0. The concentration of the Cyp18 stock solution was determined fluorimetrically as reported (18). The final concentrations were 1.15% TFE (v/v), 5 mM LiCl, and 1.1 nM (Xaa = Ala) or 3.6 nM Cyp18 (Xaa = Leu, Phe), respectively. Chymotrypsin was used as a stock solution of 10 mg/ml in 35 mM Hepes, pH 7.8, for measurements with tetrapeptide-(4-) nitroanilides. Twenty microliters of chymotrypsin was used for *cis* peptide concentrations below 400 μM and 60 μl was added at higher concentrations to give final protease concentrations of 0.15 and 0.45 mg/ml, respectively.

In a like manner, Suc-Ala-Ala-Pro-Phe-(2,4-) difluoroanilide was investigated using 20 to 60 μl subtilisin from a 20 mg/ml stock solution in 35 mM Hepes, pH 7.8, and 2.1 nM Cyp18. Subtilisin was utilized because the used oligopeptides were cleaved more efficiently by this protease than by chymotrypsin, thereby allowing the reduction of the protease concentration. Low protease concentrations are necessary for measurements at 290 nm to reduce the optical density of the assay mixture.

The wavelengths were set to 390 nm in the case of peptide-(4-) nitroanilides and 290 nm in the case of the peptide-(2,4-) difluoroanilide. A reference wavelength fixed at 510 nm was used for all measurements.

Evaluation of the progress curves, calculation of initial velocities, and estimation of Michaelis–Menten parameters were performed as described previously (17).

Measurement of PPIase Activity by the Uncoupled Assay

For assaying prolyl isomerization with the uncoupled method, progress curves were recorded, applying the experimental conditions of the isomer-specific proteolysis with the exception that the protease was omitted from the assay mixture. Instead, 1 μM bovine serum albumin was added to avoid adsorption of Cyp18 to the surface of the silica cell. SlyD was used from a 190 μM stock solution in 35 mM Hepes at pH 7.8, giving a final concentration of 870 nM. The concentration of the SlyD stock solution was estimated from the absorption at 280 nm according to the method of Gill and von Hippel (19).

The change of absorbance of the tetrapeptide-(2,4-) difluoroanilide and -(4-) nitroanilides was followed at 246 and 330 nm, respectively. At higher substrate concentrations, longer wavelengths were used to limit total absorbance below 1.5. For reference, the simultaneously measured absorbance at 510 nm was subtracted. The change of absorbance was recorded for 6 min with a cycle time of 0.5 s.

Evaluation of Progress Curves

Progress curves were converted from absorbance/time curves to conversion/time curves on the basis of Lambert–Beer's law by using

$$C_c = \frac{A_0 - \epsilon_t\cdot C_0}{\epsilon_c - \epsilon_t}, \quad [3]$$

where A_0 is the overall absorbance. C_0 and C_c are the total and the *cis* isomer concentration, respectively. In further considerations, the *cis* conformer is formally designated as substrate [S].

Converted progress curves were fitted to the rate law for reversible single substrate reactions given by

$$v = k_1\cdot[S] - k_{-1}\cdot([C]_0 - [S]) + \frac{V_{max}^{cis}\cdot[S]}{[S] + K_M^{cis}\cdot(1 + ([C]_0 - [S])/K_M^{trans})} - \frac{V_{max}^{trans}\cdot([C]_0 - [S])}{([C]_0 - [S]) + K_M^{trans}\cdot(1 + [S]/K_M^{cis})}, \quad [4]$$

where [S] is the actual concentration of the *cis* conformer, $[C]_0$ is the total peptide concentration, and k_1 and k_{-1} are the first-order rate constants for spontaneous *cis* to *trans* and *trans* to *cis* prolyl isomerization, respectively. V_{max}^{cis} and V_{max}^{trans} are the maximal velocities for catalyzed *cis* to *trans* and *trans* to *cis* prolyl isomerization, respectively, and K_M^{cis} and K_M^{trans} are the corresponding Michaelis-Menten constants. Numerical integration and least square fits were performed with the program TREND (Martin-Luther-University, Halle, Germany). The variables k_1, k_{-1}, and $[C]_0$ were measured independently and were handled as constants during the iteration procedure, whereas [S], K_M^{cis}, K_M^{trans}, V_{max}^{cis}, and V_{max}^{trans} were fit parameters. From the determined sets of parameters, initial velocities v of the enzyme catalyzed prolyl isomerization were calculated using

$$v = \frac{V_{max}^{cis} \cdot [S]}{[S] + K_M^{cis} \cdot (1 + ([C]_0 - [S])/K_M^{trans})} - \frac{V_{max}^{trans} \cdot ([C]_0 - [S])}{([C]_0 - [S]) + K_M^{trans} \cdot (1 + [S]/K_M^{cis})}. \quad [5]$$

Calculated initial velocities (v) were finally plotted versus *cis* isomer concentration. To reduce number of fitted parameters, Eq. 5 was rearranged to give Eq. 6, as devised by Segel (20),

$$v = \frac{V_{max}^{cis} \cdot ([S] - ([C]_0 - [S])/K_{eq})}{K_M^{cis} \cdot (1 + ([C]_0 - [S])/K_M^{trans}) + [S]}, \quad [6]$$

with K_{eq} as the *cis*/*trans* equilibrium constant in solution and K_M^{cis}, K_M^{trans}, and V_{max}^{cis} the parameters to be calculated by the iterative procedure.

According to Haldane (21), from

$$K_{eq} = \frac{V_{max}^{cis} \cdot K_M^{trans}}{V_{max}^{trans} \cdot K_M^{cis}} \quad [7]$$

V_{max}^{trans} could be calculated.

The turnover numbers for *cis* to *trans* and *trans* to *cis* prolyl isomerization can be obtained by division with the enzyme concentration of the respective V_{max} values.

Determination of Inhibition Constants

The inhibition constants for the inactivation of PPIase activity of Cyp18 by CsA were measured in 35 mM Hepes, pH 7.8, at 9.5°C using both the proteolytically coupled or protease-free assay. CsA was used from solutions in 50% ethanol/water (v/v) that were 1000-fold more concentrated than their final concentration. From these solutions, 1.3 μl were added to the assay mixture. Cyp18 was used at a concentration of 2 nM. Enzyme and inhibitor were incubated for 15 min prior to the start of the measurement (22). Suc-Ala-Ala-Pro-Phe-(2,4-) difluoroanilide was used as substrate at a final concentration of 25 μM. The inhibition constants were estimated as described by Morrison (23).

Stability of SlyD in the Presence of Subtilisin

SlyD was used from a 380 μM stock solution. Two microliters of this solution and 1 μl of a 1 mg/ml solution of subtilisin in 35 mM Hepes, pH 7.8, were added to 7 μl 35 mM Hepes, pH 7.8. This mixture was incubated for 20, 120, or 300 s before the protease activity was quenched by addition of 2 μl of PMSF from a 100 μM stock solution in methanol. As a control, 2 μl of SlyD solution were added to a mixture containing subtilisin already quenched by PMSF. Subtilisin was used instead of chymotrypsin because SlyD and chymotrypsin are only poorly separable on 15% SDS–PAGE. Cyp18, used from a 234 μM stock solution, was treated in a similar way.

RESULTS

Direct Assessment of Prolyl Isomerization by UV/Vis Spectroscopy

Solvent jumps, as ingeniously devised by Kofron *et al.* (17) to increase the amplitude of the signal in the proteolytically coupled assay, were performed to transiently disturb *cis*/*trans* equilibria in tetrapeptide-(4-) nitroanilides. As shown here they provided a means for direct assessment of isomer-specific spectral differences of prolyl conformers as well. Indeed, the evaluation of the time-dependent spectra revealed a slight difference in the absorbance of the peptide conformers in the wavelength range between 230 and 530 nm (Fig. 1). The maximum difference is detected at about 330 nm for the tested peptide-(4-) nitroanilides. A decrease of about 2% of initial absorbance results for the investigated peptide-(4-) nitroanilides when injected from solutions in 0.47 M LiCl/TFE into aqueous buffer solution. To optimize the magnitude of this amplitude anilide ring, substituted tetrapeptide anilides (2,4-difluoro, 4-fluoro, 4-bromo, 4-chloro, 4-cyano, 4-methyl, 4-trifluoromethyl, and 4-methoxy) were synthesized and tested. With a relative magnitude of 7.6% the highest isomer-specific spectral difference was found for Suc-Ala-Ala-Pro-Phe-(2,4-) difluoroanilide.

The change of the signal can be expressed in terms of strict first-order kinetics at any given wavelength within the absorption band of the C-terminal anilide moiety (Fig. 2). The calculated first-order rate constants represent the sum of the rate constants for spontaneous *cis* to *trans* and *trans* to *cis* prolyl isomerization. Hence, the difference of the observed rate

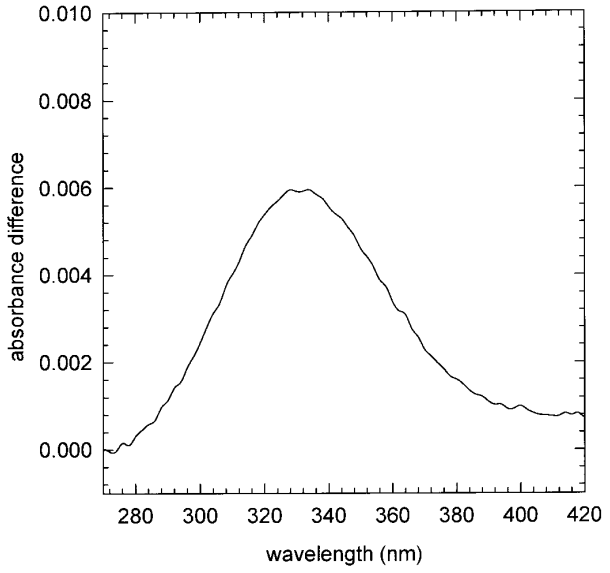

FIG. 1. Difference spectrum of 21 μM Suc-Ala-Ala-Pro-Phe-(4-) nitroanilide calculated from spectra at 30 and 600 s after starting a solvent jump. The peptide was injected from a stock solution in 0.47 M LiCl/TFE into 35 mM Hepes buffer at pH 7.8.

constants obtained from the protease-free and the proteolytically coupled assay for uncatalyzed prolyl isomerization equals the rate constant for *trans* to *cis* isomerization. Rate constants of 7.0×10^{-3} s^{-1} and 0.9×10^{-3} s^{-1} were determined for *cis* to *trans* and *trans* to *cis* isomerization of Suc-Ala-Ala-Pro-Phe-(4-) nitroanilide. From these rate constants a *cis* content of 11.4% can be calculated, which is close to the 10% level found using ^{1}H NMR spectroscopy. The absorption coefficients of the prolyl isomers were found to be independent of the peptide concentration. As was expected for prolyl isomerization, the decrease in absorbance after the solvent jump can be accelerated by addition of nanomolar amounts of Cyp18 (Fig. 2). This acceleration is sensitive to CsA and can be completely inhibited by addition of 1 μM CsA. These data suggest that the observed change in the UV/vis spectra is entirely due to the *cis*/*trans* isomerization of the peptide substrate. Hence, the *cis* and *trans* conformers of the used tetrapeptides have different absorption coefficients, thereby providing a direct measure of the prolyl isomerization processes.

The individual absorption coefficients of the conformers were calculated from sets of time-dependent UV/vis spectra combining isomer-specific proteolysis and solvent jumps at various peptide concentrations (see Material and Methods for details).

Molar absorption coefficients of 13100 ± 200 and 12500 ± 200 M^{-1} cm^{-1} were found for the *cis* and the *trans* conformers of Suc-Ala-Xaa-Pro-Phe-(4-) nitroanilides (Xaa = Ala, Leu, Phe) at 330 nm, respectively. In the limits of error the coefficients of these substrates were independent of the nature of Xaa. As a consequence of Lambert–Beer's law, the absolute amplitude of the change of absorbance, which determines the sensitivity of the uncoupled assay, depends not solely on the difference of the absorption coefficients of the two isomers but is strongly influenced by the *cis*/*trans* ratio of the respective substrates in 0.47 M LiCl/TFE and in buffer.

Comparative Analysis of Cyp18-Catalyzed Standard Substrate Isomerization

For comparison, Suc-Ala-Xaa-Pro-Phe-(4-) nitroanilides were analyzed with regard to k_{cat} and K_M values of the Cyp18-catalyzed prolyl isomerization in the coupled and the uncoupled assay. The obtained kinetic data for the *cis* to *trans* direction of catalysis could be used to validate the evaluation of enzymatic constants by the uncoupled assay (Table 1). In support of our methods, Suc-Ala-Ala-Pro-Phe-(4-) nitroanilide exhibits $K_M^{cis} = 360 \pm 80$ μM and $k_{cat}^{cis} = 9100 \pm 890$ s^{-1}, values that are comparable to $K_M^{cis} = 870$ μM and $k_{cat}^{cis} = 12700$ s^{-1}, values published for slightly different reaction conditions (17).

In addition, kinetic parameters of $K_M^{cis} = 1610 \pm 200$ μM and $k_{cat}^{cis} = 10300 \pm 700$ s^{-1} were determined by isomer-specific proteolysis for Suc-Ala-Ala-Pro-Phe-(2,4-) difluoroanilide as a novel substrate for the uncou-

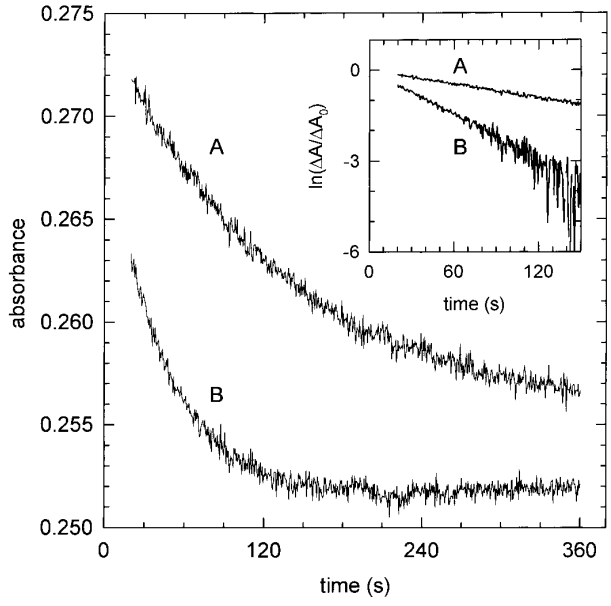

FIG. 2. Time course of the reversible first-order prolyl isomerization of Suc-Ala-Ala-Pro-Phe-(2,4-) difluoroanilide following a solvent jump from 0.47 M LiCl/TFE to 35 μM Hepes, pH 7.8, 1 μM bovine serum albumin without (A) and with 2 nM Cyp18 (B) at 9.5°C. The final dilution of the organic solvent is 88-fold. Decrease of absorbance was recorded at 246 nm. The inset shows the first-order linearization of the progress curves.

TABLE 1

Michaelis–Menten Parameters Determined from the Proteolytically Coupled and the Uncoupled Protease-Free Assay for Catalysis of *cis/trans* Isomerization of Suc-Ala-Xaa-Pro-Phe-(4-) Nitroanilides by Cyp18

	Leu		Phe		Phe
Xaa assay	Coupled[a]	Uncoupled[b]	Coupled[a]	Uncoupled[b]	NMR[c]
K_M^{cis} (μM)	110 ± 20	≈150	40 ± 20	120 ± 70	80
k_{cat}^{cis} (s^{-1})	580 ± 30	≈770	290 ± 20	400 ± 200	680
K_M^{trans} (μM)		≈230		≈500	220
k_{cat}^{trans} (s^{-1})[d]		≈140		≈600	620

[a] 35 mM Hepes, pH 7.8, 0.2 mg/ml chymotrypsin, 9.5°C.
[b] 35 mM Hepes, pH 7.8, 1 μM bovine serum albumin, 9.5°C.
[c] Performed in 10 mM sodium phosphate, pH 6.4, at 10°C (6).
[d] Calculated according to Eq. [7].

pled assay. Thus, the promising signal/noise ratio detected for the UV/vis signal of the tetrapeptide-(2,4-) difluoroanilide anilide was accompanied by a loss of affinity of Cyp18 for at least the *cis* isomer of this substrate. The difference in absorption coefficients enabled us to determine full sets of kinetic constants for Cyp18-catalyzed standard substrate prolyl isomerization under reversible conditions. The respective kinetic constants were estimated for the same substrates as those used with the proteolytically coupled test. The estimation of the specificity constant k_{cat}/K_M for the *cis* to *trans* isomerization of Suc-Ala-Ala-Pro-Phe-(2,4-) difluoroanilide revealed very similar results with 4.4 ± 0.1 $\mu M^{-1}\ s^{-1}$ obtained by isomer-specific proteolysis and 5.1 ± 0.2 $\mu M^{-1}\ s^{-1}$ by the uncoupled assay. The determination of k_{cat}/K_M for *cis* to *trans* isomerization from observed rate constants measured with the aid of the uncoupled assay was performed by use of Eq. [7].

A plot of the initial reaction rate versus substrate concentration is shown for Suc-Ala-Phe-Pro-Phe-(4-) nitroanilide in Fig. 3A. Table 1 gives a summary of the calculated Michaelis–Menten parameters.

Values of $K_M^{cis} \approx 150$ μM and $K_M^{trans} \approx 230$ μM were estimated for the *cis* and *trans* isomers of Suc-Ala-Leu-Pro-Phe-(4-) nitroanilide, respectively. The Michaelis–Menten constant K_M^{trans} was found to be 4.2-fold higher than K_M^{cis} for Suc-Ala-Phe-Pro-Phe-(4-) nitroanilide. This result is in rather good agreement with earlier reports (6). Data are summarized in Table 1. The determination of the kinetic parameters for *trans* to *cis* isomerization of Suc-Ala-Ala-Pro-Phe-(4-) nitroanilide and Suc-Ala-Ala-Pro-Phe-(2,4-) difluoroanilide was impaired at overall peptide concentrations of <2 mM due to the high K_M value found for the *cis* conformers.

Determination of K_i for Inhibition of Cyp18 by CsA

An inhibition constant K_i of 1.5 ± 0.8 nM was determined for the inactivation of 2 nM Cyp18 by CsA with the uncoupled assay at 9.5°C in 35 mM Hepes, pH 7.8. The time dependence of inhibition was taken into account by a 15-min preincubation of enzyme and inhibitor. A value of $K_i = 1.9 \pm 1.4$ nM was determined with the standard proteolytically coupled test at identical experimental conditions.

Enzymatic Characterization of SlyD

Assaying the PPIase activity of SlyD by chymotrypsin- or subtilisin-coupled reaction lead to scrambled first-order traces. Thus, the proteolytic stability of SlyD toward subtilisin was tested. SlyD at a concentration of 75 μM was incubated with 45 μM subtilisin (0.1 mg/ml), a protease concentration generally used in the PPIase standard assay. The digestion was quenched by addition of PMSF after 20 s, 2 min, and 5 min, respectively. As is evident from Fig. 4, SlyD is digested within 20 s of incubation with subtilisin. Similar results can be obtained with digestion of SlyD by chymotrypsin (data not shown). Obviously, assaying SlyD by the proteolytically coupled assay results in an artificially low enzyme activity.

The kinetic characterization of SlyD was thus performed under protease-free conditions using the uncoupled PPIase test as described above. The plot of the calculated initial rates versus substrate concentration is shown in Fig. 3B. Michaelis–Menten parameters of $K_M^{cis} = 1.1 \pm 0.6$ mM and $k_{cat}^{cis} = 16 \pm 6\ s^{-1}$ were obtained for catalysis of *cis* to *trans* isomerization of Suc-Ala-Phe-Pro-Phe-(4-) nitroanilide by 870 nM SlyD.

DISCUSSION

PPIases were shown to be involved in widespread cellular events like protein folding (24, 25) and trafficking (26, 27), immunosuppression (28), HIV virus replication (29, 30), and cell division (31). In some cases the role of enzyme activity in these processes remains to be examined. Data on enzyme kinetics may give in-

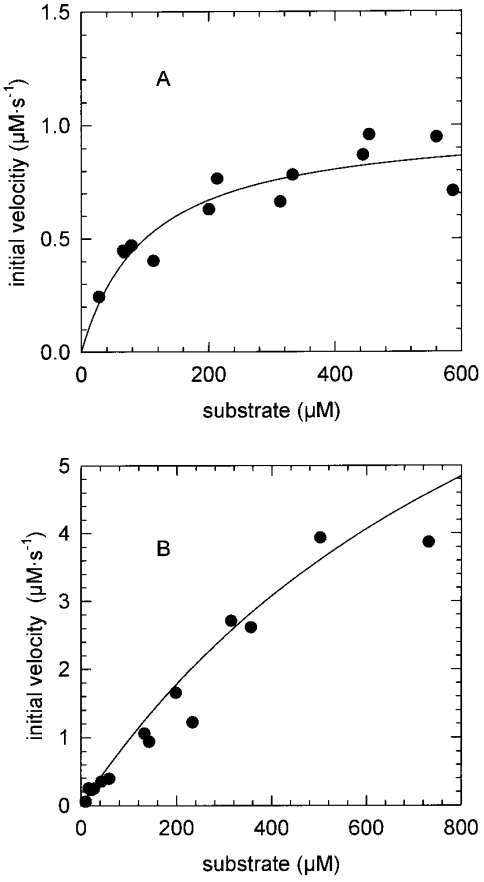

FIG. 3. Michaelis–Menten plot for the Cyp18-catalyzed (A) and SlyD-catalyzed (B) prolyl isomerization of Suc-Ala-Phe-Pro-Phe-(4-) nitroanilide. The plotted substrate concentration corresponds to the concentration of the *cis* isomer of the peptide in aqueous buffer. Initial velocities were calculated from progress curves measured at 9.5°C in 35 μM Hepes, pH 7.8, 1 μM bovine serum albumin, 1.15% TFE, 5 mM LiCl. Cyp18 was used at 3.6 nM, and SlyD was used at 870 nM. Drawn curves represent least square fits of Eq. [6].

sight into the biological function of PPIases and the mechanism of their interaction with cellular effectors and provide the precondition for screening of substance libraries for PPIase effectors. The standard spectrophotometric assay for PPIases, which uses isomer-specific proteases as helper enzymes, is simple and requires a minimum of effort for sample preparation and experimental equipment. However, only one direction of the reversible prolyl isomerization can be investigated, and the test is restricted to reaction components being stable toward digestion by the helper proteases. Therefore, the usefulness of this standard assay for the *in vitro* characterization of PPIases and their interaction with proteinaceous ligands is rather limited.

The recently proposed use of 3-nitrotyrosine as an intramolecular probe of *cis*/*trans* isomerization provides a means for direct assessment of prolyl isomerization, but requires the use of substrates containing this nonnatural amino acid (7). Indeed, an about 100-fold higher concentration of Cyp18 was used to obtain a rate of enhancement for prolyl isomerization similar to that found for the standard tetrapeptide-(4-) nitroanilides (7), suggesting a much lower specificity constant for catalysis of prolyl isomerization by Cyp18 of the nitrotyrosine containing substrates.

The protease-free assay presented in this study is based on the different absorption coefficients found for *cis* and *trans* conformers of succinyl-tetrapeptide anilides. Thus, the prolyl isomerization of tetrapeptide anilides can be followed directly without usage of proteases. Nevertheless, the same tetrapeptide-(4-) nitroanilides, as utilized in the standard isomer-specific assay, can be used with the new method as well. In an attempt to increase the signal/noise ratio observed for these model peptides, tetrapeptide anilides with different substituted anilines were synthesized and tested. Especially Suc-Ala-Ala-Pro-Phe-(2,4-) difluoroanilide fits the requirements expected for PPIase substrates used in a routine kinetic assay. It provides both a sufficient signal change of 7.6% and a specificity constant k_{cat}/K_M of about 5 $\mu M^{-1} \cdot s^{-1}$, which allows detection of nanomolar amounts of Cyp18 under standard conditions (35 mM Hepes, pH 7.8, 10°C). Thus, Suc-Ala-Ala-Pro-Phe-(2,4-) difluoroanilide is a valuable tool for detection of PPIase activity and characterization of its effectors. This peptide was used recently in kinetic competition experiments to examine the interaction of Cyp18 with a fragment of the HIV–Gag polyprotein (32).

Very similar specificity constants k_{cat}/K_M of 5.1 ± 0.2 and 4.4 ± 0.1 $\mu M^{-1} \cdot s^{-1}$ were estimated for the catalysis of *cis* to *trans* isomerization of Suc-Ala-Ala-Pro-Phe-(2,4-) difluoroanilide by Cyp18 with the uncoupled assay and with isomer-specific proteolysis, respectively. This demonstrates the stability of Cyp18 toward proteases and the equivalence of the kinetic parame-

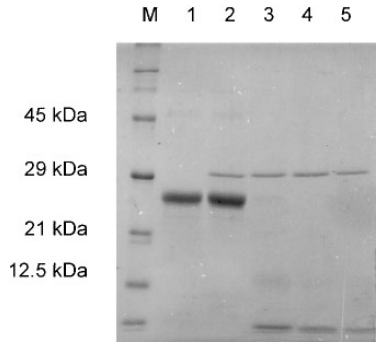

FIG. 4. The stability of 76 μM SlyD toward 36 μM subtilisin was investigated in 35 mM Hepes, pH 7.8, at 10°C and analyzed by SDS–PAGE. The gel was Coomassie blue-stained. Shown are the molecular weight marker (M), SlyD (lane 1), SlyD incubated for 5 min with PMSF-inactivated subtilisin (lane 2), and SlyD incubated with subtilisin for 20 s (lane 3), 2 min (lane 4), and 5 min (lane 5).

ters determined from both PPIase tests for the catalysis of *cis* to *trans* isomerization. Moreover, within the limits of error, similar Michaelis–Menten parameters (K_M and k_{cat}) for the Cyp18-catalyzed *cis* to *trans* isomerization were estimated with both the proteolytically coupled and the protease-free assays for Suc-Ala-Xaa-Pro-Phe-(4-) nitroanilides (Xaa = Leu, Phe). However, a threefold higher value was found for the K_M of the *cis* conformer of Suc-Ala-Phe-Pro-Phe-(4-) nitroanilide by means of the uncoupled assay in comparison with the result obtained from isomer-specific proteolysis. This difference might be caused by interactions of the oligopeptide with bovine serum albumin present in the uncoupled assay. However, the albumin cannot be omitted from the assay mixture due to the considerable adsorption of Cyp18 to the surface of silica cells (data not shown).

The Michaelis–Menten parameters for catalysis of both *cis* to *trans* and *trans* to *cis* isomerization of Suc-Ala-Xaa-Pro-Phe-(4-) nitroanilide (Xaa = Leu, Phe) by Cyp18 were determined by means of the novel uncoupled assay. In terms of K_M, a preference of the *cis* isomers of the chromogenic peptides compared with the *trans* isomers was observed for binding by Cyp18. Whereas the K_M for the *trans* isomer of Suc-Ala-Leu-Pro-Phe-(4-) nitroanilide was found to be only 1.5-fold higher than that estimated for the *cis* isomer, a 4.2-fold higher K_M^{trans} was determined for *trans*-Suc-Ala-Phe-Pro-Phe-(4-) nitroanilide. The values for K_M and k_{cat} calculated for the Cyp18-catalyzed prolyl isomerization of Suc-Ala-Phe-Pro-Phe-(4-) nitroanilide by the uncoupled assay were found to be in accordance with those determined by NMR line shape analysis (6). Parameters of K_M^{cis} = 80 μM and k_{cat}^{cis} = 620 s^{-1} for *cis* to *trans* isomerization and K_M^{trans} = 220 μM and k_{cat}^{trans} = 680 s^{-1} for *trans* to *cis* isomerization, respectively, were reported (6).

The formation of the complex of Cyp18 and CsA has been investigated by different methods, resulting in a surprising scattering of the determined binding constants. Whereas inhibition constants of about 2 nM for the competition of CsA with tetrapeptide-(4-) nitroanilides for Cyp18 binding were obtained from isomer-specific proteolysis (10–12), dissociation constants between 20 and 400 nM were estimated from fluorescence measurements (13, 14), biosensor experiments (15), or radioactive binding assays (16), which were all performed in the absence of proteases under reversible conditions. With K_i = 1.5 ± 0.8 nM and K_i = 1.9 ± 1.4 nM, very similar inhibition constants were found in this study for the inactivation of Cyp18 by CsA with the uncoupled and the proteolytically coupled assay, respectively. Hence, the observed diversity in the published binding constants is rather due to the complex conformational equilibria of CsA encountered under different experimental conditions than to the peculiarities of the quasi-irreversible prolyl isomerization used in the proteolytically coupled assay. The novel uncoupled assay enables the kinetic characterization of proteolytically sensitive PPIases by means of UV/vis spectroscopy. This has been demonstrated by the determination of the Michaelis–Menten parameters for the catalysis of *cis* to *trans* isomerization of Suc-Ala-Phe-Pro-Phe-(4-) nitroanilide by the FKBP-like *E. coli* SlyD. A Michaelis–Menten constant of K_M^{cis} = 1.1 ± 0.6 mM and a turnover number of k_{cat}^{cis} = 16 ± 6 s^{-1} were calculated for the *cis* isomer of the tetrapeptide substrate.

Taken together, the new spectrophotometric assay presented in this study provides a means for the assessment of the steady-state enzyme constants for the catalysis of the prolyl isomerization of tetrapeptide-(4-) nitroanilides by PPIases under conditions where the contribution of both forward and backward isomerization can be determined. This uncoupled assay for PPIase activity should prove to be an useful tool in the characterization of interactions of proteolytically sensitive PPIases and effectors.

ACKNOWLEDGMENTS

We thank S. Hottenrott and Dr. J. Rahfeld for providing SlyD and Cyp18, respectively. We are grateful for the excellent technical assistance of I. Kunze and B. Hökelmann. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Fi 455/1-3), the Fonds der Chemischen Industrie, and the Boehringer-Ingelheim Stiftung.

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