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Inhibition of Peptidyl-prolyl *cis/trans* Isomerase Activity by Substrate Analog Structures: Thioxo Tetrapeptide-4-nitroanilides[†]

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ABSTRACT: The ubiquitous cyclophilins belong to peptidyl-prolyl *cis/trans* isomerases (PPIases; EC 5.2.1.8). They are able to catalyze the *cis/trans* isomerization about peptidyl-prolyl amide bonds. The mode of action of human cytosolic cyclophilin (Cyp18cy) has been studied on substrate analog tetrapeptide-4-nitroanilides containing the thioxo peptidyl-prolyl bond. Five peptides of the general structure Ala-Xaa-ψ[CS-N]-Pro-Phe-NH-Np (Xaa = Gly, Ala, (*S*)-2-aminobutyric acid, Phe, and Leu) containing the thioxo peptidyl-prolyl bond were synthesized. The k_{cat} values for the chymotryptic cleavage of 4-nitroanilide bond of the thioxo tetrapeptide-4-nitroanilides ranged from 1.7 to 9.0 s^{-1} and were sufficiently high to analyze the conformational equilibria by isomer-specific proteolysis. The rate constants of the *cis/trans* isomerization of the thioxo peptidyl-prolyl bond were found to be 25–100-fold lower due to the O/S substitution. Cyp18cy binds both thioxo peptides and oxo peptides in similar manner in the active center but cannot utilize the sulfur analogs as substrates. Instead, competitive inhibition occurs, which was further characterized for Ala-Gly-ψ[CS-N]-Pro-Phe-NH-Np. The inhibition was nearly independent of the pH value in the range of pH 4.5–9, exhibiting apparent K_i values ranging from 200 to 600 μM. In comparison to Ala-Gly-*trans*-ψ[CS-N]-Pro-Phe-NH-Np, the *cis* thioxo peptide Ala-Gly-*cis*-ψ[CS-N]-Pro-Phe-NH-Np was found to possess an approximately 30-fold higher affinity for the active site of the enzyme. Thus, in the presence of stoichiometric amounts of Cyp18cy, the total amount of Ala-Leu-*cis*-ψ[CS-N]-Pro-Phe-NH-Np in solution, detectable by isomer-specific proteolysis, was considerably enhanced.

Until recently, the ubiquitous enzymes peptidyl-prolyl *cis/trans* isomerases comprise three different enzyme families not related to each other in their amino acid sequences, the parvulins (Rahfeld et al., 1994), cyclophilins, and FK506-binding proteins (reviewed by Galat, 1993; Hacker & Fischer, 1993; Fischer, 1994). As many as five cyclophilins, differing in their molecular mass, intracellular localization, or their N-terminal and/or C-terminal extensions of the cyclophilin-like part of the polypeptide chain, are known to exist in human tissues. Especially, the highly conserved cyclophilin-like core domain of the enzymes is found in every organism examined. Although precise information about the cellular functions of cyclophilins on the molecular level is lacking, they have emerged as important participants in several biological processes. For example, retroviral Pr55^{gag} and the p24 fragment (both from HIV-1) have been shown to perform specific binding to Cyp18cy[1] and Cyp23sec from a human leukemia cell line (Luban et al., 1993). It has been suggested that HIV-1 virions specifically incorporate cytosolic Cyp18 of the infected HeLa cells due to an affinity for the four-proline stretch in the region between amino acids 217 and 231 in the capsid domain of the viral Pr55^{gag} protein (Thali et al., 1994; Franke et al., 1994). The presence of Cyp18cy in the virus particle has been shown to be essential for the infectivity of the virions. The formation of the enzymatically inactive complex of Cyp18cy by either cyclosporin A or the non-immunosuppressive derivative MeIle-4 cyclosporin A biased the Pr55^{gag}/Cyp18cy interaction, leading to the formation of noninfectious particles from chronically HIV-1 infected cells (Rosenwirth et al., 1994; Thali et al., 1994; Billich et al., 1995).

Furthermore, several oligomeric complexes, such as the nonactivated estrogen receptor (Ratajczak et al., 1993; Kieffer et al., 1992) and two ligand-gated ion channels (Helekar et al., 1994), appear to require cyclophilins for proper functioning. Mutations in the eye-specific NinaA gene of *Drosophila melanogaster* lead to seriously damaged protein export machinery of the endoplasmatic reticulum providing the R1-6 photoreceptor cells with Rh1 rhodopsin (Stamnes et al., 1991). Temperature-sensitive mutant flies reporting on the dysfunction of the cyclophilin-like NinaA have been found using a genetic approach. Specific amino acid substitutions have been detected in the mutant NinaA protein that gave evidence of a catalytic role of the enzyme during Rh1 maturation. Indeed, amino acid substitution by mutation at homologous positions in the rat Cyp18cy leads to enzymes with different properties at the permissive and the restrictive temperature of the *Drosophila* experiment (Ondek et al., 1992).

In situ inhibition of PPIase activity would be helpful to study the biological function of cyclophilins. Indeed, powerful competitive inhibition of cyclophilins can be obtained by cyclosporin A (Fischer et al., 1989). However, one of

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[1] Abbreviations: All amino acids are of the L-configuration. Abu, (*S*)-2-aminobutyric acid; ACN, acetonitrile; Boc, (*tert*-butyloxy)carbonyl; Cyp18cy, human recombinant cytosolic cyclophilin with a molecular mass of 18 kDa; DCM, dichloromethane; DIPE, diisopropyl ether; DMSO, dimethyl sulfoxide; NEM, *N*-ethylmorpholine; NH-Np, 4-nitroanilide; NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography.

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the problems as a matter of principle associated with the macrocyclic cyclosporins is that the enzyme−inhibitor complex by itself is toxic. Furthermore, it does not affect signal transduction by enzyme inhibition but due to the action of the complex on further constituents of the cell (Liu et al., 1991; Hultsch et al., 1991; Friedman & Weissman, 1991; Schreiber et al., 1993). Substrate analog inhibitors would be helpful in avoiding such secondary biological effects. They may also allow access to differential inhibitors of the cyclophilins. In this case, the small but significant differences in the subsite specificity detected for cyclophilins may be utilized.

Recently, we have reported the use of thioxo[2] amino acylprolyl peptide derivatives to study the *cis/trans* isomerization of the thioxo amide moiety (Schutkowski et al., 1994). Obviously, this group mimics the natural reaction center for the catalytic action of cyclophilins and should provide a valuable tool for investigating the molecular events of catalysis by PPIases.

Here, we report the synthesis and partial conformational characterization of thioxo tetrapeptide-4-nitroanilides equipped with a thioxo peptidyl-prolyl bond. Furthermore, we were able to demonstrate that this peptide bond surrogate switches a typical substrate of Cyp18cy to a competitive inhibitor for this enzyme. The binding strength of the inhibitor was sensitive to the residue Xaa in the Ala-Xaa-ψ[CS-N]-Pro-Phe-NH-Np.[3] Additionally, the *cis*-conformer was bound stronger to the active site of the enzyme than the *trans* form. Analysis of the equilibrium composition for *cis* and *trans* Ala-Leu-ψ[CS-N]-Pro-Phe-NH-Np in mixtures containing stoichiometric amounts of Cyp18cy gives rise to changed *cis/trans* ratio in solution.

EXPERIMENTAL PROCEDURES

Chemical Studies. The ^{1}H- and ^{13}C-NMR spectra were recorded with a Bruker ARX 500 spectrometer at 500.13 and 125.27 MHz in the case of ^{1}H and ^{13}C measurements, respectively. If not otherwise noted, the measurements were performed in DMSO-d_6 at 25 °C. All chemical shifts given only for the major isomer are δ values relative to TMS. Electrospray mass spectra were recorded with a VG BIO-Q (Fisons Instruments, England). Reversed phase HPLC was performed with a Sycam apparatus (Gilchingen, Germany) on a Nucleosil C8 column (25 × 250 mm; 7 μm) using various acetonitrile−water mixtures containing 0.05% TFA. Melting points were determined on a micro hot plate according to BOETIUS. The optical rotation was measured with a Polamat A polarimeter (Carl Zeiss Jena, Germany) at 25 °C. All materials and solvents were of analytical grade and were used without further purification with the following exceptions: benzene was distilled from KOH; THF and diethyl ether were distilled from sodium and stored over KOH.

The purity of all compounds was checked by TLC and HPLC and for the deprotected substances by capillary electrophoresis. The presence of the thioxo amide group in each compound was confirmed by UV−vis spectroscopy, ^{1}H-NMR, and ^{13}C-NMR which showed the 30 ppm downfield shift in the thioxo carbonyl carbon relative to the carbonyl carbon of oxo amide analogs.

Pro-Phe-NH-Np and Phe-NH-Np were synthesized according to the literature (Fischer et al., 1984). Boc-Gly-Pro, Boc-Ala-Pro, Boc-Phe-Pro, Boc-Leu-Pro, and Suc-Ala-Ala-Pro-Arg-NH-Np were purchased from Bachem (Heidelberg, Germany). All Boc-protected amino acids were purchased from Propeptide (Vert-le-petit, France). Acetonitrile for HPLC (catalog no. 13358) was a product from Merck (Darmstadt, Germany). All deprotected compounds were characterized as the salts of hydrochloric acid and in the case of deprotected thioxo tetrapeptide derivatives as the salts of trifluoroacetic acid.

Standard Procedures. (I) Mixed Anhydride Coupling. A solution of 10 mmol of the Boc-protected amino acid dissolved in 10 mL of THF and cooled to −15 °C was treated with 1.27 mL (10 mmol) of NEM and 1.3 mL (10 mmol) of isobutyl chloroformate followed by 10 mmol of the appropriate N-terminal free peptide derivative and an additional 1.27 mL (10 mmol) of NEM after an activating time of 10 min. The reaction mixture was stirred for 1 or 2 h at −15 °C and an additional 5−15 h at ambient temperature. The solvent was evaporated and the residue taken up into ethyl acetate. The mixture was washed successively with brine, 5% $KHSO_4$, brine, 5% $NaHCO_3$, and brine, dried over Na_2SO_4, and evaporated to give a residual foam or solid. The product was either used for further modifications without additionally purification or purified by crystallization using various solvent systems.

(II) Thioxylation of the Protected Tripeptide-4-nitroanilides. To a solution of 20 mmol of Boc-protected tripeptide-4-nitroanilide in a mixture of benzene/dimethoxyethane/thiophene (25:25:1 v/v) was added 11 mmol of 2,4-bis(*p*-methoxyphenyl)-1,3-dithiadiphosphetane 2,4-disulfide (Lawesson's reagent). The resulting solution was refluxed for 10 h, yielding a mixture of Boc-Xaa-ψ[CS-N]-Pro-Phe-NH-Np and Boc-Xaa-Pro-ψ[CS-NH]-Phe-NH-Np. The solvent was evaporated and the desired product purified by flash chromatography on silica gel 60 (40−63 μm) with DCM to remove byproducts, followed by DCM/AcOEt mixtures to obtain the thioxo tripeptide derivatives.

(III) Deprotection of the Peptide Derivatives. The Boc-protected peptide derivative (1 mmol) dissolved in 3 mL of AcOH was treated with 3 equiv of 1.1 N HCl/AcOH. After 30 min at room temperature, the solvent was evaporated. The product was dissolved in EtOH and precipitated by addition of diethyl ether. The resulting precipitate was filtered off and washed carefully with diethyl ether several times.

(IV) Deprotection of the Thioxo Peptide Derivatives. The Boc-protected thioxo peptide derivative (2 mmol) was dissolved in 5 mL of dioxane, and 5 equiv of HCl/dioxane was added in the presence of 0.5 mL of thioanisole. The resulting mixture was stirred at 15 °C under argon atmosphere for 20 min. The solvent was evaporated, and the resulting residue was dissolved in EtOH and precipitated by addition of diethyl ether. The resulting product was filtered off and washed with warm diethyl ether.

Boc-Gly-Pro-Phe-NH-Np. Coupling of Boc-Gly-Pro and Phe-NH-Np (30 mmol), product precipitated using *n*-hexane, yield 14.2 g (88%), mp 124−125 °C, $[\alpha]_D = -32.3°$ (*c* 1, AcOEt).

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[2] For naming C=S, the prefix "thiono" has been frequently used in place of "thioxo". But the IUPAC nomenclature committee recommends the use of "thioxo".

[3] Alterations of a peptide bond are represented by the ψ nomenclature system. A ψ is followed by the structure of the new bond in parentheses. The nomenclature of the compounds is in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (1984) *Pure Appl. Chem. 5b*, 595.

Boc-Ala-Pro-Phe-NH-Np. Coupling of Boc-Ala-Pro and Phe-NH-Np (30 mmol), crystals from AcOEt, yield 15.6 g (94%), mp 112−114 °C, $[\alpha]_D = -27.4°$ (*c* 1, AcOEt).

Boc-Abu-Pro-Phe-NH-Np. Coupling of Boc-Abu and Pro-Phe-NH-Np (30 mmol), crystals from AcOEt/diethyl ether (1:1 v/v), yield 15.3 g (90%), mp 193−194 °C, $[\alpha]_D = -25.3°$ (*c* 1, AcOEt).

Boc-Phe-Pro-Phe-NH-Np. Coupling of Boc-Phe-Pro and Phe-NH-Np (30 mmol), product precipitated using *n*-hexane, yield 17.4 g (92%), mp 82−83 °C, $[\alpha]_D = -10.1°$ (*c* 1, AcOEt).

Boc-Leu-Pro-Phe-NH-Np. Coupling of Boc-Leu-Pro and Phe-NH-Np (30 mmol), product precipitated using *n*-hexane, yield 15.5 g (87%), mp 142−143 °C, $[\alpha]_D = +0.8°$ (*c* 1, AcOEt).

Boc-Gly-ψ[CS-N]-Pro-Phe-NH-Np. Thioxylation of Boc-Gly-Pro-Phe-NH-Np (10.8 g) according to II, flash chromatography using 40% AcOEt in DCM yielded 9.1 g (82%) of Boc-Gly-ψ[CS-N]-Pro-Phe-NH-Np and 1.2 g (11%) of Boc-Gly-Pro-ψ[CS-N]-Phe-NH-Np as colorless oily residues after evaporation of the solvent.

Boc-Ala-ψ[CS-N]-Pro-Phe-NH-Np. Thioxylation of Boc-Ala-Pro-Phe-NH-Np (11.1 g) according to II, flash chromatography using 35% AcOEt in DCM yielded 8.7 g (76%) of Boc-Ala-ψ[CS-N]-Pro-Phe-NH-Np and 2.1 g (18%) of Boc-Ala-Pro-ψ[CS-N]-Phe-NH-Np as a pale yellow foam after evaporation of the solvent.

Boc-Abu-ψ[CS-N]-Pro-Phe-NH-Np. Thioxylation of Boc-Abu-Pro-Phe-NH-Np (11.4) according to II, flash chromatography using 25% AcOEt in DCM yielded 6.1 g (52%) of Boc-Abu-ψ[CS-N]-Pro-Phe-NH-Np and 4.2 g (36%) of Boc-Abu-Pro-ψ[CS-N]-Phe-NH-Np as oily residues after evaporation of the solvent.

Boc-Phe-ψ[CS-N]-Pro-Phe-NH-Np. Thioxylation of Boc-Phe-Pro-Phe-NH-Np (12.6) according to II, flash chromatography using 15% AcOEt in DCM yielded 1.6 g (13%) of Boc-Phe-ψ[CS-N]-Pro-Phe-NH-Np and 6.4 g (54%) of Boc-Phe-Pro-ψ[CS-N]-Phe-NH-Np as oily residues after evaporation of the solvent.

Boc-Leu-ψ[CS-N]-Pro-Phe-NH-Np. Thioxylation of Boc-Leu-Pro-Phe-NH-Np (11.9 g) according to II, flash chromatography using 10% AcOEt in DCM yielded 1.2 g (10%) of Boc-Leu-ψ[CS-N]-Pro-Phe-NH-Np and 5.9 g (48%) of Boc-Leu-Pro-ψ[CS-N]-Phe-NH-Np as pale yellow oily residues after evaporation of the solvent.

Gly-Pro-Phe-NH-Np. Deprotection of Boc-Gly-Pro-Phe-NH-Np according to III (5 mmol), yield 2.2 g (94%), mp 146 °C, $[\alpha]_D = -13.7°$ (*c* 1, MeOH).

Ala-Pro-Phe-NH-Np. Deprotection of Boc-Ala-Pro-Phe-NH-Np according to III (5 mmol), yield 2.2 g (90%), mp 153−154 °C, $[\alpha]_D = -42.8°$ (*c* 1, MeOH).

Abu-Pro-Phe-NH-Np. Deprotection of Boc-Abu-Pro-Phe-NH-Np according to III (5 mmol), yield 2.2 g (88%), mp 172 °C, $[\alpha]_D = -15.2°$ (*c* 1, MeOH).

Phe-Pro-Phe-NH-Np. Deprotection of Boc-Phe-Pro-Phe-NH-Np according to III (5 mmol), yield 2.6 g (92%), mp 148−150 °C, $[\alpha]_D = -9.2°$ (*c* 1, MeOH).

Leu-Pro-Phe-NH-Np. Deprotection of Boc-Leu-Pro-Phe-NH-Np according to III (5 mmol), yield 2.1 g (81%), mp 166−168 °C, $[\alpha]_D = -1.4°$ (*c* 1, MeOH).

Boc-Ala-Gly-Pro-Phe-NH-Np. Coupling of Boc-Ala and Gly-Pro-Phe-NH-Np (0.5 mmol), crystals from AcOEt, yield 265 mg (87%), mp 104−105 °C, $[\alpha]_D = -98.2°$ (*c* 1.9, AcOEt).

Boc-Ala-Ala-Pro-Phe-NH-Np. Coupling of Boc-Ala and Ala-Pro-Phe-NH-Np (0.5 mmol), crystals from AcOEt, yield 253 mg (81%), mp 190−191 °C, $[\alpha]_D = -80.1°$ (*c* 1, MeOH).

Boc-Ala-Abu-Pro-Phe-NH-Np. Coupling of Boc-Ala and Abu-Pro-Phe-NH-Np (0.5 mmol), crystals from AcOEt/light petroleum (4:1 v/v), yield 255 mg (80%), mp 182−183 °C, $[\alpha]_D = +0.6°$ (*c* 1.1, AcOEt).

Boc-Ala-Phe-Pro-Phe-NH-Np. Coupling of Boc-Ala and Phe-Pro-Phe-NH-Np (0.5 mmol), product precipitated using *n*-hexane, yield 315 mg (90%), mp 99−101 °C, $[\alpha]_D = -4.0°$ (*c* 1, AcOEt).

Boc-Ala-Leu-Pro-Phe-NH-Np. Coupling of Boc-Ala and Leu-Pro-Phe-NH-Np (0.5 mmol), product precipitated using *n*-hexane, yield 253 mg (76%), mp 111−113 °C, $[\alpha]_D = -4.5°$ (*c* 1, AcOEt).

Boc-Ala-Gly-ψ[CS-N]-Pro-Phe-NH-Np. Deprotection of Boc-Gly-ψ[CS-N]-Pro-Phe-NH-Np according to IV (5 mmol). The crude product (1.6 g, 65%) was coupled without further purification to Boc-protected alanine (2 mmol) according to I, yielding a yellow powder after precipitation with DIPE. The product was purified by flash chromatography on silica gel with benzene/acetone/acetic acid (50:20:1 v/v/v), yielding 915 mg (73%) of a white solid after removing the solvent from the collected thioxo peptide containing fractions, mp 92−93 °C, $[\alpha]_D = -36.2°$ (*c* 1, AcOEt).

Boc-Ala-Ala-ψ[CS-N]-Pro-Phe-NH-Np. Deprotection of Boc-Ala-ψ[CS-N]-Pro-Phe-NH-Np according to IV (5 mmol). The crude product (1.7 g, 71%) was coupled without further purification to Boc-protected alanine (2 mmol) according to I, yielding an amorphous powder after precipitation with DIPE. Collection of the product-containing fractions and evaporation of the solvent after silica gel chromatography with DCM/AcOEt (4:1 v/v) yielded 782 mg (61%) of a white solid, mp 102−103 °C, $[\alpha]_D = -42.3°$ (*c* 1, AcOEt).

Boc-Ala-Abu-ψ[CS-N]-Pro-Phe-NH-Np. Deprotection of Boc-Abu-ψ[CS-N]-Pro-Phe-NH-Np according to IV (5 mmol). The crude product was purified by silica gel chromatography using chloroform/methanol (9:1 v/v) to remove byproducts and pure methanol to obtain the desired thioxo peptide derivative. Product-containing fractions were collected, and the solvent was stripped off. The remaining oil (yield 1.2 g, 48%) was coupled without further purification to Boc-protected alanine (1 mmol) according to I, yielding 543 mg (83%) of an amorphous powder after precipitation with DIPE, mp 63−64 °C, $[\alpha]_D = -21.1°$ (*c* 1.5, AcOEt).

Boc-Ala-Phe-ψ[CS-N]-Pro-Phe-NH-Np. Deprotection of Boc-Phe-ψ[CS-N]-Pro-Phe-NH-Np according to IV (2 mmol). The crude product was purified by silica gel chromatography using chloroform/methanol (9:1 v/v) followed by pure methanol. Product-containing fractions were collected, and the solvent was evaporated. The remaining oil (yield 629 mg, 54%) was coupled without further purification to Boc-protected alanine (1 mmol) according to I, yielding an oily residue after precipitation with *n*-hexane, which was purified by preparative HPLC (53% ACN, 0.05% TFA); lyophilization yielded 588 mg (82%) of the thioxo tetrapeptide derivative, mp 106−108 °C, $[\alpha]_D = -16.3°$ (*c* 1, AcOEt).

Boc-Ala-Leu-ψ[CS-N]-Pro-Phe-NH-Np. Deprotection of Boc-Leu-ψ[CS-N]-Pro-Phe-NH-Np according to IV (1.5 mmol). The remaining yellow powder (yield 560 mg, 68%) was coupled without further purification to Boc-protected alanine (1 mmol) according to I, yielding an oil after precipitation with *n*-hexane, which was purified by preparative HPLC (60% ACN, 0.05% TFA); lyophilization yielded 492 mg (72%) of the desired product, mp 113−115 °C, $[\alpha]_D = -22.1°$ (*c* 1, AcOEt).

Ala-Gly-Pro-Phe-NH-Np. Deprotection of Boc-Ala-Gly-Pro-Phe-NH-Np according to III (0.3 mmol), yield 148 mg (90%), mp 174−176 °C, $[\alpha]_D = -45.8°$ (*c* 1.1, MeOH), calcd for $C_{25}H_{30}N_6O_6$ 510.22, found $(M + H)^+$ 511.39; ^{1}H NMR δ = 1.35 (d, 3H), 1.65 (m, 2H), 1.81 (m, 1H), 1.97 (m, 1H), 3.03−3.22 (m, 2H), 3.43 (q, 1H), 3.59 (m, 1H), 3.94 (m, 1H), 4.06 (d, 2H), 4.27 (m, 1H), 4.67 (m, 1H), 7.12−7.41 (m, 5H), 7.93 (m, 2H), 8.19−8.27 (bd + m, 3H), 8.46 (d, 1H), 8.67 (t, 1H), 10.72 (s, 1H); ^{13}C-NMR δ = 17.4, 23.9, 29.1, 36.7, 41.6, 46.1, 48.1, 55.4, 59.9, 119.2, 125.0, 126.5, 128.2, 129.3, 137.6, 142.3, 145.1, 167.2, 169.8, 171.1, 171.7.

Ala-Ala-Pro-Phe-NH-Np. Deprotection of Boc-Ala-Ala-Pro-Phe-NH-Np according to III (0.3 mmol), yield 158 mg (94%), mp 202−204 °C, $[\alpha]_D = -11.5°$ (*c* 1, MeOH), calcd for $C_{26}H_{32}N_6O_6$ 524.24, found $(M + H)^+$ 525.36; ^{1}H-NMR δ = 1.22 (d, 3H), 1.32 (d, 3H), 1.67 (m, 1H), 1.82 (m, 2H), 1.98 (m, 1H), 2.95 (dd, 1H), 3.13 (dd, 1H), 3.55 (m, 1H), 3.61 (m, 1H), 3.83 (q, 1H), 4.58 (q, 1H), 4.62 (m, 1H), 4.65 (m, 1H), 7.16−7.34 (m, 5H), 7.94 (m, 2H), 8.17 (bd, 1H), 8.23 (m, 2H), 8.34 (d, 1H), 8.70 (d, 1H), 10.73 (s, 1H); ^{13}C-NMR δ = 16.8, 17.3, 24.4, 28.9, 36.7, 46.6, 46.8, 48.0, 55.3, 59.8, 118.9, 124.9, 126.4, 128.1, 129.2, 137.4, 142.3, 145.0, 169.2, 170.5, 171.0, 171.6.

Ala-Abu-Pro-Phe-NH-Np. Deprotection of Boc-Ala-Abu-Pro-Phe-NH-Np according to III (0.3 mmol), yield 150 mg (87%), mp 168−169 °C, $[\alpha]_D = -5.8°$ (*c* 1, MeOH), calcd for $C_{27}H_{34}N_6O_6$ 538.25, found $(M + H)^+$ 539.34; ^{1}H-NMR δ = 0.87 (t, 3H), 1.33 (d, 3H), 1.53 (m, 1H), 1.72 (m, 2H), 1.83 (m, 2H), 1.99 (m, 1H), 2.92 (dd, 1H), 3.11 (dd, 1H), 3.43 (m, 1H), 3.61 (m, 1H), 3.89 (q, 1H), 4.29 (m, 1H), 4.43 (m, 1H), 4.63 (m, 1H), 7.13−7.35 (m, 5H), 7.92 (m, 2H), 8.21 (m, 2H), 8.28 (bd, 1H), 8.36 (d, 1H), 8.65 (d, 1H), 10.83 (s, 1H); ^{13}C-NMR δ = 10.0, 17.3, 24.2, 24.4, 28.8, 36.7, 46.9, 47.8, 52.1, 55.4, 59.6, 118.9, 124.8, 126.3, 128.1, 129.2, 137.3, 142.2, 145.1, 169.3, 170.3, 171.1, 171.6.

Ala-Phe-Pro-Phe-NH-Np. Deprotection of Boc-Ala-Phe-Pro-Phe-NH-Np according to III (0.3 mmol), yield 168 mg (88%), mp 162−164 °C, $[\alpha]_D = -4.9°$ (*c* 1, MeOH), calcd for $C_{32}H_{36}N_6O_6$ 600.27, found $(M + H)^+$ 601.43; ^{1}H-NMR δ = 1.38 (d, 3H), 1.71 (m, 1H), 1.84 (m, 2H), 2.02 (m, 1H), 2.78−3.18 (m, 4H), 3.61 (m, 1H), 3.79 (m, 1H), 4.21 (q, 1H), 4.31 (dd, 1H), 4.65 (m, 1H), 4.78 (m, 1H), 7.24−7.38 (m, 10H), 7.91 (m, 2H), 8.21 (d, 1H), 8.26 (m, 2H), 8.39 (d, 1H), 8.82 (d, 1H), 10.81 (s, 1H); ^{13}C-NMR δ = 17.1, 24.4, 28.8, 36.1, 36.8, 46.9, 47.8, 52.8, 55.4, 59.8, 118.9, 124.8, 126.3, 128.0, 128.2, 129.2, 137.4, 137.6, 142.2, 145.1, 169.4, 169.9, 170.9, 171.4.

Ala-Leu-Pro-Phe-NH-Np. Deprotection of Boc-Ala-Leu-Pro-Phe-NH-Np according to III (0.3 mmol), yield 148 mg (82%), mp 108−110 °C, $[\alpha]_D = -1.3°$ (*c* 1, MeOH), calcd for $C_{29}H_{38}N_6O_6$ 566.28, found $(M + H)^+$ 567.47; ^{1}H-NMR δ = 0.88 (d, 6H), 1.32 (d, 3H), 1.45 (m, 2H), 1.66 (m, 1H), 1.75 (m, 1H), 1.84 (m, 2H), 2.01 (m, 1H), 3.02 (dd, 1H), 3.14 (dd, 1H), 3.54 (m, 1H), 3.61 (m, 1H), 3.86 (m, 1H), 4.31 (q, 1H), 4.54 (m, 1H), 4.61 (m, 1H), 7.19−7.35 (m, 5H), 7.91 (m, 2H), 8.19 (m, 2H), 8.23 (bs, 1H), 8.34 (d, 1H), 8.61 (d, 1H), 10.78 (s, 1H); ^{13}C-NMR δ = 17.1, 21.2, 23.2, 23.9, 24.4, 28.8, 36.7, 46.7, 47.8, 49.0, 55.4, 59.5, 118.9, 124.8, 126.4, 128.0, 129.2, 137.3, 142.2, 145.0, 169.3, 170.2, 171.0, 171.6.

Ala-Gly-ψ[CS-N]-Pro-Phe-NH-Np. Deprotection of Boc-Ala-Gly-ψ[CS-N]-Pro-Phe-NH-Np according to IV (0.25 mmol), crude product was purified by preparative HPLC (32% ACN, 0.05% TFA), yield 86.7 mg (57%), mp 163−165 °C, $[\alpha]_D = -36.8°$ (*c* 1, MeOH), ultraviolet absorbance (water): λ_{max} (log ϵ) = 278 nm (4.13), calcd for $C_{25}H_{30}N_6O_5S_1$ 526.19, found $(M + H)^+$ 527.18; ^{1}H-NMR δ = 1.41 (d, 3H), 1.82 (m, 2H), 1.92 (m, 1H), 2.09 (m, 1H), 2.99−3.14 (m, 2H), 3.72 (m, 1H), 3.86 (m, 1H), 3.95 (q, 1H), 4.16 (m, 1H), 4.32 (m, 1H), 4.70 (m, 1H), 4.89 (m, 1H), 7.13−7.35 (m, 5H), 7.85 (m, 2H), 8.01−8.28 (bd + m, 3H), 8.51 (d, 1H), 8.68 (t, 1H), 10.65 (s, 1H); ^{13}C-NMR δ = 17.4, 24.0, 29.1, 36.9, 47.1, 48.2, 50.3, 55.1, 66.4, 119.5, 124.9, 126.4, 128.1, 129.2, 137.3, 142.3, 144.9, 169.3, 170.0, 170.8, 196.1.

Ala-Ala-ψ[CS-N]-Pro-Phe-NH-Np. Deprotection of Boc-Ala-Ala-ψ[CS-N]-Pro-Phe-NH-Np according to IV (0.1 mmol), crude product was purified by preparative HPLC (35% ACN, 0.05% TFA), yield 37.9 mg (61%), mp 132−134 °C, $[\alpha]_D = -23.2°$ (*c* 1, MeOH), ultraviolet absorbance (water): λ_{max} (log ϵ) = 280 nm (4.17), calcd for $C_{26}H_{32}N_6O_5S_1$ 540.22, found $(M + H)^+$ 541.32; ^{1}H-NMR δ = 1.27 (d, 3H), 1.34 (d, 3H), 1.75 (m, 1H), 1.91 (m, 2H), 2.14 (m, 1H), 2.99 (dd, 1H), 3.21 (dd, 1H), 3.80−3.91 (m + q, 3H), 4.62 (q, 1H), 4.81 (m, 1H), 4.93 (m, 1H), 7.21−7.34 (m, 5H), 7.79 (m, 2H), 8.08 (bs, 1H), 8.21 (m, 2H), 8.43 (d, 1H), 8.71 (d, 1H), 10.59 (s, 1H); ^{13}C-NMR δ = 16.7, 19.7, 24.2, 28.9, 36.9, 47.7, 50.7, 50.9, 55.1, 65.5, 118.9, 124.9, 126.4, 128.0, 129.1, 137.0, 142.3, 144.8, 168.4, 169.8, 170.9, 201.9.

Ala-Abu-ψ[CS-N]-Pro-Phe-NH-Np. Deprotection of Boc-Ala-Abu-ψ[CS-N]-Pro-Phe-NH-Np according to IV (0.1 mmol), crude product was purified by preparative HPLC (38% ACN, 0.05% TFA), yield 40.7 mg (64%), mp 117−119 °C, $[\alpha]_D = -12.1°$ (*c* 1, MeOH), ultraviolet absorbance (water): λ_{max} (log ϵ) = 279 nm (4.26), calcd for $C_{27}H_{34}N_6O_5S_1$ 554.23, found $(M + H)^+$ 555.29; ^{1}H-NMR δ = 0.91 (t, 3H), 1.36 (d, 3H), 1.58 (m, 1H), 1.74 (m, 2H), 1.87 (m, 2H), 2.02 (m, 1H), 2.97 (dd, 1H), 3.13 (dd, 1H), 3.46 (m, 1H), 3.65 (m, 1H), 3.92 (q, 1H), 4.27 (m, 1H), 4.63 (m, 1H), 5.04 (m, 1H), 7.11−7.32 (m, 5H), 7.90 (m, 2H), 8.23 (m, 2H), 8.30 (d, 1H), 8.38 (d, 1H), 8.69 (d, 1H), 10.64 (s, 1H); ^{13}C-NMR δ = 10.0, 17.1, 24.3, 24.1, 28.5, 36.8, 46.8, 47.7, 52.2, 55.4, 67.2, 118.8, 124.8, 126.4, 128.1, 129.1, 137.2, 142.2, 145.0, 169.4, 170.3, 171.1, 202.6.

Ala-Phe-ψ[CS-N]-Pro-Phe-NH-Np. Deprotection of Boc-Ala-Phe-ψ[CS-N]-Pro-Phe-NH-Np according to IV (0.1 mmol), crude product was purified by preparative HPLC (42% ACN, 0.05% TFA), yield 33.5 mg (48%), mp 131−132 °C, $[\alpha]_D = -8.9°$ (*c* 1, MeOH), ultraviolet absorbance (water): λ_{max} (log ϵ) = 284 nm (4.09), calcd for $C_{32}H_{36}N_6O_5S_1$ 616.25, found $(M + H)^+$ 617.13; ^{1}H-NMR δ = 1.37 (d, 3H), 1.95 (m, 1H), 2.07 (m, 2H), 2.31 (m, 1H), 2.91−3.21 (m, 4H), 3.63 (m, 1H), 3.81 (m, 1H), 3.95 (q, 1H), 4.17 (m, 1H), 4.68 (m, 1H), 5.16 (dd, 1H), 7.27−7.41 (m, 10H), 7.91 (m, 2H), 8.27 (m, 2H); ^{13}C-NMR δ = 17.2, 24.3, 29.0, 36.4, 36.8, 46.7, 47.4, 53.1, 55.1, 64.6, 119.1, 124.7, 126.4, 128.2, 128.3, 129.2, 137.3, 137.5, 142.3, 145.1, 169.3, 169.9, 171.3, 202.1.

Ala-Leu-ψ[CS-N]-Pro-Phe-NH-Np. Deprotection of Boc-Ala-Leu-ψ[CS-N]-Pro-Phe-NH-Np according to IV (0.2 mmol), crude product was purified by preparative HPLC (41% ACN, 0.05% TFA), yield 90.4 mg (68%), mp 139−141 °C, $[\alpha]_D = -11.7°$ (*c* 1, MeOH), ultraviolet absorbance (water): λ_{max} (log ϵ) = 280 nm (4.12), calcd for $C_{29}H_{38}N_6O_5S_1$ 582.26, found $(M + H)^+$ 583.31; ^{1}H-NMR δ = 0.89 (d, 6H), 1.31 (d, 3H), 1.36 (m, 1H), 1.61 (m, 2H), 1.81–2.06 (m, 3H), 2.16 (m, 1H), 2.97 (dd, 1H), 3.20 (dd, 1H), 3.78 (m, 1H), 3.91 (m, 2H), 4.60 (q, 1H), 4.79 (m, 1H), 4.98 (m, 1H), 7.18–7.32 (m, 5H), 7.79 (m, 2H), 8.11 (bs, 1H), 8.20 (m, 2H), 8.51 (d, 1H), 8.64 (d, 1H), 10.63 (s, 1H); ^{13}C-NMR δ = 16.7, 21.2, 23.2, 24.0, 24.2, 28.8, 36.8, 42.8, 47.7, 50.6, 53.8, 55.2, 65.4, 118.9, 124.9, 126.4, 128.0, 129.1, 137.0, 142.2, 144.9, 168.8, 169.8, 171.0, 201.6.

Kinetic Studies. (I) Enzymes. Pancreatic α-chymotrypsin A4 was purchased from Merck (Darmstadt, Germany), and the content of active enzyme was determined by titration using 4-nitrophenyl acetate. The protease subtilisin Carlsberg type VIII and the buffer salts used were products from Sigma Chemical Co. (St. Louis, MO). Trypsin was a product from Roth (Karlsruhe, Germany). Human recombinant Cyp18cy was from Boehringer-Mannheim (Mannheim, Germany). The concentration of active PPIase was determined using fluorimetric titration with cyclosporin A assuming that inhibitor binding reflects an unperturbed active site (Handschumacher et al., 1984). Typical purity was above 70% active enzyme, leading to a k_{cat}/K_m value of 1.5×10^7 s^{-1} M^{-1} for Suc-Ala-Ala-Pro-Phe-NH-Np on standard conditions.

(II) Enzyme Assays. All measurements were performed by monitoring the absorbance of released 4-nitroaniline at 390 nm (ϵ = 11 814 M^{-1} cm^{-1}) on a Hewlett-Packard 8452A diode array spectrophotometer with a thermostated cuvette holder. Constant temperature was maintained within the cell (d = 1 cm) by water circulated from a Cryostat Haake D8 (Haake Fisons, Germany). The Hewlett-Packard 89531A MS-DOS-UV/VIS operation software or SigmaPlot Scientific Graphing System Vers. 5.01 (Jandel Corp., USA) were used for data analysis.

Determination of the kinetic constants for the α-chymotrypsin mediated cleavage of the tetrapeptide- and thioxo tetrapeptide-4-nitroanilides was performed using 0.035 M HEPES buffer, pH 7.8 at 25 °C. Peptide derivatives (8–9 mg) were dissolved in DMSO (100 μl) and diluted with 7–8 mL of degassed buffer. Any insoluble material was removed by centrifugation for 5 min at 7000*g*. The supernatant served as substrate stock solution and was diluted with the appropriate volume of buffer to yield a total volume of 900 μL. The enzyme stock solution was prepared by dissolving 3 mg of α-chymotrypsin in 100 μL of 1 mM HCl and diluting the resulting mixture with buffer. Insoluble material was separated from the enzyme solution by centrifugation at 4 °C for 5 min at 10000*g*. The reaction was initiated by the addition of 5 μL of protease stock solution.

Measurement of the *cis/trans* isomerization of the compounds Ala-Gly-Pro-Phe-NH-Np and Ala-Gly-ψ[CS-N]-Pro-Phe-NH-Np was performed using the coupled α-chymotrypsin assay devised by Fischer et al. (1984) at 10 °C for the peptide derivatives and at 25 °C for the thioxo peptide-4-nitroanilides. In a typical experiment, x μL of buffer and y μL of α-chymotrypsin stock solution (50 mg/mL) prepared as described above ($x + y$ = 1.2 mL) were preincubated for 5 min. After the solution had reached thermal equilibrium, the reaction was initiated by the addition of 2–3 μL of the stock solution of the peptide (25 mg/mL in DMSO). The final concentration of α-chymotrypsin was 4.5 mg/mL. Other peptide derivatives were analyzed as described for Ala-Gly-Pro-Phe-NH-Np but using subtilisin as an isomer-specific protease. The final subtilisin concentration was 2.6 mg/mL except for Ala-Ala-ψ[CS-N]-Pro-Phe-NH-Np and Ala-Abu-ψ[CS-N]-Pro-Phe-NH-Np (1.3 mg/mL). Analysis of the temperature dependence of the rate constant $k_{cis\rightarrow trans}$ was performed as described above. The temperature was varied between 25 and 50 °C for the thioxo peptides and between 5 and 20 °C for Ala-Xaa-Pro-Phe-NH-Np. The thermodynamic parameters of these interconversions were computed by linear regression analysis of the Eyring plot data. Measurement of the bimolecular rate constants k_{cat}/K_m for the PPIase-catalyzed isomerization was performed according to the literature (Fischer et al., 1984). Typically, a mixture of $(1000 - x)$ μL of buffer and 200 μL of protease stock solution was treated with x μL of PPIase solution, reaching a final Cyp18cy concentration up to 2 μM. Reactions were started by addition of 3 μL of the peptide stock solutions mentioned above. Since $[S]_0 \ll K_m$ was valid, absorbance data points were fitted to a first-order kinetics. The k_{cat}/K_m values were calculated according to the equation $k_{cat}/K_m = (k_{obs} - k_u)/[\text{PPIase}]$ (Harrison & Stein, 1990b), where k_u = first-order rate constant for spontaneous *cis/trans* isomerization and k_{obs} = pseudo-first-order rate constant for *cis/trans* isomerization in the presence of PPIase.

Experiments in the presence of thioxo peptide derivatives to determine the K_i values of these compounds were performed with Suc-Ala-Ala-Pro-Arg-NH-Np in a coupled trypsin assay at 10 °C. This assay has to be used, because the Ala-Xaa-ψ[CS-N]-Pro-Phe-NH-Np is degraded by α-chymotrypsin. Cyp18cy catalyzes the isomerization of this substrate with $k_{cat}/K_m = 9.3 \times 10^6$ s^{-1} M^{-1}.

The substrate was dissolved at 18 mM in anhydrous trifluoroethanol and 0.5 M LiCl. Thioxo peptide stock solutions (70 mg/mL DMSO) were prepared as described above. In a typical experiment, 5 μL of PPIase solution was added to a mixture of 1200 μL of buffer and 4 μL of trypsin solution (3.5 mg/mL). The final concentrations for the enzymes were 4 and 300 nM, respectively. After addition of x μL of DMSO and y μL of thioxo peptide stock solution ($x + y$ = 5 μL), the reaction was initiated by injection of 1.5 μL of substrate (30 μM stock solution). The inhibitor concentration was calculated from the final absorbance of 4-nitroaniline at 37 °C after prolonged reaction time. The absorbances of the time course monitored were fitted to a first-order rate equation. In the case of competitive inhibition and if $[S]_0 \ll K_m$, the obtained pseudo-first-order rate constant k is equal to $k_u + k_{cat}[E_0]/K_m(1 + [I_0]/K_i)$. Plots of $k - k_u$ versus inhibitor concentration were used to calculate K_i.

In order to gain access to the individual K_i values of the *cis-* and the *trans*-isomer, the determination of the K_i value was performed, starting the reaction with dissolved peptide exhibiting various *cis/trans* ratios. This could be obtained by using different solvents for the stock solution of the peptide derivative. The data obtained for the *cis* content were as follows: 33% in DMSO, 3.7% in trifluoroethanol, and 11.1% in HEPES buffer for Ala-Gly-ψ[CS-N]-Pro-Phe-NH-Np. We can extend the model for competitive inhibition, with respect to conformer-specific inhibition of Ala-Xaa-ψ-[CS-N]-Pro-Phe-NH-Np, to:

$$v = \frac{V_{max}[S_0]}{[S_0] + K_m(1 + [I_0]^{cis}/K_i^{cis} + [I_0]^{trans}/K_i^{trans})} \quad (1)$$

For $[S_0] \ll K_m$ we can derive the equation:

$$k_i = \frac{k_e}{1 + [I_0]^{cis}/K_i^{cis} + [I_0]^{trans}/K_i^{trans}} \quad (2)$$

$[I_0]^{cis}$ and $[I_0]^{trans}$ are independent variables in this model. The dependent variable k_i is the observed pseudo-first-order rate constant in the presence of inhibitor and Cyp18cy when the first-order rate constant for uncatalyzed isomerization k_u was subtracted. For determination of k_e the same assay was used but without the inhibitor.

The steady-state kinetic parameters k_{cat} and K_m in the absence and the presence of thioxo peptide inhibitor became available by the improved coupled assay devised by Kofron (Kofron et al., 1991). An increase in the *cis* content (46% in total) of Suc-Ala-Ala-Pro-Arg-NH-Np (25 mg) was monitored when dissolved in 180 μL of anhydrous trifluoroethanol and 0.5 M LiCl. Insoluble material was removed by centrifugation at 7000*g* for 10 min. Thioxo peptide (Ala-Gly-ψ[CS-N]-Pro-Phe-NH-Np) stock solution (69 mg/mL) was prepared in DMSO as described above. In a typical experiment, a mixture of 1200 μL of buffer and 12 μL of trypsin solution (17 mg/mL in buffer) incubated at 10 °C for 4 min was supplemented with 5 μL of PPIase solution (34 μg/mL). Subsequently, x μL of DMSO and y μL of thioxo peptide stock solution ($x + y = 5$ μL) were added and incubated at 10 °C for 6 min. The measurement of the *cis* to *trans* isomerization was initiated by the addition of 1 μL of Suc-Ala-Ala-Pro-Arg-NH-Np solution. Absorbance data points from each progress curve were analyzed by nonlinear least-squares optimization of K_m and k_{cat} in the differential equation $d[C]/dt = -k_0[C] - V_{max}[C]/(K_m+[C])$ using the software TREND Vers. 1.2 (Martin-Luther-University Halle-Wittenberg, Germany). The resulting initial rates were fitted 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 determination of k_{cat} and K_m was performed in the absence of thioxo peptide inhibitor and repeated using the competitive inhibitor at final concentrations of 89 μM (1 μL of inhibitor stock solution) and 392 μM (4.4 μL of inhibitor stock solution). Unlike the Cyp18cy inhibitor cyclosporin A, no time dependence of the inhibition by thioxo peptide derivatives was observed.

Determination of the pH dependence of K_i and of k_{cat}/K_m was performed using a series of constant ionic strength buffers containing 50 mM sodium acetate/0.2 M KCl (4.5 ≤ pH ≤ 6), 50 mM Bis-Tris propane/0.2 M KCl (6 ≤ pH ≤ 8), and Bis-Tris propane (6 ≤ pH ≤ 9.0). The pH of each sample was determined after the end of the reaction and found to be unchanged during the reaction. The final PPIase concentration was varied between 5.8 nM (pH 9) and 82 nM (pH 4.5). The final concentration of trypsin (stock solution: 1 mM in 50 mM sodium acetate, pH 5) ranged from 2 μM (pH 9) to 100 μM (pH 4.5). The inhibitor stock solution (Ala-Gly-ψ[CS-N]-Pro-Phe-NH-Np) was prepared in DMSO at 100 mM. The thioxo peptide derivatives were chemically stable in the used pH range at 10 °C. The substrate (Suc-Ala-Ala-Pro-Arg-NH-Np) stock solution was prepared as described above. All measurements were performed at 10 °C. The reaction was initiated by addition of 2 μL of substrate.

Determination of the pH dependence of k_{cat} and K_m was carried out using a modified coupled assay with subtilisin as the auxiliary protease. Reactions were conducted over a pH range of 5.5−8.5 in buffered solutions containing 50 mM piperazine (pH 5.5), 65 mM Bis-Tris propane (pH 6.1), 65 mM Bis-Tris propane (pH 6.8), 50 mM HEPES (pH 7.5), and 50 mM Bicine (pH 8.5) at 10 °C. The substrate (Suc-Ala-Phe-Pro-Phe-NH-Np) was dissolved in DMSO at 10 mg/mL. The reaction was initiated by the addition of 30 μL (pH 8.5) or 60 μL (pH 5.5) of subtilisin stock solution. Immediately after the rapid cleavage of the Suc-Ala-Phe-*trans*-Pro-Phe-NH-Np (25−50 s), 5−20 μL of the PPIase stock solution was added. The final concentration of Cyp18cy ranged from 7 to 30 nM, and the final concentration of subtilisin ranged from 20 to 100 μM. The *cis* to *trans* isomerization of the substrate was followed spectrophotometrically at 390, 446, or 456 nm. The final absorbance remained below a value of 1.6 in any case.

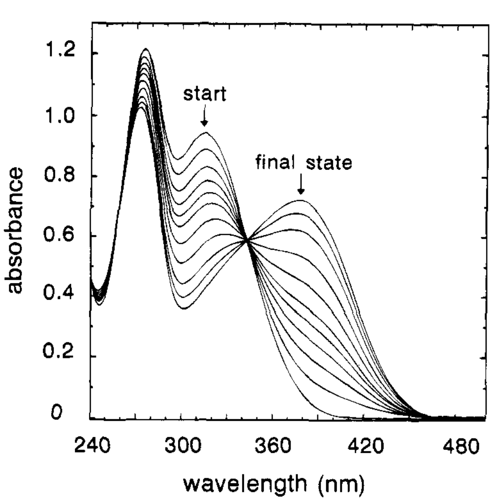

FIGURE 1: UV−vis spectra of the cleavage of Ala-Gly-ψ[CS-N]-Pro-Phe-NH-Np by subtilisin at zero time and after 5, 10, 15, 20, 25, 33, 43, 66, 100, and 166 min of proteolysis; 35 mM HEPES pH 7.8, 10 °C; for details, see Experimental Procedures. The two isosbestic points are at 258 and 346 nm.

Determination of the dependence of the percentage of the *cis*-isomer of thioxo peptide derivatives from the PPIase concentration was performed by incubation of Ala-Leu-ψ-[CS-N]-Pro-Phe-NH-Np (stock solution in DMSO, 70 mg/mL) and Cyp18cy in 35 mM HEPES, pH 7.8 at 8 °C, for 3 h. The isomer-specific hydrolysis of Ala-Leu-ψ[CS-N]-Pro-Phe-NH-Np was started by adding 10 μL of subtilisin stock solution in 35 mM HEPES, pH 7.8, to 90 μL of the thioxo peptide derivative/cyclophilin mixture after incubation for 5 min at 10 °C. Final concentrations in the reaction solution were 70 μM for the thioxo peptide derivative and 200 μM subtilisin and varied between 0 and 100 μM for Cyp18cy.

RESULTS

Isomer-Specific Proteolysis. In principle, alternative reaction pathways may be possible for the cleavage of the thioxo peptide derivatives by subtilisin and α-chymotrypsin. In aqueous solution, either direct hydrolysis of the peptide bond to give the free thioxo tetrapeptide and 4-nitroaniline or fast desulfurization of the substrate followed by rapid cleavage of the resulting oxo peptide derivative can occur. The isosbestic points at 258 and 346 nm (Figure 1) gave the first indications of a kinetically simple reaction progress. Destruction of the thioxo amide chromophore that should lead to a distinct decrease of absorbance in the 270−290 nm spectral region was not observed. Additionally, the lack of desulfurization during proteolysis received support from the observation that the reaction mixture was devoid of sulphide ions even at prolonged incubation time.

At very high concentrations of chymotrypsin and subtilisin used in ISP the quantitative amount of 4-nitroaniline released from Ala-Xaa-ψ[CS-N]-Pro-Phe-NH-Np agreed with a single cleaveage site. Both enzymatic and alkaline hydrolysis of

Table 1: Kinetic Constants for the Hydrolysis of Ala-Xaa-Yaa-Phe-NH-Np by Pancreatic α-Chymotrypsin, pH 7.8, 25 °C

-Xaa-Yaa-	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
-Gly-Pro-	0.28 ± 0.041	4.3 ± 0.13	5.2
-Ala-Pro-	0.45 ± 0.057	28.1 ± 2.94	62.4
-Abu-Pro-	0.21 ± 0.019	26.2 ± 2.30	124.8
-Leu-Pro-	0.17 ± 0.053	4.4 ± 1.31	25.9
-Phe-Pro-	0.08 ± 0.013	4.5 ± 0.54	56.2
-Gly-ψ[CS-N]-Pro-	0.53 ± 0.037	1.7 ± 0.01	3.2
-Ala-ψ[CS-N]-Pro-	0.13 ± 0.008	9.0 ± 0.87	69.2
-Abu-ψ[CS-N]-Pro-	0.07 ± 0.021	3.1 ± 0.95	44.3
-Leu-ψ[CS-N]-Pro-	0.06 ± 0.019	3.5 ± 1.12	58.3
-Phe-ψ[CS-N]-Pro-	0.04 ± 0.011	4.1 ± 1.27	102.5

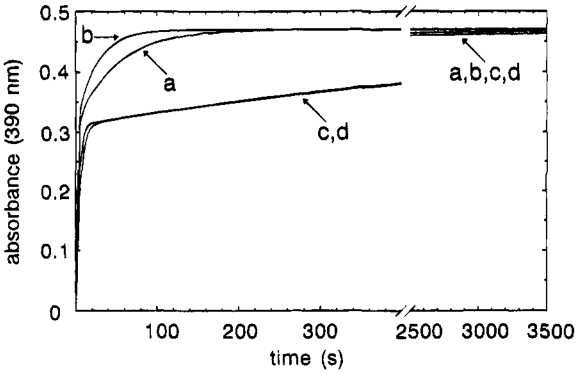

FIGURE 2: Progress curves for the α-chymotrypsin mediated hydrolysis of Ala-Gly-Pro-Phe-NH-Np (a, b) and of Ala-Gly-ψ-[CS-N]-Pro-Phe-NH-Np (c, d) in the absence and the presence of Cyp18cy; data were collected at pH 7.8 in buffered solution containing 35 mM HEPES at 25 °C; for details, see Experimental Procedures. (a) no Cyp18cy, $\tau = 34$ s; (b) 3 nM Cyp18cy, $\tau = 28$ s; (c) no Cyp18cy, $\tau = 772$ s; (d) 2μM Cyp18cy, $\tau = 781$ s.

the thioxo peptides yielded equal amounts of 4-nitroaniline. This observation indicates the stereochemical purity of the thioxo peptide derivatives.

Studies of the steady-state kinetics of chymotryptic hydrolysis (Table 1) for each substrate examined were performed at pH 7.8 and 25 °C. The measurements gave rise to the assumption that the resulting second-order rate constants k_{cat}/K_m and the turnover number k_{cat} were sufficiently high to allow very fast cleavage of the *trans*-isomer in the protease-coupled experiments (Fischer, 1994) and complete separation of the kinetic phases in the progress curves. A comparison of the enzymatic constants of oxo and thioxo substrate series indicated that the sulfur atom at the backbone of the P_3 subsite does not considerably influence chymotrypsin mediated peptide bond hydrolysis.

Thioxo Peptide cis/trans Isomerization. As has already been visible on the endoproteolytic cleavage of oxo tetrapeptide-4-nitroanilides with a proline residue in the P_2 position (Fischer et al., 1984), the time course of proteolysis with chymotrypsin of thioxo peptide derivatives also showed biphasic behavior (Figure 2). The slow phase was insensitive to the concentration of the protease up to 0.2 mM. Assuming conformational specificity of chymotrypsin toward the thioxo peptides, the ratio of amplitudes extrapolated to zero time should characterize the isomeric composition in the stock solution of the peptide dissolved in DMSO (88 mM). The percentage of the slow phases characterized by a rate constant of about 10^{-4} s^{-1} generally agreed in the limits of error with the percentage of *cis*-isomer found in the ^{1}H-NMR spectra. As exemplified for Ala-Leu-ψ[CS-N]-Pro-Phe-NH-Np (12 mM in DMSO-d_6), the ratio of intensity was 13.7 in favor of *trans* as measured on the NH signal of the 4-nitroanilide residue ($\delta_{trans} = 10.78$ ppm; $\delta_{cis} = 11.16$ ppm). This value agreed with the ratio of 14.4 ± 0.9 as measured for the ratio of both phases in proteolysis experiments.

These investigations demonstrated the requirement for the *trans* conformation of the nonscissile P_3-P_2 peptide bond also in the case of thioxo substrates for the α-chymotrypsin and subtilisin mediated hydrolysis.

Thus, the first-order rate constants representing the kinetics of the slow phase can be attributed to the rate constant $k_{cis\rightarrow trans}$ of the thioxo peptidyl-prolyl bond. Together with data for the corresponding oxo derivatives, the values characterizing the *cis/trans* isomerization of thioxo peptidyl-prolyl bonds of a number of tetrapeptide-4-nitroanilides are summarized in Table 2.

At 10 °C the *cis* to *trans* isomerization of the thioxo peptide derivatives is 25−125-fold slower compared to the corresponding oxo peptides (Table 2 and Figure 2). The reason for this retardation becomes obvious from the linear Eyring plots of the rate constants $k_{cis\rightarrow trans}$ and the activation parameters derived from these plots (Table 2). Due to the unchanged or even decreased $\Delta H^{\ddagger}$ values of thioxo peptides, a highly unfavorable entropic contribution has to occur. It was most pronounced for peptides having an amino acid Xaa with an extended, hydrophobic side chain.

Inhibition of Cyp18cy by Thioxo Peptides. An acceleration of the *cis* to *trans* isomerization of the thioxo peptide derivatives by Cyp18cy could not be detected up to 2 μM enzyme in the assay (Figure 2). Thus, they would have at best k_{cat}/K_m values 4 orders of magnitude lower than those of the corresponding oxo compounds. Experiments were performed to determine whether thioxo peptides can still bind to the active center of the enzyme and can influence the catalytic action of Cyp18cy on substrates. The ability of trypsin to perform isomer-specific proteolysis (Lin & Brandts, 1985) allowed us to monitor the *cis* to *trans* isomerization of Suc-Ala-Ala-Pro-Arg-NH-Np catalyzed by Cyp18cy. In the presence of thioxo tetrapeptide-4-nitroanilides, being uncleaved by trypsin, competitive inhibition of Cyp18cy could be obtained (Figure 3). In terms of K_i values which could be calculated from the observed rates at different concentrations of thioxo peptide, the binding to Cyp18cy is quite weak. The amino acid adjacent to proline (Xaa-Pro) is of minor influence on K_i. Even in dependence on the nature of Xaa, the variation of K_i exactly mirrored that of k_{cat}/K_m of the oxo substrates (Table 2).

pH Dependence of Cyp18cy Reactions. Dependence of K_i on pH would be indicative for ionizable groups of enzyme and inhibitor on the binding pathway. Thus, we examined the interaction of thioxo peptide Ala-Gly-ψ[CS-N]-Pro-Phe-NH-Np with Cyp18cy as a function of pH in the range of 5.5−9.0 at 10 °C. In addition, the estimation of the pK_a value of the N-terminal amino group of Ala-Gly-ψ[CS-N]-Pro-Phe-NH-Np gave 8.27 ± 0.32 and signalizes a small influence of the remote thioxo carbonyl when compared to those of the parent oxo peptide Ala-Gly-Pro-Phe-NH-Np (8.42 ± 0.27). As depicted in Figure 4, any pronounced influence of pH on thermodynamics of inhibitor binding is lacking.

In contrast, the k_{cat}/K_m value for the Cyp18cy-catalyzed isomerization of Suc-Ala-Ala-*cis*-Pro-Arg-NH-Np (Figure 5) showed a clear decrease in magnitude below pH 6.5. A pK_a of 5.8 ± 0.08 has been found by fitting the data to the model $(k_{cat}/K_m)_{obs} = (k_{cat}/K_m)_{lim}/(1 + [H^+]/K_a)$ for a single kinetically

Table 2: Kinetic and Thermodynamic Constants of the Noncatalyzed *cis* to *trans* Isomerization and Kinetic Constants of the rhCyp18cy Mediated Acceleration of the *cis* to *trans* Isomerization of Ala-Xaa-Yaa-Phe-NH-Np, pH 7.8, 10 °C

-Xaa-Yaa-	$k_{cis\rightarrow trans} \times 10^5$ (s^{-1}) at 10 °C[a,b]	$\Delta S^{\ddagger}$ (J mol^{-1} K^{-1})	$\Delta H^{\ddagger}$ (kJ mol^{-1})	*cis*-content (%)[a,d]	k_{cat}/K_m (μM^{-1} s^{-1})[a]	$K_{i,obs}$ (mM)[a]
-Gly-Pro-	610 ± 20	−23.4 ± 1.89	74.7 ± 0.81	33.0 ± 1.0	0.98 ± 0.13	
-Ala-Pro-	690 ± 30	−2.7 ± 0.56	79.8 ± 0.76	9.8 ± 0.3	5.10 ± 0.09	
-Abu-Pro-	650 ± 20	3.3 ± 0.62	81.7 ± 0.80	9.2 ± 0.1	3.73 ± 0.07	
-Leu-Pro-	620 ± 20	25.1 ± 2.24	88.5 ± 0.72	8.1 ± 0.3	3.77 ± 0.08	
-Phe-Pro-	360 ± 10	22.8 ± 1.94	89.0 ± 0.65	12.6 ± 0.3	1.90 ± 0.05	
-Gly-ψ[CS-N]-Pro-	25 ± 3	−81.1 ± 1.72	65.8 ± 0.93	33.0 ± 0.5		0.23 ± 0.04
-Ala-ψ[CS-N]-Pro-	7[c]	−41.9 ± 1.37	79.7 ± 1.06	8.5 ± 0.4		0.58 ± 0.07
-Abu-ψ[CS-N]-Pro-	5[c]	−41.4 ± 1.16	80.8 ± 0.88	9.6 ± 0.5		0.32 ± 0.08
-Leu-ψ[CS-N]-Pro-	8[c]	−94.9 ± 3.52	64.5 ± 0.89	6.5 ± 0.2		0.26 ± 0.07
-Phe-ψ[CS-N]-Pro-	11[c]	−114.0 ± 5.38	80.8 ± 0.92	10.5 ± 0.6		0.31 ± 0.12

[a] Error limits are standard deviations of the means of 3 or 4 determinations. [b] All measurements using subtilisin. [c] The data were obtained by extrapolation from the Eyring plot because of the sluggish *cis* to *trans* isomerization of the thioxo peptide derivatives. [d] In DMSO.

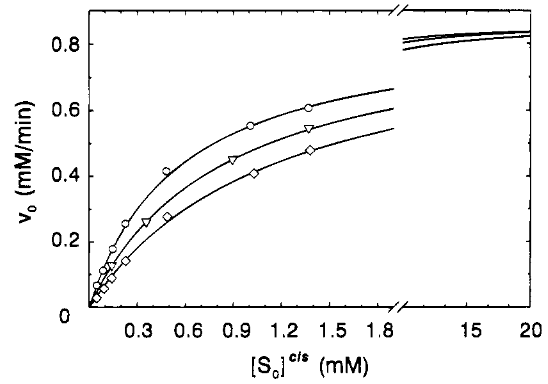

FIGURE 3: Determination of k_{cat} and K_m for the Cyp18cy-catalyzed *cis* to *trans* isomerization of Suc-Ala-Ala-Pro-Arg-NH-Np; the data were fitted according to Kofron et al. (1991) by nonlinear least-squares optimization in the presence of: (○) 0 μM, (△) 89 μM, and (◇) 392 μM of thioxo peptide inhibitor Ala-Gly-ψ[CS-N]-Pro-Phe-NH-Np.

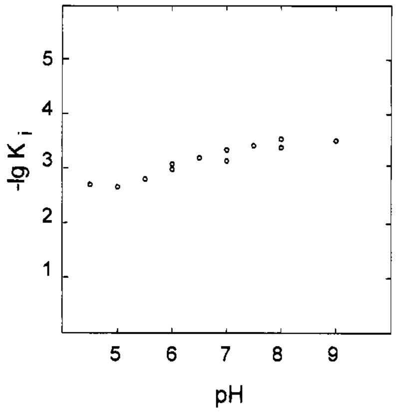

FIGURE 4: pH dependence of the K_i value of the thioxo peptide derivative Ala-Gly-ψ[CS-N]-Pro-Phe-NH-Np; K_i values were determined using the trypsin-coupled assay and Suc-Ala-Ala-Pro-Arg-NH-Np in buffered solutions containing 50 mM sodium acetate/0.2 M KCl (4.5 ≤ pH ≤ 6), 50 mM Bis-Tris propane/0.2 M KCl (6 ≤ pH ≤ 8), and Bis-Tris propane (6 ≤ pH ≤ 9.0) at 10 °C.

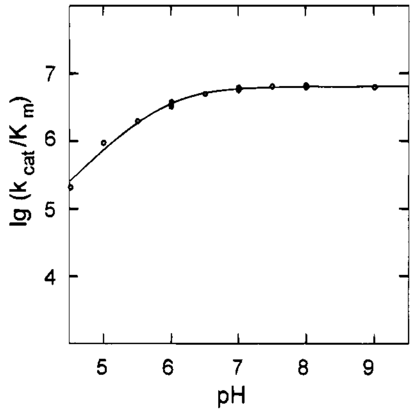

FIGURE 5: pH dependence of the second-order rate constant k_{cat}/K_m for the Cyp18cy-catalyzed isomerization of Suc-Ala-Ala-Pro-Arg-NH-Np; the enzymatic reaction was performed over a pH range of 4.5–9 in buffered solutions containing 50 mM sodium acetate/0.2 M KCl (4.5 ≤ pH ≤ 6), 50 mM Bis-Tris propane/0.2 M KCl (6 ≤ pH ≤ 8), and Bis-Tris propane (6 ≤ pH ≤ 9.0) at 10 °C.

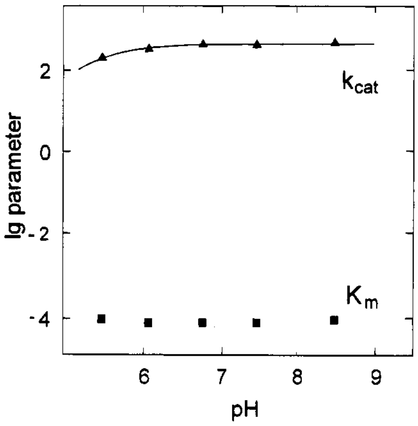

FIGURE 6: pH dependence of k_{cat} and K_m for the Cyp18cy-catalyzed isomerization of Suc-Ala-Phe-Pro-Phe-NH-Np; k_{cat} and K_m were estimated in buffered solutions containing 50 mM piperazine (pH 5.5), 65 mM Bis-Tris propane (pH 6.1), 65 mM Bis-Tris propane (pH 6.8), 50 mM HEPES (pH 7.5), and 50 mM Bicine (pH 8.5) at 10 °C.

significant dissociation, already used by Harrison and Stein (1990a). For the pH range of 4–10 a similar picture resulted for the pH dependence of k_{cat}/K_m of Suc-Ala-Ala-*cis*-Pro-Phe-NH-Np (data not shown). In the latter case, the calculated pK_a value of 5.9 ± 0.1 is slightly higher than the value of 4.92 ± 0.09 previously found for authentic calf thymus enzyme (Harrison & Stein, 1990a). However, whether the ionizable group is localized either in reaction steps contributing to K_m or k_{cat} still remained unknown. This prompted us to measure the full Michaelis–Menten dependences of Suc-Ala-Phe-*cis*-Pro-Phe-NH-Np at five different pH values.

Thus, the feature of the pH profiles of Figure 6 is the insensitivity of the K_m value to pH. In general, this is reminiscent of what has been observed for the K_i value of thioxo peptide. This result may reflect the molecular background of the dissociation step characterized by a pK_a <6. The event probably takes place during the interconversion of the Michaelis complex E*[*cis*] to the transition state of the catalysis.

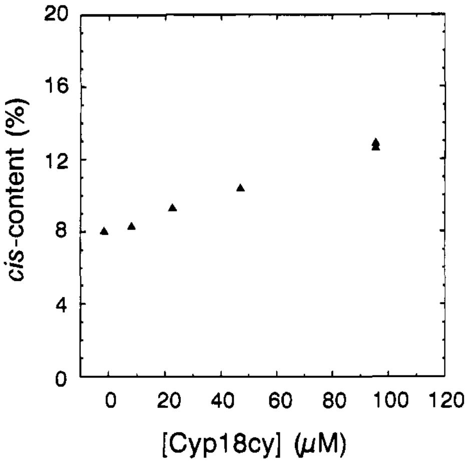

FIGURE 7: Dependence of the percentage of *cis*-isomer of Ala-Leu-ψ[CS-N]-Pro-Phe-NH-Np on the Cyp18cy concentration; the thioxo peptide derivative was preincubated with Cyp18cy for 3 h at 8 °C before starting the protease-coupled assay for determination of the *cis* content; for details, see Experimental Procedures.

Isomer-Specific Thioxo Peptide Recognition. Since PPIases interconvert conformers of peptides, isomer-specific recognition of Ala-Gly-ψ[CS-N]-Pro-Phe-NH-Np by Cyp18cy should occur. Our approach to determine separately K_i^{trans} and K_i^{cis} took advantage of changed *cis/trans* ratios inducible by solvents.

The inhibitor dissolved in different solvents was added to the incubation mixture. The *cis/trans* isomerization of Ala-Gly-ψ[CS-N]-Pro-Phe-NH-Np is very slow at 10 °C (Table 2). Therefore, the equilibration of the disturbed *cis/trans* equilibrium during the measurement time can be neglected and the concentration of $[I_0]^{cis}$ and $[I_0]^{trans}$ assumed to be constant.

The experimental data were analyzed by nonlinear least-squares optimization of K_i^{cis} and K_i^{trans} in eq 2. In this manner, we obtained a K_i^{cis} value of 82 μM and a K_i^{trans} value of 2500 μM for the thioxo peptide derivative Ala-Gly-ψ-[CS-N]-Pro-Phe-NH-Np. Obviously, with respect to Cyp18cy, this isomer-specific recognition resulted from steady-state conditions typical for very low enzyme concentrations.

Therefore, an about 30-fold higher affinity of *cis*-isomer of the thioxo peptide to the active site of Cyp18cy prompted us to investigate whether stoichiometric amounts of Cyp18cy should be able to shift the percentage of *cis*-isomer in solution. The data of Ala-Leu-ψ[CS-N]-Pro-Phe-NH-Np depicted in Figure 7 clearly indicated the postulated effect.

DISCUSSION

With a single exception, peptide bond surrogates have not been used as a probe to elucidate PPIase catalysis. A tetrapeptide fluoroolefin peptide isoster mimicking the imino form of the peptidyl-prolyl bond was reported to inhibit cyclophilin, but quantitative data have not been given (Boros et al., 1994). An approach more closely related to natural substrates of PPIases may be developed when using peptides thioxylated at the Xaa-Pro bond because the reactive peptide bond of interest is labeled in this case.

The objective of analyzing the reaction pathway and steady-state kinetics of the hydrolysis of Ala-Xaa-ψ[CS-N]-Pro-Phe-NH-Np was to explore the magnitude of the rate limiting step of chymotryptic hydrolysis. The knowledge of these values is a prerequisite for evaluating *cis/trans* isomerization from the progress curves of isomer-specific proteolysis. Sufficiently rapid cleavage at high protease concentrations would be signalized by high turnover numbers.

Our results show that the thioxylated peptide bond surrogate is well tolerated (Table 1) although the extended binding sites of chymotrypsin include important S_3-P_3 contacts on the level of an antiparallel β-sheet structure (Polgar, 1989). For several examples, concerning the O/S pair of substrates, the second-order rate constants are even in favor of the thioxo compound. Small effects of the O/S substitution, seen as a general decrease of k_{cat}, are compensated for k_{cat}/K_m by favorable K_m values. Surprisingly, the carbonyl of the Xaa-Pro bond was found to be involved in a quite strong hydrogen bond to a backbone NH group of the protease. Examination of the three-dimensional structure of the α-chymotrypsin/eglin C complex indicates a short N,O distance (2.87 Å) of the Trp_{215}-Gly_{216} peptide nitrogen to the Val_{143} carbonyl of the protease inhibitor (Frigerio et al., 1992). It was suggested that Val_{143} represents the P_3 subsite of eglin C. In a similar manner, the inhibitory potency to γ-chymotrypsin of *N*-acetyl-Leu-Phe-$COCF_3$ was accounted for by an acetyl carbonyl to Trp_{215}-Gly_{216} peptide nitrogen hydrogen bond, leading to 2.6 Å of N,O distance (Brady, 1990). The steady-state constants summarized in Table 1 imply that either the particular hydrogen bond tolerates thioxylation or loss of binding energy is compensated.

However, with respect to their k_{cat} values, which would considerably exceed the *cis/trans* isomerization rates even of oxo peptidyl-prolyl bonds, compounds depicted in Table 1 could be applied to conformational analysis using chymotryptic proteolysis. A comparable set of data could be obtained for subtilisin Carlsberg (data not shown) that allows usage of this protease for purpose of isomer-specific proteolysis, too.

With respect to the rotational barrier in nonaqueous solvents of the C−N bond, there is a linear correlation of the form $\Delta G^{\ddagger}_{[CS\text{-}N]} = 1.13 + 1.11(\Delta G^{\ddagger}_{[CO\text{-}N]})$ (kcal mol^{-1}) in a series of nonpeptidic N,N-disubstituted amides and their corresponding thioxo amides (Piccini-Leopardi et al., 1977). By this equation a difference (in terms of $\Delta\Delta G^{\ddagger}$) of about 12 kJ mol^{-1} can be calculated for our compounds. In aqueous solution, we measured an average difference $\Delta\Delta G^{\ddagger}$ of the *cis* to *trans* interconversion of 10.9 kJ mol^{-1} for the compounds summarized in Table 2. Keeping in mind the very different conditions used for the measurement of $\Delta\Delta G^{\ddagger}$ values, the above correlation describes very well the effect of O/S substitution on aminoacyl-prolyl bonds (Schutkowski et al., 1994) and the peptidyl-prolyl bonds investigated here. The relative insensitivity of the $\Delta\Delta G^{\ddagger}$ values to solvents and structural constraints placed by adjacent moieties could be accounted for using a model of bond rotation which includes a bulky transition state of rotation. This would imply that the bulkiness of the sulfur atom in *N,N*-dialkyl thioxo amides interferes with adjacent atoms. This would occur most likely when the plane of the alkyl groups is arranged perpendicularly to the thioxo carbonyl and should apply an approximately constant factor to the increase of rotational barrier. Apparently, in the magnitude of $\Delta\Delta G^{\ddagger}$ chain-specific features were absent, which may result from the stabilization of the rotational transition state of either the oligopeptide structure or the proline ring. This specific interactions include both the control function of the angle ψ_{Pro} on the energy of the ground state and the stabilization of the nitrogen lone pair in the transition state by polar effects of remote parts of the peptide (Fischer et al., 1994). Indeed, the main reason for the higher barrier of rotation about the thioxo peptide bond could be attributed to the much more unfavorable $\Delta S^{\ddagger}$ values throughout the thioxo peptides of Table 2.

Surprisingly, inspection of Figure 2 suggested that the *cis* to *trans* isomerization of Ala-Xaa-ψ[CS-N]-Pro-Phe-NH-Np cannot be catalyzed by Cyp18cy. On the other hand, there is a decrease in rate of the catalyzed isomerization with respect to increased thioxo peptide concentration in a Cyp18cy assay with substrate and thioxo peptide simultaneously present. Data of Figure 3 indicated competitive inhibition and thus thioxo peptide binding to the active center of Cyp18cy. The transfer of the thioxo peptides to the nonpolar active site of Cyp18cy can be quantitated by the K_i values shown in Table 2. On further considerations, the K_i value of 580 μM for the thioxo peptide inhibitor Ala-Ala-ψ[CS-N]-Pro-Phe-NH-Np should be compared to the K_m value of 870 μM for the structurally related substrate Suc-Ala-Ala-Pro-Phe-NH-Np (Kofron et al., 1991). This becomes possible due to comparable *cis/trans*-isomer ratios for both types of compounds. Since both constants were found in the same order of magnitude, structural factors determining ground-state binding of substrates to the enzyme are preserved in the thioxo compound. Furthermore, the relation $K_m > K_i$ allows the assignment of the PPIase kinetics to the Briggs–Haldane type. Thus, the rate constant for the decay of the Michaelis complex E*[*cis*] to the product state may be equal to the rate constant for the back-reaction to the *cis*-isomer state.

The question arose whether the binding step of the thioxo peptide inhibitors has further properties also found for the binding step measured with a related substrate. Obviously, the nearly invariant K_i value when determined at different pH values (Figure 4) mirrored the behavior of the K_m value of a tetrapeptide-4-nitroanilide substrate (Figure 6). The small decrease of K_i at high pH values may indicate the increasing amount of the uncharged amino form of the thioxo peptide ($pK_a = 8.27$) which may bind more strongly. The single dissociation step reflected by the pH dependence of k_{cat}/K_m (Figure 5; Harrison & Stein, 1990a) is mostly localized in reaction steps contributing to k_{cat} (Figure 6). The minimal kinetic model for Cyp18cy catalysis in the protease-coupled assay contains free enzyme and free *cis*-conformer as well as the Michaelis complexes E*[*cis*] and E*[*trans*]. Together with the large normal substrate deuterium isotope effects (Harrison et al., 1990), the above results give rise to the assumption that the protonation step responsible for inactivation does completely suppress the formation of the perpendicularly rotated state from the Michaelis complex E*[*cis*].

The essential role of the carbonyl group of the peptidyl-prolyl bond during interconversion of the Michaelis complexes E*[*cis*] and E*[*trans*] could not be easily extrapolated from the current models of PPIase catalysis. Stabilization by a appropriate environment of desolvated twisted state relative to the more polar ground state was thought to contribute to PPIase catalysis in a considerable part (Rosen et al., 1990; Wolfenden & Radzicka, 1991; Radzicka et al., 1992; Park et al., 1992). Obviously, in apolar solvents simulating the hydrophobic binding pocket, acceleration of the *cis/trans* isomerization can be obtained for oxo amides up to 100-fold (Drakenberg et al., 1972; Eberhardt et al., 1992) as well as for the photoinduced *cis* to *trans* isomerization of *N*-methylthioxoacetamide (Ataka et al., 1984; Kato et al., 1985).

However, more specific contribution of the active site of Cyp18cy such as hydrogen bonding to an intermediate state of rotation (Ke et al., 1993) should also be provided by the enzyme. This reasoning is consistent with the crystal structure of *cis* Ala-Pro (Ke et al., 1993) bound to Cyp18cy, representing the minimal competitive inhibitor of this enzyme (Fischer et al., 1994). The only strong polar interaction of the peptidyl-prolyl bond carbonyl probably comes from a hydrogen bond (N,O distance 2.26 Å) with the backbone NH of Asn_{102}. This contact increases the rotational barrier and should be removed on the catalytic pathway. The thioxo carbonyl was thought to have an acceptor capacity for hydrogen bonding that equals that of the carbonyl group, at least (Hollósi et al., 1990). In this case, the ground-state binding of the *cis*-isomer may be quite similar. However, the enhanced hydrogen bond accepting capacity of the thioxo peptide moiety is at variance with previous results on thioxo amides (Sherman & Spatola, 1990; Kessler et al., 1992).

For FKBP12 a catalytic term attributed to an intramolecular participation of the NH group of the P_2' amino acid of the substrate in stabilizing the twisted nitrogen of the proline ring of the enzyme-bound substrate was discussed. Consequent to a different spatial orientation of the groups involved, this interaction was omitted for Cyp18cy (Fischer et al., 1993). Interestingly, the *cis* to *trans* isomerization of Ala-Leu-ψ[CS-N]-Pro-Phe-NH-Np in the presence of FKBP12 was slightly accelerated (data not shown).

The peptide conformers cannot equilibrate with the enzyme during the quasi-irreversible conditions utilized for determining steady-state parameters of PPIase catalysis by the protease-coupled assay. Application of this technique will monitor enzymatic constants for the *cis*-isomer only. This does not hold true for the thioxo peptides in our experiments, because there is a mixture of *cis*- and *trans*-isomer in solution ready to equilibrate with Cyp18cy. Thus, the situation is not quite clear with the isomeric distribution of the bound thioxo peptide. In the crystal structure of the Cyp18cy/Ala-Pro complex only the *cis*-isomer was visible. In the three-dimensional X-ray structure of *N*-acetyl-Ala-Ala-Pro-Ala-amidomethylcoumarin bound to Cyp18cy, the torsional angle ω of the peptidyl-prolyl bond has values between 20° and 40°, which are still close to the *cis* value (Kallen & Walkinshaw, 1992). Transferred nuclear Overhauser effect measurements with Suc-Ala-Phe-Pro-Phe-NH-Np, closely related in its structure to the thioxo peptides discussed here, gave rise to the assumption of an exclusive binding of the *cis*-isomer to Cyp18cy (Kakalis & Armitage, 1994).

On the other hand, Cyp18cy can express exclusive affinity to the *trans* form of the MeLeu^9-MeLeu^{10} peptide bond in cyclosporin A (Fesik et al., 1990; Kofron et al., 1992; Braun et al., 1995). Furthermore, Park et al. have supposed that a preference for binding of the *cis*-isomer of a substrate to a member of the FKBP family of PPIases might exist (Park et al., 1992). For the first time, the obtained K_i^{cis} value of 82 μM and the K_i^{trans} value of 2500 μM for the thioxo peptide derivative Ala-Gly-ψ[CS-N]-Pro-Phe-NH-Np give quantitative access to the difference in the binding energies of both conformers to Cyp18cy, which approaches as much as 8.2 kJ mol^{-1}. Thus, Ala-Leu-*cis*-ψ[CS-N]-Pro-Phe-NH-Np represents the most favorable competitive inhibitor among the examined thioxylated compounds when the different *cis* contents of the various thioxo peptides were taken into account (Table 2). The active site of Cyp18cy shows a strong conformational specificity. Consequently, this enzyme was able to shift the percentage of the *cis*-isomer of Ala-Leu-ψ[CS-N]-Pro-Phe-NH-Np after preincubation of the thioxo peptide with stoichiometric amounts of Cyp18cy. Thus, besides the ability to accelerate the *cis/trans* isomerization of both peptides and proteins, Cyp18cy possesses another interesting property. A concentration of Cyp18cy in great excess to its ligands can shift the *cis/trans* equilibrium (Figure 7). The enzyme extracts the *cis*-conformer from solution, forming a reservoir of *cis*-conformer reversibly bound to the enzyme. Considering the high intracellular concentration (Ryffel et al., 1991; Lad et al., 1991), it may be hypothesized that Cyp18cy is able to perform this function within the cell as well.

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