Photoregulation of Enzyme Activity. Photochromic, Transition-State-Analogue Inhibitors of Cysteine and Serine Proteases

Pamela R. Westmark, John P. Kelly, and Bradley D. Smith∗

Contribution from the Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556

Received September 28, 1992

Abstract: Two azobenzene derivatives, the aldehyde 1 and the boronic acid 2, were synthesized and shown to be reversible, photoregulatable inhibitors of the cysteine protease, papain, and the serine proteases, chymotrypsin and subtilisin. trans-1 was found to be a potent, reversible inhibitor of papain (Ki = 2.1 µM) while its cis isomer was about 40 times weaker. Irradiation of a solution containing papain and trans-1 with 330 > λ > 370 nm light resulted in a photostationary state mixture of 83% cis-1 and a corresponding 500% increase in enzyme activity. The original activity level was subsequently restored by irradiation with λ > 400 nm light which converted the inhibitor back to its trans configuration. The cycle was repeated to give an identical change in enzyme activity. Similar results were obtained with 2 as a photoswitchable inhibitor of chymotrypsin although the change in enzyme activity was much smaller (13%). In the case of subtilisin, the relative inhibitory abilities of trans-2 and cis-2 were found to be dependent upon the conditions of the experiment, particularly the concentration of KCl. In the absence of KCl, cis-2 was a better inhibitor of subtilisin than trans-2, but in the presence of 0.5 M KCl the reverse trend was observed.

Introduction

General methods that simplify the technique of protein affinity chromatography would be of practical value, especially in the area of enzyme purification.1 Work on the purification of cobalamin-binding proteins has demonstrated that affinity adsorption ligands that can be photoconverted from a configuration with strong affinity for a desired enzyme to a configuration with a weak affinity provide a novel approach to enzyme purification under mild conditions.2,3 Since transition-state-analogue enzyme inhibitors can be utilized as enzyme affinity adsorption ligands,4 it is possible that appropriately immobilized photosomerizable transition-state-analogue enzyme inhibitors may act as photoswitchable affinity ligands. An initial requirement is the development of such a series of photochromic enzyme inhibitors.

Here we report on the novel photochromic, transition-state-analogue protease inhibitors, 1 and 2, and their ability to reversibly bind and inhibit the activity of certain cysteine and serine proteases in a photoregulatable manner.5

The general design of these inhibitors can be considered as the structural sum of two components, a head and a tail. The head consists of a primary recognition moiety known to have a propensity for reversible binding in the enzyme active site. The tail is a photochromic moiety whose molecular shape can be controlled in a photoswitchable manner. Photomodulation of the shape of the tail results in regulation of the inhibitory ability of the inhibitor and therefore enzyme activity. Such a strategy should be applicable to other enzyme/reversible-inhibitor systems.


0002-7863(93)1515-3416$04.00/0 © 1993 American Chemical Society
Photoregulation of Enzyme Activity

Figure 1. Half-reciprocal plots for the mixed inhibition of papain (12 μM), in 100 mM potassium phosphate buffer, pH 7.4; (O) no inhibitor, (●) trans-1 (9 μM), (□) cis/trans photostationary mixture of 1 (9 μM) obtained by UV irradiation.

Figure 2. Absorption spectrum of (a) trans-1 in 100 mM potassium phosphate buffer, pH 7.4, and (b) the photostationary, 83:17, cis/trans mixture of 1 achieved after UV (330 > λ > 370 nm) irradiation.

trans-1

trans-2

to allow λ > 400 nm) was observed to result in complete restoration of the trans configuration. In situ photoregulation of papain activity was accomplished in the following manner. Addition of trans-1 (18 μM) to a solution of papain (12 μM) was found to lower its ability to hydrolyze N-benzoyl-DL-arginine-p-nitroanilide from a rate of 480 to 18 nM min⁻¹. UV irradiation of this sample raised the hydrolysis rate to 114 nM min⁻¹. Subsequent visible irradiation returned the hydrolysis activity to 17 nM min⁻¹. The cycle was repeated again and an identical change in activity levels was observed (Figure 3). Thus, the hydrolytic activity of papain in the presence of inhibitor 1 could be reversibly switched from a relative activity of 1.0 after irradiation with visible light to an activity of 6.3 after UV irradiation.

A similar study was conducted with compound 2 as a photoregulatable inhibitor of the serine proteases, chymotrypsin and subtilisin. trans-2 was a competitive inhibitor of chymotrypsin, Kᵢ = 11 μM. Photosomerization with UV light produced a photostationary state of 80% cis-2 and an apparent Kᵢ = 41 μM. With this difference in Kᵢ's, a 2- to 3-fold change in enzyme activity can be predicted upon photoswitching. Attempts to produce an in situ photoswitchable inhibitor/chymotrypsin system, however, were hindered by problems maintaining enzyme activity and inhibitor solubility over the time period of the experiment.

These problems were solved by including potassium chloride (KCl) and bovine serum albumin (BSA) as solution additives. Unfortunately, this resulted in a 10-fold increase in Kᵢ which attenuated the range over which enzyme activity could be modulated. Addition of trans-2 (42.3 μM) to a pH 8.4 solution of chymotrypsin (50 nm), BSA (4 μM), and KCl (0.5 M) lowered the rate of MeO-Suc-Ala-Ala-Pro-Phe-p-nitroanilide hydrolysis from 716 to 590 nM min⁻¹. UV irradiation of this sample raised the hydrolysis rate to 670 nM min⁻¹. Subsequent visible irradiation returned the hydrolysis activity to 600 nM min⁻¹. Further cycling of the irradiation wavelength produced virtually identical changes in activity levels (Figure 4). Thus, the hydrolytic activity of chymotrypsin in the presence of inhibitor 2 could be reversibly switched from a relative activity of 1.00 after irradiation with visible light to an activity of 1.13 after irradiation with UV light.


experiment from being attempted. Current efforts are focussed supports and determining their ability to act as photoswitchable The modest inhibition this reversal in irradiation with visible irradiation (Figure 4). The reason for and cis-2 were found to be dependent on the conditions of the acetate (20 mL). A precipitate formed immediately and after 2 h, TLC hydrolysis activity for chymotrypsin and subtilisin control solutions kept in the dark.

In the case of subtilisin, the relative inhibitory abilities of trans-2 and cis-2 were found to be dependent on the conditions of the experiment. When the sample included the additives described in the chymotrypsin experiments above, trans-2 (visible irradiation, \( K_i = 0.9 \text{ mM} \)) was observed to be a slightly better subtilisin inhibitor than cis-2 (UV irradiation, \( K_i = 1.4 \text{ mM} \)). However, in the absence of KCl, which was not necessary for the enzyme's stability in this case, trans-2 (\( K_i = 0.5 \text{ mM} \)) was a weaker subtilisin inhibitor than cis-2 (after UV irradiation, \( K_i = 0.3 \text{ mM} \)). Irradiation of the enzyme–inhibitor system with UV light resulted in a decrease in hydrolysis activity of 12% which was restored by visible irradiation (Figure 4). The reason for this reversal in \( K_i \) upon KCl addition is not clear; speculative explanations include an enzyme conformational change or a relative change in inhibitor partition coefficients.

Since aldehydes are known to act as inhibitors of serine proteases,1 I was briefly examined as a chymotrypsin inhibitor. The modest inhibition (\( K_i = 0.15 \text{ mM} \)) observed, combined with poor inhibitor solubility prevented the in situ photoregulation experiment from being attempted. Current efforts are focussed on attaching structural analogues of these inhibitors to solid supports and determining their ability to act as photoswitchable enzyme affinity adsorption ligands.

**Experimental Section**

**Materials.** The following reagents were obtained commercially: 4-(phenylazo)benzoic chloride (Eastman Kodak); 4-(phenylazo)-benzenesulfonyl chloride (TCI America); N-benzoyl-L-arginine 4-nitroanilide hydrochloride, MeO-succinyl-Ala-Pro-Phe 4-nitroanilide, and 4-nitrophenyl butyrate (Sigma); BSA (Serva). All enzymes were obtained commercially and were used without further purification: subtilisin BPN' and \( \alpha \)-chymotrypsin (Sigma); papain (2 × recrystallized, Worthington).

**Synthesis.** N-(4-(Phenylazo)benzoyl)aminoacetaldehyde (1). Aminoacetaldehyde diethyl acetal (0.59 mL, 4.08 mmol) was added dropwise to a stirring, room temperature solution of 4-(phenylazo)benzoic chloride (1.00 g, 4.08 mmol) and triethylamine (5.70 mL, 4.08 mmol) in ethyl acetate (20 mL). A precipitate formed immediately and after 2 h, TLC indicated the reaction was complete. The precipitate was collected, rinsed with water, and recrystallized twice from aqueous ethanol to give N-(4-(phenylazo)benzoyl)aminoacetaldehyde diethyl acetal as an orange solid (1.20 g, 73%); mp = 109–110°C; TLC (silica, 1:1 ethyl acetate/hexanes), \( R_f \) 0.82; 1HNMR (300 MHz, CDCl3) 7.93 (m, 7 H), 7.55 (d, 2 H, \( J = 9 \text{ Hz} \)), 6.43 (br t, 1 H, NH), 4.64 (t, 1 H, J = 5.5 Hz, CH2), 3.77 (m, 2 H), 3.63 (m, 4 H), 1.25 (t, 6 H, \( J = 7 \text{ Hz} \), CH3), m/e (FAB) 342 (M + 1), 296 (M – OEt). Anal. Calcd for C16H13NO4: C, 66.84; H, 6.79; N, 12.31. Found: C, 66.65; H, 6.74; N, 12.26.

Removal of the acetal was achieved by stirring in a 50% solution of tetrahydrofuran and 0.1 M HCl until TLC indicated consumption of the protected material (usually 24 h). Rotary evaporation of the tetrahydrofuran produced a precipitate which was collected, rinsed with water, and dried via desiccation. The product 1 was purified by preparative TLC (silica gel, 1:1 ethyl acetate/hexanes), \( R_f \) 0.62, and shown to be pure by HPLC analysis (silica, 1:1 hexanes/ethyl acetate); \( t_{R} = 20\% \); mp = 124–126°C; 1HNMR (300 MHz, CDCl3) 9.20 (s, 1, H, OCH), 8.00 (s, 5, H, Ph-N), 7.96 (d, 2 H, \( J = 9 \text{ Hz} \)), 7.56 (d, 2 H, \( J = 9 \text{ Hz} \)), 6.98 (br t, 1 H, NH), 4.50 (2 H, J = 4 Hz); m/e (FAB) 268 (M + 1), 209 (M – NHCH2COH); UV \( \lambda_{max} \) = 324 nm (\( t = 15 \text{ 864 cm}^{-1} \text{ M}^{-1} \text{ in 100 mM potassium phosphate, pHe 7.4} \)).

N-(4-(Phenylazo)phenylsulfonyl)-3-aminobenzeneboronic Acid (2). To an ice-cooled solution of 3-aminobenzeneboronic acid monohydrate (0.50 g, 2.33 mmol) in 50% aqueous acetone, \( J_\text{H}_{2} \text{O} \) and water, adjusted to pH 8 with NaOH, 4-(phenylazo)benzenesulfonyl chloride (0.90 g, 3.23 mmol) in acetone (10 mL). After stirring for 1 h, the acetal was removed by rotary evaporation. The residual alkaline layer was washed twice with methylene chloride, acidified to pH 5 with 20% HCl, and extracted with methylene chloride. After drying, the organic solvent was evaporated to leave 2 as an orange solid which was recrystallized twice from aqueous ethanol (1.12 g, 80%). mp = 147–149°C; TLC (silica, 25:25:2, ethyl acetate/hexanes/ethanol); \( R_f \) trans = 0.66, cis = 0.54; 1HNMR (300 MHz, CDCl3) 9.07 (s, 1, H, NH), 7.95 (m, 7 H), 7.71 (s, 1, H), 7.58 (m, 5 H), 7.26 (2 H, m); m/e (positive ion FAB in glycerol matrix) 438 (M + 57); molecular weight via NaOH titration = 381 (calcd 389); \( \lambda_{max} \) = 323 nm (\( t = 21 \text{ 440 cm}^{-1} \text{ M}^{-1} \text{ in 100 mM potassium phosphate, pH 7.4} \)). Anal. Calcd for C25H19N2O4S: C, 65.71; H, 4.23; N, 11.02; S, 8.41. Found: C, 56.93; H, 4.33; N, 10.98; S, 8.61.

**Instrumentation.** UV spectra were obtained on a computer driven Perkin-Elmer Lambda 2 spectrophotometer, equipped with a constant temperature cell-holder. The kinetic data were collected using Perkin-Elmer Computerized Spectroscopy Software and analyzed graphically using Cricket Graph Software. Irradiation experiments utilized a 150 W Oriel Xe arc lamp equipped with a housing condensor to collimate the beam. An Oriel 59810 filter was used for UV (330–430 nm) light and a 59494 filter for visible (\( \lambda > 400 \text{ nm} \) ) light. Organic sample was placed in glass containers and enzyme solutions in plastic containers were held two inches away from the lamp output lens; control experiments showed there was no sample heating due to the irradiation.

**Determination of Inhibition Constants.** The trans inhibitors were added to the enzyme solutions as concentrated organic solutions (1 in methanol and 2 in acetone) protected from the light. The cis inhibitors were obtained by irradiating the organic solutions until photostationary states had been achieved. In the dark, the cis isomers spontaneously converted to the trans isomers with half-lives of about 8 h at room temperature in organic or aqueous solutions. In the presence of excess \( \beta \)-mercaptoethanol or cysteine this process was accelerated up to 100-fold. The enzyme/inhibitor solutions were incubated for 5 min before the substrate was added.

Papain was freshly activated using 8-mercaptoethanol and EDTA, at pH 6, and its ability to hydrolyze N-benzoyl-L-arginine-4-nitroanilide was determined by the rate of 4-nitroaniline production observed at 410 nm.11 Inhibition assays consisted of papain (12 \( \mu \text{g} \)) in 1 mL of potassium phosphate solution (100 mM, pH 7.35), EDTA (1 mM), \( \beta \)-mercaptoethanol (6 mM), KCl (300 mM), and 3% organic solvent v/v. Inhibition of chymotrypsin activity was determined using MeO-Suc-Ala-Ala-Pro-Phe 4-nitroanilide as the substrate14 in solutions containing chymotrypsin (100 nM), BSA (4 \( \mu \text{g} \)), potassium phosphate (40 mM, pH 8.4), KCl (500 mM), and 3% organic solvent v/v. The same conditions, with and without added KCl, were used to determine subtilisin (250 nM) inhibition which was monitored via hydrolysis of 4-nitrophenyl butyrate.17 Background hydrolysis of the substrate at this pH was negligible.
Photoregulation of Enzyme Activity

Enzyme/Inhibitor Irradiation Experiments. A magnetically stirred solution (10 mL) of papain (12 μM), 1 (18 μM), β-mercaptoethanol (6.7 mM), EDTA (1 mM), and KCl (300 mM) in 3% methanol/potassium phosphate buffer (100 mM, pH 7.35) was alternately irradiated by UV (usually 20 min to reach photostationary state) and then visible light. After each irradiation, a 1-mL aliquot was withdrawn and its ability to hydrolyze N-benzoyl-dl-arginine-4-nitroanilide was determined. The results were compared to an identical control solution that remained in the dark. Similarly, a 10-mL solution of chymotrypsin (50 nM), 2 (42 μM), BSA (4 μM), and KCl (500 mM) in 3% acetonitrile/potassium phosphate buffer (40 mM, pH 8.4) was assayed for MeO-Suc-Ala-Ala-Pro-Phe-4-nitroanilide hydrolysis activity after successive irradiations by UV and visible light. In the case of subtilisin, aliquots from a 10-mL solution of subtilisin (250 nM), 2 (220 μM), and BSA (4 μM) in 7% acetonitrile/potassium phosphate buffer (40 mM, pH 8.4) were assayed for 4-nitrophenyl butyrate hydrolysis activity after successive irradiations by UV and visible light.

Acknowledgment. Acknowledgment is made to the donors of The Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research. J.P.K. is an American Chemical Society–Petroleum Research Fund Undergraduate Scholar. We are grateful to Professors A. M. Trozzolo and D. J. Pasto for use of equipment.